Using an aerosol human BCG infection model to define the early immune response in the lung and systemic circulation



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Using an aerosol human BCG infection model to define the early immune response in the lung and systemic circulation

Julia Marshall, St Anne's College, The University of Oxford
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There remains much that we do not understand about the interaction between Mycobacterium tuberculosis (*M.tb*) and the host immune response. Development of an effective vaccine is hampered by an incomplete understanding of the immunoprotective mechanisms, no biomarker of infection and no validated correlate of protection. Initial immune events driven by the innate system play an important role in the development of adaptive immunity and protection. This thesis describes the development of an aerosol BCG human infection challenge and demonstrates that this model is safe and well tolerated in BCG and *M.tb* naïve UK adults. This novel challenge model is then used to describe the initial innate and adaptive immune events at the site of infection, the airway parenchymal interface. The response is characterised by a local innate and adaptive lymphocytosis by day 7, and rapid upregulation of MHC-II antigen presentation differential gene expression pathways and Th1 effector function. There is a corresponding peak in antigen-specific IFN-y production at day 7 in the peripheral circulation, and improved mycobacterial growth control by peripheral blood mononuclear cells at day 14 on an *ex-vivo* functional assay. By describing underlying early immune responses to a mycobacterial airway infection in humans, this thesis provides an invaluable contribution to the field. This body of work has created a rich resource of early immune variables from which new hypotheses can be drawn for further investigation. Novel immune mechanisms, if correlated with growth control, could be used for rational tuberculosis vaccine design, to help tackle this pervasive disease.

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And lastly to my Australian family, despite the distance, I could not have written this without you all. Thank you for your unconditional support and belief in me.

List of Abbreviations

95% CI	95% confidence interval
AE	Adverse event
AJV	AJ Vaccines
ALF	Alveolar lining fluid
AM	Alveolar macrophage
ATAC-Seq	Assay for transposase-accessible chromatin sequencing
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BCG	Bacillus Calmette–Guérin
CCVTM	The Centre for Clinical Vaccinology and Tropical Medicine
CFP-10	10 kDa culture filtrate protein
CFU	Colony forming units
DCs	Dendritic cells
DGE	Differential gene expression
DMSO	Dimethylsulphoxide
DN	Double negative
DP T cells	Double positive (CD4+ CD8+) T cells
DURTs	Donor-unrestricted T cells
EDTA	Ethylenediaminetetraacetic acid solution
ELISpot	Enzyme-linked immune absorbent spot
ESAT-6	6 kDa early secretory antigenic target
FACS	Flow cytometry
FEV1	Forced expiratory volume in one second
FMO	Fluorescence minus one
FVC	Forced vital capacity
GCP	Good clinical practice
HRA	Health Research Authority
ID	Intradermal
IFN	Interferon
IGRA	Interferon gamma release assay
IQR	Interquartile range
IV	Intravenous
KCO	Carbon monoxide transfer coefficient
LAM	Lipoarabinomannan
LN	Lymph node
logfc	Log fold change
LTBI	Latent TB infection
M.tb	*Mycobacterium tuberculosis*
MAITs	Mucosal-associated invariant T cells
MGIA	Mycobacterial growth inhibition assay
MGIT	Mycobacterial growth indicator tube using the BACTEC system
MHC	Major histocompatibility complex
MHRA	Medicines and Healthcare Products Regulatory Agency

MMP	Matrix metalloproteinase
MoAMs	Monocyte derived AMs
MTBC	*Mycobacterium tuberculosis* complex
NETs	Neutrophil extracellular traps
NF-KB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHPs	Non-human primates
NK cells	Natural killer cells
NKT cells	Natural killer T cells
NO	Nitric oxide
NODs	Nucleotide-binding oligomerization domains
NTM	Non-tuberculous mycobacteria
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered solution
PFT	Pulmonary function test
POI	Prevention of infection
PPD	Purified protein derivative
PRRs	Pattern recognition receptors
R10NS	R10 no streptomycin
RBC	Red blood cell
RD1	Region of difference 1
REC	Research ethics committee
ROS	Reactive oxygen species
RT	Room temperature
SAE	Serious adverse event
SEB	Staphylococcus enterotoxin B
SEM	Standard error of the mean
SFC	Spot forming cells
SmPCs	Summary of product characteristics
SOPs	Standard operating procedures
SSI	Statens Serum Institut
SUSARs	Suspected unexpected serious adverse reactions
TB	Tuberculosis
TCR	T cell receptor
TEM	Tetramer
TLCO	Transfer capacity of the lung for carbon monoxide uptake
TLRs	Toll-like receptors
TNFα	Tumour necrosis factor alpha
Treg	Regulatory helper CD4+ T cell
TRM	Tissue resident memory T cell
TST	Tuberculin skin test
TTP	Time to positivity
UMAP	Uniform manifold approximation and projection
VA	Alveolar volume
WB	Whole blood

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Chapter 1.

Introduction

1 Introduction

1.1 Tuberculosis today

In 2019, Tuberculosis (TB), caused predominantly by the *Mycobacterium tuberculosis* (*M.tb*) bacterium, led to disease in 10 million people, with 1.4 million deaths.[1] This is despite antibiotic therapy, targeted public health measures and widespread use of the only licenced vaccine against TB, Bacille Calmette–Guérin (BCG), which had its 100th anniversary this year.[2] The development of an effective vaccine against TB is essential if we are to meet the WHO End TB Strategy.[3] Over the millennia of co-evolution with Homo sapiens, *M.tb* has developed numerous mechanisms to evade host detection and elimination[4] and to date research vaccine candidates have failed to improve on BCG efficacy. We therefore need a different approach.

1.2 Vaccinations against TB

1.2.1 BCG as a vaccine

BCG vaccine offers protection against disseminated TB in childhood, but is less effective against pulmonary TB in many endemic areas, ranging from 0-80% protection in adults.[5-7] The reason for this difference is not completely understood. BCG is less effective at lower altitudes, in equatorial regions or areas endemic for non-tuberculous mycobacteria (NTM), and in people with previous mycobacterial sensitisation.[8-10] By contrast, BCG vaccination in UK adolescents was shown to confer 84% efficacy against *M.tb* disease at five years and 77% at 20 years.[11] As lower altitude tropical countries tend to also be endemic for NTM spp., poor BCG efficacy may be caused in part by immune masking or blocking. Historic NTM exposure might result in an attenuated “masking” immune response, as demonstrated in two studies in

UK and Malawi BCG-naïve adolescents.[12] Alternatively a robust cross-reactive immune response on BCG vaccination in NTM-primed people might clear the BCG before BCG-specific immune responses can develop ("immune blocking").[8,13] Other reasons for varied BCG efficacy might be due to strain diversity, genetic differences in human populations, nutritional status or helminth exposure.[8,14] Knowledge in this field is hampered by studies with contradictory findings, likely due to varied study design endpoints and the inherent limitations in observational field studies.[15,16] A controlled human infection study could contribute to our understanding of what factors affect the development of a protective immune response following BCG exposure.

1.2.2 Vaccine development strategies

Historically, vaccine development has largely been empirical and hence vaccine development for diseases such as HIV or TB, where we need to improve upon natural immunity, remain a challenge. There are currently 21 vaccines against *M.tb* in various stages of development, using four broad strategies, the majority designed as a BCG boost.[17] A gold standard vaccine would aim to prevent initial *M.tb* infection (POI) and create sterilising immunity.[18] Whether this is achievable is debated.[19,20] Further, it is unclear whether the immune mechanisms of protection for infection and disease are the same and hence whether protection against infection is predictive of protection against disease.[17] Given approximately 25% of the world population is already infected with *M.tb,*[1] vaccines which aim to prevent progression to disease (POD) are also an important public health target.[17,21] Other vaccines in the design pipeline aim to prevent TB recurrence.[1,17] To date, six vaccine candidates have progressed to Phase 2b/3 field trials.[22] Despite encouraging evidence for protection in NHP models and human immunogenicity and safety trials, the MVA85A trial in South African infants did not improve protection beyond that conferred by BCG alone.[23-25] In 2018, again despite promising

pre-clinical animal models, a POI trial in adolescents in an endemic setting showed no difference between H4:IC31 subunit candidate vaccine (containing TB10.4 and Ag85B *M.tb* antigens) and BCG alone.[26] However, in this study, intradermal (ID) BCG revaccination resulted in 45.4% efficacy against sustained Interferon Gamma Release Assay (IGRA) conversion, a putative marker for TB infection state (see discussion below).[26] BCG revaccination did not stop initial colonisation of *M.tb* but may have led to bacterial clearance. This trial differed in design and population to previous trials in Brazil and Malawi which had showed no effect of BCG revaccination on POD.[6,27] A second trial is underway to confirm and expand on these findings. In addition, the POD adjuvant recombinant protein vaccine candidate (M72/AS01E) has demonstrated 50% protection against progression to TB in subjects with latent *M.tb* infection (LTBI) for 3 years.[21] Whether this vaccine can confer protection against initial infection and if it protects against disease in those without LTBI warrants further investigation.[28]

An in depth understanding of the underlying immune processes which confer protection against TB is essential. Follow on work from these large scale Phase 2 and 3 trials allows for important interrogation of immune responses and will continue to aid the field in understanding potential protective mechanisms.[21,29] However, deciding on candidates for Phase 2, and long term financially-draining Phase 3 trials, remains problematic due to the poor predictive value of animal models.[23,30] The TB field is even more dependent on the outcome of these Phase 2 and 3 trials as we do not have a validated correlate of protection. Further, all these vaccine strategies are hampered by the fact that there is no definitive test for *M.tb* infection.

1.2.3 Poor predictive value of animal models

The predictive value of *M.tb* animal models for human protection is limited, due to differences in disease pathology and immune systems. For example BALB/c and C57BL/6 mice do not develop structured caseating granulomas and don't have Group 1 CD1-restricted T cells.[31,32] Guinea pigs and non-human primates (NHPs) do develop granulomas, however research with guinea pigs is restricted by the lack of immunological reagents.[33,34] NHPs can act as a useful model of *M.tb* infection however cost and strict animal welfare standards limits the flexibility of this model.[35,36] Furthermore, the protection gained from BCG vaccination and the susceptibility to *M.tb* infection varies considerably between species and the direct comparability to humans is unclear.[37] Despite this, animal models do retain an important role in TB research. They provide a wider range of tissues for investigation and are able to be manipulated through genetic engineering. Further, the tractable animal model allows confirmation and elucidation of immune mechanisms identified from human studies.

1.3 Pathogenesis of TB

1.3.1 Mycobacterium spp.

Mycobacteria are gram positive, aerobic, slow growing, non-spore forming bacteria, classified as *Mycobacterium tuberculosis* complex (MTBC), NTM or leprosy bacilli.[38] The majority are saprophytes living harmlessly in the environment, a few species have evolved to become obligate pathogens.[39]

1.3.1.1 NTMs

NTMs are widely distributed in soil, vegetation and water.[39] Colonisation and disease seems to be dependent on exposure levels and the immunocompetence of the patient.[39,40] NTM can

cause disease in the lung and other tissues, though colonisation is often asymptomatic.[38] NTMs tend to be more common in tropical environments and have a certain amount of immune cross reactivity with *M.tb*.[38]

1.3.1.2 MTBC

The MTBC have 99.99% genome sequence homology, are obligate pathogens and cause TB-like disease (lung tubercle-nodules) in humans and other mammals.[4,41]
Some have host tropism exclusively to humans (*M.tb*, *M. canettii* or *M. africanum)*. Others, such as *M.bovis,* have a primary host, in this case cattle, but cause TB-like disease in a range of animals, including humans.[42,43] In humans, TB is primarily caused by *M.tb*, with an uncertain percentage caused by *M.bovis*.[38]

The MTBC are weakly gram positive and are identified microbiologically using acid fast staining such as the Ziehl-Neelsen.[38] Their resistance to decolourisation by weak acids is due to their thick waxy cell wall, the mycolyl-arabinogalactan-peptidoglycan complex.[44] This wall is essential to the bacteria's ability to resist killing within the lysosome of a macrophage and enables dormancy.[45] Cell wall components such as lipomannan, lipoarabinomannan and phosphatidylinositol mannosides are used by the host as early warning signals of infection (PAMPs or DAMPs).[44] The bacteria is able to remodel the peptidoglycan and mycolic acid structure during infection, resulting in a heterogenic population better able to avoid host clearance.[46]

1.3.2 *M.tb* infection

Despite our long history with the disease, surprisingly little is known about the mechanism of *M.tb* infection in humans. In the field, pinpointing exact timing of infection is difficult and so understanding of the initial immune response against *M.tb* infection must be extrapolated from animal studies, *in-vitro* human models or studies of TB disease once infection is well established.[4,5] The exact sequence of early pathogenesis and immune mechanisms in the human is unknown.

TB is spread through inhaled or contact-touch respiratory droplets. If the bacteria avoids removal by innate physical defences such as mucosal ciliated clearance, sneezing or coughing, it establishes infection in the alveolar spaces of the lung. From *ex-vivo* studies we know that *M.tb* is capable of invading many human cells including neutrophils and dendritic cells (DCs), however its adaptive niche appears to be the alveolar macrophage.[47-50] Mononuclear phagocytes (MPs) can detect the Pathogen-Associated Molecular Patterns (PAMPs) on the *M.tb* cell wall and phagocytose the bacterium. Ordinarily the macrophage would then fuse the phagosome with the acidic lysosome resulting in effective killing of the microorganism, followed by processing of the foreign peptides and mounting them on major histocompatibility complex class II (MHC-II) molecules, thus stimulating a targeted inflammatory immune response. However, *M.tb* evades destruction and creates a niche within the phagosome.[51]

In mice, the early immune response is marked by an influx of neutrophils, interstitial macrophages, inflammatory monocytes and DCs. With other pathogens this response might contain the infection, however the *Mycobacterium* has evolved various strategies to manipulate the innate immune response.[52] The influx of innate cells to the site of

inflammation allows for additional niches in which the bacteria can multiply and *M.tb* appears to attenuate the immune response, delaying T-cell recruitment.[53,54] Bacterial replication therefore continues unabated until the activation of the adaptive immune system, particularly of Th1 CD4+ T cells, where in most cases the infection can be contained.[6] In mice and NHPs, the development of this adaptive response is delayed compared with other pathogens, with influx into the lungs seen around 3 weeks.[54]

In most humans with a robust Th1 CD4+ axis, mounting an adaptive immune response to *M.tb* leads to control but not necessarily clearance, resulting in latent *M.tb* infection (LTBI). The human immune system attempts to wall off the infection through development of granulomas in the lung.[55-57] Cytokines and chemokines produced by activated macrophages, DCs, granulocytes and lymphocytes maintain the inflammation.[81] These cells transform and organise themselves into granulomas around the bacteria, which goes into either a dormant or slowly replicating state.[58,59]

There is a 5-10% lifetime risk of reactivation of LTBI leading to active disease; marked by uncontrolled replication within the lung or, in 30% of active cases, dissemination throughout the body.[60,61] Active disease classically presents with the canonical symptoms of weight loss, productive cough and night sweats.[62] Clinical diagnosis is made by detection of mycobacteria in the sputum or via bronchoalveolar lavage (BAL). *M.tb* and the resultant vociferous immune response results in devastating destruction of local lung tissue which can be seen radiologically on plain chest x-ray as cavitating lesions.[63] Breakdown of the lung architecture allows the bacteria to enter the alveolar spaces where it is expelled out of the airway in infected respiratory droplets through cough, primed to infect a new host.[64]

1.4 Natural sterilising immunity, does it occur?

Primary lung infection triggers a mucosal immune response which has four hypothesised outcomes based on our current clinical detection methods.

- Early Clearance: Clearance of the infection without a detectable adaptive immune response
- Delayed Clearance: Clearance of the infection with sustained or transient antigen-specific immunity; detectable on Tuberculin skin test (TST) or Interferon Gamma Release Assay (IGRA)
- LTBI: Asymptomatic containment of infection within a granuloma; detected as sustained IGRA or TST and/or calcified granulomas visible on chest x-ray[57]
- Active pulmonary and/or disseminated disease; detected by live mycobacterial culture/detection and clinical disease.[62]

1.4.1 Latency – a protective immune state against M.tb?

Studying the differences between LTBI and active TB disease is one method used to interrogate the protective immune requirements against TB. The exact reasons for conversion from latency to TB disease is poorly understood but the risk is increased with factors affecting immune competence such as nutrition, age, and comorbidities such as diabetes.[65,66]

However studying LTBI as a marker of protective immunity is problematic as latency is likely the preferred state of the bacterium.[59] LTBI is not a dormant inert state. Rather it appears to be a constant balancing act between the *Mycobacterium* and the host, with bacterial clearance and healing granulomas in some areas, and in other parts of the lung bacterial growth with new granuloma formation.[67] It may also be the bacteria's preferred state, as

Mycobacteria readily enter dormancy through the DosS-DosT/DosR system.[45] Granulomas with the resultant intense immune cell aggregation may be a virulence strategy to find new niches to invade.[55,56] Further, immune responses are localised and appear to vary significantly between granulomas.[68] In addition, *M.tb* may manipulate the host immune response to allow a switch to active TB disease, in order to spread to other hosts, as a critical part of the bacterium's survival.

Further, defining latency, and indeed presence of infection at all, is difficult due to the limited tools available. Traditionally a tuberculin skin test (TST) has been used, which measures a delayed type IV hypersensitivity response against a broad range of mycobacteria including NTMs.[69,70] Increasingly, infection is diagnosed by IGRA (QuantiFERON®-TB Gold or T-SPOT®) which detect IFN-y production by PBMCs following incubation with the *M.tb*-specific antigens ESAT-6, CFP-10 +/- TB-7.7.[71,72] However, as these tests are immune based they do not directly detect *M.tb.*[73] They also do not distinguish between LTBI and active disease, nor are they able to clearly define the point of bacteria clearance.[74,75]

1.4.2 Delayed Clearance with sustained or transient adaptive immunity

Because we don't have a definitive marker of infection it is unclear how many people spontaneously clear infection, or even when treatment has been successful. IGRA reversion may not be a marker of infection clearance, but merely reflect a reduction in effector T cells, perhaps due to reduced *M.tb* antigen production as the bacterium goes into dormancy,[76,77] or in patients with immune anergy.[78,79] Conversely, false positives may occur, as antigen-specific T cells have been shown to persist after bacterial clearance.[80]

However, as anti-TB treatment in humans does usually lead to IGRA reversion, and in mice, there is a positive correlation between the *M.tb*-specific IFN-y response and the bacterial load,[77,81] a positive IGRA is generally accepted as a marker of infection.[74,82,83]

Based on a mathematical model from post mortem and IGRA reversion data on Indian, Chinese and Japanese cases, Emery and colleagues estimated that 24.4% people clear *M.tb* infection within 10 years and 73% over a lifetime.[45] Further, LTBI confers 79% protection against reinfection.[84] Taken together, this data suggests that humans can develop a natural adaptive immune response capable of clearing *M.tb* infection. However, the precise nature of this immune response is unknown.

1.4.3 Early Clearance

Not everyone exposed to *M.tb* seems to establish an infection. Case-contact studies suggest 30-50% of people exposed to *M.tb* are protected from infection as they do not develop an adaptive immune response, as defined by a positive IGRA.[10] In one systematic review of 203 studies, prevalence of LTBI on IGRA or TST, following contact with an active case was 51.5% (95% confidence interval (95%CI) 47.1–55.8%), where high exposure correlated with conversion risk.[85,86] Persistent IGRA negative people have low risk of progression to active TB disease, supporting the hypothesis that *M.tb* is being cleared.[77,85,87] Whether this is before or after infection is established, is unclear.

"Early clearance" could be evidence of a protective innate immune mechanism, either via muco-ciliary-mediated clearance prior to the establishment of any infection, or due to a sterilising innate cellular immune response.[3,12] As historic BCG vaccination reduces the risk of IGRA conversion, clearance may be due to trained innate immunity (discussed later).[88]

While early clearance is defined as *M.tb* infection clearance without the development of an adaptive immune response,[89] this phenomenon has not been proven and an explanation for persistently negative IGRA could be an efficient protective adaptive immune response where

rapid elimination also results in loss of detectable effector adaptive immune responses, with IGRA reversion as discussed above.[75,90] This has been reported in NHPs, with low or undetectable T-cell and antibody responses after *M.tb* clearance in IV BCG-primed animals.[91]

Another explanation is that an adaptive response develops, and infection may even persist, but that this is never detected on IGRA, either because of T-cell anergy, IFN-y independent adaptive immune mechanisms, or because the adaptive response is localised to the lung mucosa.

As early clearance is a model of protective immunity, understanding this phenomenon could provide new opportunities for vaccine targets. A major impediment to elucidating the underlying mechanisms is the inherent limitations of field studies where defining the dose and the timing of infection is not possible.[85]

My thesis aims to advance understanding of the early immunological events at the local mucosal level and relate them to detectable systemic adaptive immune responses. Findings from my study could help shed light on some of the underlying mechanisms of early clearance.

1.5 Potential players in early immune defence

Plants and invertebrates successfully ward off pathogens without an adaptive immune system, however the innate response in mammals has been believed to be limited and static. Work highlighting different classes of pattern recognition receptors (PRRs) has demonstrated pathogen-specific variable innate responses in humans.[92] Understanding innate response plasticity and its impact on developing a protective adaptive response is vital for optimal

vaccine development, however the role of the innate immune response in protection against TB has traditionally been restricted to the ability of MPs to prime T and B cells. More recently, the concept that innate cells such as NK cells mature into variable effector subclasses, influenced by environmental exposure,[93] as well as recent work in epigenetic imprinting of bone-marrow derived monocytes, termed training,[94] suggests that a memory-like phenomenon in innate cells may exist, independent of the adaptive response, opening up a new potential area for vaccine targets. Here I review the current understanding of the innate immune system's role in TB protection and highlight the gaps in our knowledge.

1.5.1 Non-cellular innate immune defence

Lung tissue is uniquely vulnerable to the billions of daily inhaled foreign particles.[95] Gas exchange occurs by inhaled air passing through the bronchial tree into alveolar sacs, traversing the interstitium and entering the blood stream. Lung parenchyma is made up of these alveoli, interstitium and pulmonary vasculature. The majority of foreign matter is caught in the hairs lining the nasal passages, or in antibacterial mucous excreted by secretory/goblet bronchial epithelial cells lining the bronchus, where ciliated cells then clear the airway.[95] If pathogens reach the alveoli, surfactant contains antimicrobial substances, such as peptidases, which can kill or attenuate the pathogen, while complement facilitates opsonisation and inflammation regulation.[96,97] Commensal microorganisms reside in the alveoli which may also help control pathogens.[98]

This alveolar lining fluid (ALF) may play a critical role in *M.tb* defence. ALF modifies the lipid wall through hydrolytic enzymes, which has been demonstrated *in-vitro* to restrict the ability of *M.tb* to enter alveolar macrophages (AMs) and survive in the host.[99] In addition, surfactant protein enhances *M.tb* phagocytosis, phagolysosomal fusion, cell clearance and regulates inflammation.[100,101] Neutrophils exposed to ALF-modified *M.tb* exhibit increased intracellular

killing, and tumour necrosis factor (TNFα) and IL-8 expression, while simultaneously reducing potentially harmful extracellular inflammatory responses such as extracellular trap release.[100] The potential role of ALF is often overlooked in models of TB virulence, emphasising the importance of infection challenge models at the natural site of infection.

1.5.2 Epithelial cells

In mouse models, *M.tb* infects epithelial cells within 48 hours of aerosol infection and *M.tb* have been found in type II alveolar cells, endothelial cells and fibroblasts from LTBI patients post mortem.[102,103] Epithelial cells express MHC-I, MR1 and CD1d and can present *M.tb* antigen to classical CD8+ T cells[104] and donor-unrestricted T-cells (DURTs) such as mucosal-associated invariant T cells (MAITs).[105] They can recognise *M.tb* via PRRs, secrete reactive oxygen species (ROS), Th1 and immune modulatory cytokines, are a source of chemokines and produce ALF, hence they are likely important players in the early response against *M.tb* infection.[106,107]

1.5.3 Alveolar Macrophages (AMs)

1.5.3.1 Effector mechanisms

The role AMs play in the control of TB is likely multifaceted. They are professional phagocytes, degrading bacteria and dead tissue, maintaining homeostasis, coordinating immunologic response (pro inflammatory or immune modulatory), presenting antigen on MHC-II to activate the adaptive immune response and remodelling lung tissue architecture.[50] They are also major players in granuloma formation and the main niche in which the obligate intracellular mycobacteria reside.[49,50]

Tissue resident macrophages inhabit the lung tissue for life having been populated soon after birth and, in steady state, self-regenerate, independent of bone marrow stem cells.[108] AMs reside in the alveoli and are regulated by epithelial cells, where they are trained to a non-inflammatory homeostasis on exposure to harmless foreign particles, while still responding appropriately to pathogens.[109,110] They are also found in the lung interstitium (interstitial alveolar macrophages), along with undifferentiated monocytes, resident T cells, DCs, and B cells.[109,111]

AMs are supplemented by bone-marrow derived monocytes which travel into the lung following an inflammatory insult and differentiate into macrophages (Monocyte derived AMs, MoAMs, or non-resident AMs).[112] Other mucosal MPs include a CD14+ monocyte population, either resident or recently blood derived, and DCs.[113]

AMs are traditionally classified by activation state, a polarisation that mimics Th1 and Th2 function. “M1” or Classically Activated AMs, induced by IFN-y and TLR ligands are high expressers of CD86hi (TCR co-receptor) and HLA-DR (MHC-II antigen presentation), release pro-inflammatory cytokines and chemokines and display enhanced bacterial killing.[111,114] “M2” or Alternatively Activated AMs, defined by strong expression of two PRRs, $CD206^{hi}$ (C-type lectin mannose receptor) and CD14, are induced by Th2 cytokines, express anti-inflammatory cytokines IL-10 and TGF-β, and promote tissue remodelling and repair.[110,111,115]

AM phenotype has largely been defined by mouse studies, however with the advent of transcriptomics, the origin of human alveolar macrophages and the differing lineages in human populations are being explored.[116-118] Importantly, while the M1 M2 paradigm has been useful in understanding the varied functions of AMS, in reality, macrophages seem to switch between these two phenotypes or display mixed morphology depending on the

environmental cues.[116,119,120] In addition, macrophages derived from monocytes *in-vitro*, where most of our understanding of human macrophage biology derives, display different morphology than *in-vivo* tissue resident macrophages.[118]

1.5.3.2 Response to *M.tb*

In the mouse model, lung resident macrophages are the initial cells that *M.tb* encounters.[103] PRRs on the AM recognise PAMPS on *M.tb* and phagocytose the bacteria. The main PRRs include C-type lectins (mannose receptor (CD206), DC-SIGN), opsonising receptors (complement and Fc receptors) and others such as Toll like receptors (TLRs), NODs, CD36 and MARCO.[57]

Ordinarily, after phagocytosis the phagosome matures and fuses with the lysosome. Intralysosomal acidic hydrolases, antimicrobial peptides and oxygen and nitrogen metabolites kill the organism.[121] *M.tb* ligands are subsequently processed and loaded onto MHC-II or CD1, to activate CD4+ helper T cells, or non-classically restricted T cells, respectively. Phagocytosis also activates the macrophage in a positive feedback loop. For example, TLR-2 recognises *M.tb* cell wall glycolipids, inducing AM-derived IL-1β, which aids intracellular *M.tb* growth inhibition *in-vitro*; [122] while CD206 recognises lipoarabinomannan, which, in concert with TLR-2 activation, leads to production of IL-8, responsible for neutrophil recruitment, while also regulating inflammation through COX-2 production.[50] Ligation of AM receptors results in downstream activation of MyD88 and IRAK cascades leading to transcription of interferon regulatory factor (IRF) and nuclear factor (NF)-kb, with production of IL-1β, IL-12 and TNFα.[123] Other PRRs such as Minicle, which recognises *M.tb* glycolipid trehalose dimycolate, can result in Th17 polarised cytokine production and induces Nitric oxide (NO) production and antimicrobial peptides (a-defensins, matrix metalloproteases, lactoferrin and b defensins).[124] In addition, complement receptor 3 is able to directly detect *M.tb* antigen, further activating

phagocytosis.[125,126] If phagolysosomal killing is not possible, the bacterium may also undergo apoptosis, aided by TNFα, which enables mycobacterial killing.[126,127] At this point, effective elimination of the bacteria is presumably reliant on the bactericidal action of the macrophages.

1.5.3.3 *M.tb* immune evasion of AMs

In most people *M.tb* establishes chronic infection, and in some cases active disease, despite a robust Th1 CD4+ T cell response. An inability to clear infection may therefore not be due to a lack of an effective Th1-mediated immune response, but rather perhaps an intrinsic deficiency in macrophages to respond appropriately to Th1 signals.[34] Understanding the role of these cells, which are the primary infective niche of the pathogen is critical.

Animal models demonstrate that *M.tb* evades killing and successfully resides within a non-acidic late endosomal vacuole, replicating within the macrophage. *M.tb* prevents maturation of the phagosome and fusion with the lysosome.[128,129] The bacterium either lies dormant, or freely replicates within the phagosome, and ultimately escapes the phagosome through lysis.[129] Once it enters the cytosol, detection of foreign antigen PAMPs triggers cell necrosis with subsequent expulsion of pathogenic bacteria free to repeat the cycle in other macrophages.[129,130] Here the bacterium takes advantage of the recruitment of monocytes from chemokines derived from activated AMs. Monocytes are less able to kill intracellular *M.tb,* and hence infection of monocytes by *M.tb* promotes *M.tb* dissemination.[131] In addition, in their tagged *M.tb* mouse infection model, Cohen and colleagues demonstrate that *M.tb* manipulate infected AMs through the *M.tb* early secretory antigenic target (ESAT-6), to carry the bacteria into the interstitium where they are able to establish infection.[103]

ESAT-6 also inhibits the lysosome fusion of the phagosome in the AM.[132,133] In their SMARTPool human whole-genome small interfering RNA screen of human macrophages co-cultured with virulent *M.tb* at 6, 24 and 48 hours, Kumar and colleagues demonstrated that this impacts the ability of the macrophage to undergo autophagy.[132] Autophagy is a key immune strategy in the hosts defence against intracellular pathogens and restoration of autophagy drastically reduces survival of *M.tb in-vivo*.[134] By allowing processing of foreign antigens through lysosome fusion, autophagy upregulates antigen presentation on MHC-II molecules, stimulating CD4+ T cell responses.[135] In addition, it appears that autophagy may also facilitate MHC-I and CD1 antigen presentation thereby inducing CD8+ T cell and CD1-restricted DURT activation.[136]

Apart from inhibiting phagolysosomal fusion, *M.tb* also reduces antigen presentation by manipulating intracellular processing and downregulation of antigen molecule trafficking and MHC transcription.[137,138] Further, *M.tb* and BCG can inhibit cellular responses to IFN-y and reduce recognition by downregulating expression of PAMPs like cell wall glyoclipids.[139]

1.5.3.4 AMs and TB protection

The evidence for the role of macrophages in protection and *M.tb* clearance is therefore mixed.[140] In some mouse models, AMs are essential for control of *M.tb*. Csf2-/- mice, which lack AMs due to an inability to produce GM-CSF, succumb to *M.tb* infection.[141,142] This appears to be partially through a CD4+ T cell dependent manner as CD4+ transfer conferred some protection. In one model of CD4+ and CD8+ T cell depleted mice, subcutaneous BCG vaccination led to protective immunity against *M.tb* challenge.[143] This occurred within 7 days, and vaccinated mice had higher levels of tissue homing macrophages.[143] In another study of T cell knockout mice, mucosal vaccination with the adenoviral vector candidate vaccine Ad-TB

led to an increase in activated AMs compared with intramuscular vaccination, which corresponded with improved *M.tb* control on challenge.[144] However AM may also worsen control. AM knockout mice infected with *M.tb* display reduced bacterial burden and robust local recruitment of cytotoxic cells, despite worse granuloma formation.[145]

It may be that only certain effector AM function is protective. Mice, which succumb to *M.tb,* only express M1 AMs 10 days after infection.[146] *M.tb* modulates macrophage response towards a more permissive M2 phenotype.[147] Mouse spleen or bone-marrow derived macrophages co-cultured with *M.tb* resulted in polarisation towards an M2 Th2 response.[148,149] Evidence from mouse and macaque *M.tb* challenge models show variable protection from AMs, likely dependent on AM effector function. Depletion of total AMs increases animal survival, but depletion of M1 AMs reduces survival and/or limits the granuloma's ability to contain the organism.[150,151] These classically activated macrophages are able to overcome the *M.tb's* strategy to inhibit lysosome fusion and successfully kill the bacteria through the combined effect of NO, exposure to the acidic environment in the lysosome and autophagy.[121] In humans, polymorphisms in the Vitamin D Receptor ApaI Gene reduces risk of TB disease. Activation of the receptor leads to transcription of genes mostly involved in macrophage activation and killing of intracellular bacteria.[152] Further, CD206 (upregulated in M2 AMs) has been associated in TB patients with poor prognosis.[153] However as CD206 is also used by the *Mycobacterium* to enter AMs, this may explain the association.

M2 polarisation may also be important in reducing pathology. A classic pathological consequence of *M.tb* infection is macrophage-mediated destruction of local lung tissue. Matrix metalloproteinases (MMPs) are responsible for degrading the pulmonary extracellular matrix which allows *M.tb* to evade the immune system, disseminate from the lung into

extra-pulmonary tissues and also enter the airway for dissemination to a new host.[154] *M.tb* induces expression of MMPs by infected macrophages *in-vivo* and are detected next to areas of tissue destruction in patients with active TB.[155,156] BCG has also been shown to induce MMP-7.[156] IL-10 or Th2 polarisation reduces MMP secretion.[155]

Hence it may be that an early robust activated M1-type phenotype is protective, while longer term chronic infection favours M2. Early elimination of *M.tb* seems critical, while the bacteria is still in the airways, before it accesses the interstitium, activates the adaptive immune response and establishes infection.[157] Hence the early macrophage airway response may influence the course of infection. What this response is in humans is yet to be elucidated.

1.5.4 Monocytes

The mononuclear phagocytes (MPs) are bone-marrow derived circulating monocytes, DCs and resident macrophages.[158,159] With the advent of RNA-seq technology this system is being re-classified and MPs may in fact come from multiple different subset progenators.[160] Three subsets of circulating monocytes are defined based on the expression of CD14 (a co-receptor for TLR4) and CD16 (the Fc gamma receptor IIIa) on flow cytometry. These are classical (CD14++, CD16−), intermediate (CD14+, CD16+), and non-classical (CD16++ CD14+, or CD16+ $CD14^{dim}$) monocytes.[161,162] Classical monocytes make up 80-95% of the circulating monocyte population.[160] They secrete proinflammatory cytokines and ROS, and migrate to inflamed tissues down the CCL2, CCL3 and CCL4 gradient (known as monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1α, and MIP-1β respectively).[160,163] Intermediate and non-classical monocytes each make up 2-10% of circulating monocytes.[161] Intermediate monocytes are specialised in antigen presentation and are migratory, but may also play a role in immune modulation.[160] Non-classical monocytes

are patrolling, terminally differentiated CX3CR1+ resident peripheral blood monocytes.[164] With their CD16+ expression, intermediate and nonclassical monocytes are involved in Fc-mediated phagocytosis.[117,165] However as a range of definitions are used for monocyte subsets, such as identifying all CD16+ monocytes together, or using other markers such as CCR2, CD36, HLA-DR, and CD11c and Tie-2, functional comparison across studies is problematic.[117,166] Work is also ongoing investigating the relationship between monocyte signatures defined by flow cytometry, mass spectroscopy or transcriptomics with *in-vivo* function.[117,165,167]

1.5.4.1 Monocytes and TB protection

An increased monocyte lymphocyte (ML) ratio in the peripheral blood is associated with risk of developing TB disease.[168-171] It is unclear if this association is due to a direct detrimental effect of monocytes on *M.tb* control. Alternatively, it could be a marker of other immunologic changes which might affect TB risk such as migration of lymphocytes away from the systemic circulation to the infection site or changes in the bone marrow increasing myelocytosis.[172,173] The proportion of CD16+ monocytes increases to 40% of total monocytes in TB patients.[174] After *M.tb* phagocytosis, non-classical monocytes upregulate CD11b and lose their HLA-DR *in-vivo*, which might suggest that *M.tb* is driving this effect to hide within these cells.[175,176] In addition, TGF-β which is increased in the blood of active TB patients and which induces CD16+ monocyte differentiation, might be being released by the host in response to a chronic infection to modulate the inflammatory response and reduce tissue destruction.[175,176] Further elucidation of these mechanisms will be discussed in Chapter 5.

1.5.5 Dendritic cells

DCs are the most efficient at antigen presentation of the MPs and hence are classically viewed as key cells connecting the innate and adaptive immune response.[177] Immature DCs which reside in tissue sites and are proficient at endocytosis, mature on detection of PAMPs by upregulating HLA-DR for efficient MHC loading and antigen presentation.[178-180] These DCs migrate to the draining lymph nodes to present antigen to the residing T cells and simultaneously produce cytokines to polarise CD4+ T cells.[181] While the cytokine milieu of the inflammatory site is known to influence Th outcome, causal mechanisms are not yet fully described.[182] DCs also produce pro-inflammatory cytokines which recruit and activate cells.[183] DCs are identified on flow cytometry by robust expression of MHC-II and CD11c, though many other cells also express these markers.[182] The classical DC subtype (cDC; also "conventional" or "myeloid") is identified on flow cytometry by CD141/CLEC9 (cDC1); or, CD1c/CCL17 (classical; cDC2); and plasmacytoid DCs (pDC) by CD123.[182] All subtypes are found in the lung, though pDCs are more common in the blood and secondary lymphoid tissue.[177,184,185]

While the subsets derive from distinct lineages, they appear to be plastic in their effector function and these different functionalities are still being explored.[184,186] cDC1s may preferentially polarise to Th1, while cDC2s appear more plastic and are able to polarise CD4+ T cells to Th1, Th2 and Th17, though they are often found in a regulatory role.[177,182] pDCs are characterised by high IFN-α/β production.[184,186]

There is limited information on the phenotype and function of DCs in humans as they are in low frequency in BAL fluid, and peripheral blood DCs may not represent what occurs in the lungs, especially as DCs reside more in tissues than the periphery.[50]

1.5.5.1 Early DC responses against *M.tb*

DCs likely play an essential role in *M.tb* control. Human DCs are activated and matured by co-culture with *M.tb* or BCG.[174,187] In addition, *M.tb* infected DCs are an important source of chemokines shown to induce NK and T cell migration to the infection site[188] and BCG-infected human DCs induced NK cell proliferation and activation, which in turn lysed DCs *in-vitro*.[187] In mice depleted of DCs, the CD4+ T cell response was impaired, subsequently resulting in an overwhelming *M.tb* infection.[189] In humans, monocyte and DC primary immunodeficiency or abrogation, such as transcriptional regulator IRF-8 mutations, results in disseminated BCG infection on intradermal vaccination.[190]

Further, *M.tb* appears to target DC function in mice. The delay in the development of a robust adaptive immune response in mouse models, postulated as a key strategy by *M.tb* to establish infection, is considered to be due to aberrant DC-mediated antigen presentation and delayed migration to the draining LNs.[48,54] In resistant C57BL/6 mice, bacteria reach the LN earlier than the susceptible C3H strain suggesting that early antigen presentation by DCs to CD4+ T cells is important for control.[191] Further, mice depleted of DCs in the spleen, resulted in delay of *M.tb*-specific CD4+ T cell activation and poorly controlled *M.tb* infection,[189] and following murine *M.tb* aerosol infection, *M.tb*-infected dendritic cells have slower tracking times to the lymph nodes than PPD-activated DCs.[148,192] Infected DCs are also poor antigen presenters, despite no apparent decrease in the amount of surface MHC-II expression.[48] This was shown to be mediated by inhibition of autophagosome-lysosome fusion by ESAT-6 with resultant reduction in IL-12 secretion and poor CD4+T cell activation.[133,193]

Whether DCs also show impaired function and delayed LN tracking following mycobacterial aerosol infection in humans is unknown but would be a key factor in our understanding of TB immune response.

1.5.6 Granulocytes

Granulocytes, are short lived innate cells defined by their large cytoplasmic granules and multilobular nucleus.[194] Eosinophils are associated with parasite defence and allergy via Th2 mechanisms, as are mast cells and basophils.[195] Mast cells and eosinophils make up 1% of leucocytes, and basophils are even less common. [194] Neutrophils comprise 50-70% of all circulating leucocytes and are an integral part of the early inflammatory immune response.[194,196]

1.5.6.1 Neutrophils

Neutrophils are professional phagocytes and migrate to the infection site within minutes, where they are able to respond to a primary *M.tb* infection by recognising bacteria coated with complement or by direct recognition of PAMPS.[197,198] On *in-vitro M.tb* co-culture, neutrophils release granules containing potent antimicrobial enzymes which kill and digest pathogens and also promotes apoptosis.[198-200] Neutrophils also express IFN-y and TNFα which enables further cell recruitment.[218]

However, the role of these neutrophil responses in the control of *M.tb* infection in humans is unclear, made more difficult as they are notoriously difficult to study due to their fragility.[201] Traditional blood processing retains only PBMCs and discards granulocytes, while their low RNA content and presence of endonucleases makes transcriptomic analysis problematic.[202,203]

After BCG intradermal or aerosol instillation in mice, neutrophils rapidly track to the site of infection and are a major early source of IFN-y and chemokines.[143,204,205] In addition, three days following BCG lobar instillation in *M.tb*-exposed volunteers, neutrophils increased in the lung.[206] Neutrophil depletion in mice leads to prolonged tracking of dendritic cells to the lymph nodes, delayed initiation of the adaptive immune response[207] and increased *M.tb* bacteria load.[208] *In-vitro*, DCs which ingest neutrophils infected with *M.tb* were better at antigen presentation than those that had ingested free bacteria.[205] Depletion or enhanced recruitment of neutrophils in mice also leads to increased or decreased BCG bacterial burden respectively.[209] Human whole blood depleted of granulocytes (CD15+), poorly controlled *M.tb* and BCG, compared with whole blood depleted of CD4+, CD8+ or CD14+ cells and this effect was reversed by adding live granulocytes.[210] Further, human neutrophils control the growth of *M.tb* within one hour of co-culture *in-vitro* with or without TNFα co-stimulation.[211,212]

This protective effect may only be early in infection. Lung neutrophilia is a hallmark of *M.tb* infection in susceptible mice.[213-215] Mice with CD4+ T cells unable to express IFN-y, overexpress IL-17 and exhibit hyperactive neutrophilia following *M.tb* aerosol challenge, with resultant tissue destruction.[216,217] Active TB in humans is defined by neutrophilia systemically and in the BAL and sputum, which reverts on treatment.[47,218] Further, IL-8, a potent neutrophil recruiter, is associated with poor outcome in TB disease[219,220] and neutrophil count is associated with worse prognosis in patient with established TB disease.[221] Hence neutrophils late in infection may display a more aberrant phenotype which correlates with risk. In a human whole blood assay, the addition of necrotic neutrophils worsened *M.tb* control and also reduced phagocytosis of BCG by macrophages, whereas live neutrophils improved control.[210] This finding has been supported in other studies.[222,223]

By contrast, a higher initial neutrophil count is protective against risk of developing LTBI in healthy contacts of TB patients, supporting the hypothesis of a protective effect early in infection.[211] These contacts also have higher neutrophil counts than unexposed controls. Additionally, neutrophil counts fall by 2-6 weeks post-exposure in healthy TB "resistors".[89,211] Hence neutrophil tracking to the lung may be essential for early control of disease, but prolonged inflammation with an accumulation of necrotic hyperactivated neutrophils associated lung tissue destruction may facilitate the bacteria's ability to persist. Early neutrophil responses in the naïve human airway have yet to be described.

1.5.6.2 Eosinophils

The evidence of any role for eosinophils in TB is limited. Eosinophils don't seem to be infected with *M.tb* and the biological mechanism for eosinophil effector function against this intracellular organism, if any, remains unclear.[103,224] However a recent review of 3 case reports and 3 independent clinical studies of active TB patients demonstrated depletion in the periphery of eosinophils compared with LTBI patients and an association between uncontrolled TB disease and a decreased systemic eosinophil to neutrophil ratio.[225] The same group showed that mice depleted of eosinophils had higher *M.tb* bacterial loads at 2 months compared to wildtype mice. An influx of eosinophils into the airways and lungs of *M.tb*-infected guinea pigs has also been described, and eosinophils are present in TB granulomas in humans and NHPs.[226,225] If eosinophils are enriched in the lung tissue of active TB patients and are active in granulomas this could indicate a tissue destructive, necrotic, non-protective role for eosinophils locally, and perhaps explain the association with low systemic eosinophil count and a controlling host response in LTBI.

1.5.7 NK cells

Natural killer (NK) cells are cytotoxic CD3- innate lymphocytes, from the same lymphoid progenitor as T and B cells.[227] They play an important role in defence against cancers, viruses and intracellular pathogens.[227] $CD56^{dim}CD16^{+}$ are more cytotoxic against *M.tb* while $CD56^{bright}CD16^{-}$ cells are robust cytokine producers.[228,229] Human NK cells recognise *M.tb* infected cells *in-vitro* and induce apoptosis within 24 hours, independent of IFN-γ, using perforin and granzyme proteases.[230,231] NK cell activation on *M.tb* co-culture is via MP-derived IL-12, IL-18 and IL-15,[229] and the balance between recognition of cognate MHC-I by the NK cell inhibitory Killer-cell immunoglobulin receptors (KIRs) and ligation of as yet unknown *M.tb* ligands and/or IgG by the NK cell's natural cytotoxicity receptors (NCRs) NKp46, NKp44 and NKp30, or by CD16/FcγIIIA.[232,233] NK cells can also bind directly to *M.tb* via TLR2.[234] NK cells secrete Th1 cytokines IFN-γ and TNFα, which activate AM effector function; and NK cells produce IL-22 which controls *M.tb* growth by facilitating phagolysosomal fusion.[232,235] NK cells are also able to expand other cell types such as γδ T cells and CD8+ T cells and they can restrict expansion of T regs via the NKG2D ligand.[227]

NK cells share similar markers and effector functions as Type 1 innate lymphoid cells (ILC1).[236] ILC1 however seem to be restricted to local mucosal tissues whereas NK cells are also able to circulate.[237] Both cell types release IFN-γ, however ILCs have a limited capacity to kill cells as they lack granzyme and perforin.[238] For the purposes of my thesis I have defined all CD56+ CD3- cells as NK cells, though these populations may also include ILC1.

1.5.7.1 NK cells and TB protection

While NK cells alone do not seem to be necessary for protection against *M.tb* they do seem to play an important role. In mouse studies IFN-y+ NK cells appear in the lung 10-15 days following primary infection with *Mycobacteria* spp. and 7 days in mice primed with BCG.[143,239] In a C57BL/6 mice *M.tb* challenge model, depletion of NK cells alone did not increase susceptibility, however depletion of both NK cells and T cells increased susceptibility to TB compared with depletion of T cells alone.[239] Adoptive transfer of NK cells reduced bacterial load.[239]

In humans, risk of progression to active TB disease is correlated with preceding low circulating NK cells and dysfunctional peripheral NK cells (defined as low levels of $CD56^{bright}$ NK cells, reduced IFN-y production and decreased NKp30 and NKp46 expression), which improved on treatment.[240-243] By contrast, healthy persistently IGRA- contacts had more cytotoxic NK cells than IGRA+ contacts.[244]
However, locally, the reverse is seen. NK cells are increased in the lung of active TB patients[240,245] and NK cells in pleural fluid are the major source of IFN-y and IL-22 production on *ex-vivo M.tb* stimulation.[235] Whether this demonstrates a protective immune response, given these are patients with active TB disease, needs to be determined.

In mouse models, NK cells seem necessary in *M.tb* control, but only in the context of IFN-y+ CD4+ T cells, and in TB patients, functional NK cells in the periphery may be important. However what role NK cells play in the lung early in infection is unknown.

1.5.8 Donor unrestricted T cells (DURTs)

DURTs are a group of innate CD3+ T cells which respond to MHC-independent non-peptide epitopes and hence can recognise the glycolipid rich *M.tb* wall and other PAMPS.[246] They could be an important player in early immune protection, acting as the gap between the innate and adaptive response, however their protective potential in *M.tb* is unclear.[247] They include Mucosal-associated invariant T (MAIT) cells and the CD1-restricted T cells Natural Killer T (NKT) cells and γδ T cells. [248,249]

1.5.8.1 DURTs: MAIT cells

MAIT cells typically express a semi-invariant TCRα, with Vα7.2 (TRAV1-2) paired with a variable J and variable β chain. They recognise riboflavin metabolites from bacteria such as *M.tb,* presented on the MHC-I related-1 molecule, MR1.[248,249] They can also respond in an MR1 independent manner via cytokine stimulation.[250] MAIT cells are prevalent in humans but are less common in mice hence research into this cell type is difficult.[251] MAIT cells are enriched in mucosal compartments (20% of CD8+ T cells in the airway) and so they are well placed to act early against *M.tb* infection.[252] CD8+ MAITs are the most common of the CD4+/CD8+ MAIT subsets in blood.[249] MAITs have Th1, Th17 and cytotoxic effector function and can recruit innate and adaptive cells to infection site.[249] In *in-vitro* experiments, human MAIT cells inhibit the growth of BCG within AMs in an MR1 and Th1 cytokine dependent manner and also lyse *M.tb* infected cells via perforin and granzyme.[105]

1.5.8.1.1 MAIT cells and TB protection

In mice and NHPs, there is a transient accumulation of MAITs in the airway by 10-15 days after *M.tb* or BCG airway infection.[253,254] A recent study by Sakai et al. investigated a 5-OP-RU

vaccination, which drives MAIT expansion.[255] MAITs reduced mouse survival early in infection, by contributing to delayed CD4+ T cell priming. However, MAITs may reduce bacterial load in chronic disease, in an IL-17 dependent manner.[255]

There are variable reports about the frequency of MAIT cells in active TB and LTBI patients with some studies finding an inverse correlation with TB risk and others reporting no association.[256-258] The inconsistencies in findings may be due to classification of controls or differences in populations studied. Further, MAITs appear enriched in the lung in active TB but may have blunted effector function.[257,259]

Gene association studies also suggest a role for MAITs in *M.tb* infection. The MR1 polymorphism rs1052632 is associated with increased susceptibility to TB in a Vietnamese cohort,[260] while patients completely lacking RORC, which results in deficiency of MAIT, iNKT and Th17 cells are susceptible to BCG-osis and *M.tb* disease.[261]

Exposed but uninfected household contacts of active TB patients have been variably reported as having decreased circulating IL-17+CD8+ MAIT cells or increased Granzyme B and IFN-γ+ effector MAITs compared to contacts who converted.[262,263]

Studies in TB patients, early clearers and from population genetics therefore indicate a role for MAITs in *M.tb* infection, though whether MAITs confer protection is unclear.

1.5.8.2 DURTs: iNKTs

Cluster of Differentiation 1 (CD1) molecules are a group of MHC-like lipid or glycolipid antigen presenting, limited-polymorphic molecules which are recognised by CD1-restricted T cells and are mainly expressed on MPs.[246,264] Some of these CD1-restricted T cells express γδ TCRs, though the majority express αβ TCRs. All Group 1 CD1 molecules (CD1a, CD1b and CD1c) bind to *M.tb* lipid antigens *in-vitro*.[265,266] Group 1 CD1-restricted T cells have adaptive-like

functionality and expand in patients with mycobacterial infection or following BCG vaccination.[267]

However as Group 1 CD1 molecules are not found in mice, and guinea pigs express limited isoforms,[268] research has focussed on the Group 2 CD1d restricted T cells, which are abundant in mice.[269-271] Natural Killer T cells (NKTs) are innate non-classically restricted CD3+ CD56+ T cells.[296] The invariant or Type 1 "iNKT" expresses the TCR Vα24-Jα18 with Vβ11 and recognises CD1d presenting glycolipids, a component of the mycobacterial cell wall.[265,296] They are more frequent in mice than humans, making up as low as 0.2% circulating human T cells.[298] Of the CD8/CD4 iNKT subsets, CD4+ and double negative (DN) NKTs are the most prevalent in the blood.[271] NKTs mirror classic Th cell effector function, can display a cytotoxic phenotype and can be activated in a TCR-independent manner.[269-271] They tend to be non-circulating tissue resident cells, enriched in the liver and lung.

CD4+ and CD4- iNKTs, derived from mouse splenocytes, recognise *M.tb*-infected macrophages and restrict mycobacterial growth, which may be through CD1d dependent GM-CSF production.[272,273]

1.5.8.2.1 CD1 cells and TB protection

While there is no difference in survival to *M.tb* challenge in CD1d-/- mice compared with wildtype; purified iNKT adoptive transfer, or iNKTs activated by the super glycolipid antigen α-galactosylceramide confers protection.[272,274] Further, iNKT cells are enriched in early granuloma formation in mice.[275] In NHPs, CD8+ iNKTs are correlated with protection against *M.tb* challenge.[276]

In humans, iNKTs are decreased in the blood of active TB patients compared to healthy contacts or LTBI patients[170,277] and iNKT levels are present at even lower frequencies in the pleura of active TB patients than in the periphery.[278] However both peripheral and pleural

iNKTs from active TB patients display increased *M.tb*-specific Th1, Th17 and effector memory phenotypes compared with healthy controls.[278-280] By contrast, early in infection there is no difference seen in peripheral blood iNKT frequency or activation in contacts (IGRA- or IGRA converters) compared with healthy controls.[263]

Overall while there may be an association between iNKTs and TB in humans, the evidence for protection is lacking. This may be because there is a paucity of information about lung mucosal iNKTs responses, especially early in infection.

1.5.8.3 DURTs: γδ T cells

γδ T cells make up 5% of all CD3+ T cells in human peripheral blood.[281,282] The antigens γδ T cells recognise are largely unknown, but they can recognise antigens presented independent of the MHC receptor, are able to recognise lipids presented by MPs via CD1d and may be able to respond to whole antigen protein.[306] γδ T cells can express Th1 or Th17 cytokines and kill cells via perforin and granzyme.[307] They have been shown to interact with epithelial cells, AMs, neutrophils and B cells and can induce antigen presentation by DCs.[308] They may also be able to phagocytose.[283,284] They are classified into two main subsets. Vδ2Vγ9 cells are the most common type in human peripheral blood, have an innate-like and T-effector memory (TEM) phenotype, and can recognise phosphoantigen from *M.tb*.[285,286] The non-Vδ2s, Vδ1s and Vδ3s, display a more adaptive phenotype.[287]

γδ T cells recognise a range of *M.tb* and BCG antigens and the TCR-γ and TCR-δ repertoire expands in patients with LTBI or active TB compared to healthy controls.[288]

1.5.8.3.1 γδ T cells and TB protection

In the murine model, Th1 and Th17 γδ T cells track to the lung and draining lymph nodes following aerosol *M.tb* infection.[289] Mice depleted of γδ T cells have an initial high *M.tb* load but control infection better at later timepoints than wildtype mice.[290] Chen and colleagues demonstrated an increase of Vδ2Vγ9 T cells in lung and peripheral blood following IV BCG in NHPs at 4 weeks.[291] This was associated with protection to subsequent *M.tb* challenge.[291] Further, artificial expansion of this cell population resulted in Vδ2Vγ9 T cells tracking to the lung and reducing *M.tb* burden in NHPs.[292] These cells produced IFN-γ, perforin, granulysin, IL-12 and enhanced *M.tb*-specific CD4+ and CD8+ α/β Th1 T cells in the lung.[292]
In infants following BCG intradermal vaccination, NK cells and DN γδ T cells were the highest producers of BCG-specific IFN-γ in blood at 10 weeks.[293] Peripheral cytotoxic γδ T cell response is associated with TB disease risk and progression in healthcare workers compared with healthy contacts; while IL-17+ γδ T cells are increased in active TB patients compared with controls.[294,295] CD4+ γδ T cells expand in latency compared with IGRA- contacts and controls and these cell types have reduced activation on *ex-vivo* stimulation.[263] In this study, there was no difference in γδ T cells between IGRA- contacts and controls.

Taken together, the evidence for a possible role for γδ T cell protection in animal and human models is mixed. As with iNKTs, information about early γδ T cell responses in the human airways is lacking.

1.5.9 Trained immunity

Epigenetic re-programming is an evolutionarily conserved phenomenon present in every higher order living organism from plants through to mammals, which occurs through DNA

transcriptional start site methylation and histone changes.[296,297] Due to an association between BCG vaccination and reduced childhood mortality, BCG has long been studied for its putative role in conferring protection against unrelated pathogens.[298] More recently, the potential for innate "trained immunity" to confer homologous protection against *M.tb* has undergone intense scrutiny. In a series of elegant experiments, Netea's group demonstrated that BCG confers training on mouse and human monocytes, and that subsequent protection against *M.tb* or unrelated pathogens was independent of an adaptive response.[94,299] There is also some evidence for BCG-induced training of NK cells and long term expansion of DURT effector subsets.[94,300]

However to my knowledge there is currently no evidence of trained human lung resident macrophages following mycobacterial exposure.[301] Given the short life span of circulating MPs, epigenetic changes seem dependent on bone marrow progenitor cells and may rely on the presence of BCG in the bone marrow.[302] AMs are long lived and self-perpetuate hence different mechanisms may apply.[303] In fact in a recent analysis of the training effect of protective low dose aerosol BCG in NHPs, BAL cells failed to show evidence of training.[304] A recent study of BCG aerosol infection in mice demonstrated improved killing by AMs on second exposure.[305] Training of mouse AMs has been demonstrated from 4 weeks following viral infections however this was sustained independent of bone marrow-derived or circulating monocytes and was dependent on T cell-derived IFN-y.[306]

While the exact mechanisms by which this trained immunity might confer protection are unclear it does appear this may be a mechanism through which "early clearers" may be protected against *M.tb* infection, and could be an important target for vaccine design. The development of trained immunity seems to peak between 4-12 weeks and has only been

investigated from 2 weeks following Mycobacterial exposure.[94,302] What early immune processes might contribute to the development of this training are as yet unknown and warrant further investigation.

1.6 Cytokines

1.6.1 Th1 and Th2 axis

Animal and human studies have demonstrated the critical function of cytokines in *M.tb* disease, though the role in early infection is less clear. A seminal study in Africa showed that patients with active disease were polarised towards a Th2 response.[307] Conversely household contacts who did not become infected and hence presumably were able to clear or control the infection had low levels of Th2 and high levels of Th1 compared to both infected patients and uninfected controls.[307] CD4+ T cells polarise to five principle effector lineages; Th1, Th2, Th17, Tregulatory (Treg) or Tfollicular (Tfh); with subsequent downstream adaptive cellular responses (also see Table 1).[308] IL-12 from MPs co-presenting antigen on MHC-II switches naïve CD4+ T cells to a Th1 helper phenotype which produce large amount of IFN-y, TNFα and IL-2, with subsequent activation of CD8+ cytotoxic T cells. Conversely IL-6 production induces a Th2 helper profile, with IL-4, IL-5 and IL-13 production and induction of mast cell, eosinophil, basophil activation and IgE, traditionally thought to be protective against helminths, and also implicated in allergy.[309] FoxP3+ Tregs produce IL-10 and TGF-β1 and are associated with immune dampening and self-tolerance, while Tfh stimulate B cells and antibody production.[308,309]

Table 1.1. Current understanding of CD4+ T cell lineages. Reproduced with permission from [308]

T cell subset	Phenotype	Characteristic cytokines	Characteristic transcription factors	Function
Naïve	$CD45RA^+CCR7^+$	IL-2		Precursor cells, protection against new pathogens
T_{CM} (central memory)	$CD45RA^-CCR7^+$	IL-2, IL-21		Secondary expansions, help
T_{EM} (effector memory)	$CCR7^-$	IFN-γ, IL-4, IL-5, IL-17		Protection in tissues, help
T_{RM} (tissue-resident memory)	$CD103^+CD69^+$	IFN-γ		Immediate protection in tissues
T_{FH} (follicular helper)	$CXCR5^+ICOS^+$	IL-21	BCL6	B cell help
Th1	$CXCR3^+$	IFN-γ	T-bet	Protection against intracellular pathogens
Th2	$CRTH2^+$	IL-4, IL-5, IL-13	GATA-3	Protection against extracellular parasites
Th9	?	IL-9	PU.1	Protection against extracellular parasites
Th17	$CCR6^+CD161^+$	IL-17, IL-22, IL-26	RORC2	Protection against extracellular bacteria and fungi
Treg	$CD25^+CD127^-$	TGF-β	FOXP3	Maintenance of self-tolerance
Tr1 (type 1 regulatory)	$CD25^-CD127^-$ or $CD49b^+LAG3^+$	IL-10	?	Inhibition of immunopathology

However Th1 cytokines are also secreted by the innate DURTs, NK cells, T-bet+ Type I innate like lymphocytes (ILCs), and to a lesser capacity, MPs and epithelial cells, on stimulation with IL-12, IL-18 and/or IL-15.[310,311] It is unclear what contribution these cells play in this protective Th1 response against *M.tb*.

1.6.2 Th1 cytokines

1.6.2.1 IFN-γ

IFN-γ plays a critical role in TB control, independent of CD4+ T cells. Mice deficient in IFN-γ quickly succumb to *M.tb* disease, characterised by less NO2 production, indicating an essential role for macrophages.[312] Human Mendelian susceptibility to mycobacterial disease is characterised by an inability to control Mycobacterial infection, with disseminated BCG or NTM disease.[313,314] The cause has only been identified in half the cases, however all identified causes are due to a defect in one or more of 9 separate genes, each of which are involved in IFN-γ production (e.g. IRF8, IL12B, IL12RB1, NEMO which encodes Nuclear factor-kappa B (NF-κB) and CYBB which encodes NADPH) or the IFN-Receptor (e.g. STAT1, IRF8,

IFNGR1, IFNGR2).[315] However, IFN-y responses alone do not correlate with protection in mice and humans.[316,317] It is possible that the protective effect of IFN-y acts as a goldilocks phenomenon as in preclinical mouse models too much IFN-y worsens the outcome,[318] and high serum levels of IFN-y in patients with TB predict poor outcome.[319] In mice, *M.tb*-specific IFN-y CD4+ T cell production is detected 12 days after aerosol infection in the draining LNs and 14 days in the lung.[191] At what timepoint after aerosol infection this occurs in naïve humans is currently unknown.

1.6.2.2 TNFα

TNFα is essential for the production of reactive nitrogen spp in the mouse macrophage, and also aids in chemokine production, apoptosis and granuloma development in mouse and human *M.tb*-infected cells.[320-322] However *in-vitro*, TNFα may promote *M.tb* growth in human macrophages, especially in the absence of iron.[323,324] As with IFN-y, mice deficient in TNFα rapidly succumb to *M.tb* infection.[322] In humans anti-TNFα monoclonal antibody treatment results in progression to active disease from latency.[325,326] Loss of TNFα increases Treg frequency and depletes CD8+ CD45A effector memory cells (TEMRA) in patients.[327,328] While TNFα is clearly critical in controlling *M.tb*, its role in humans early in infection is yet to be established.

1.6.2.3 IL-2

IL-2 promotes T cell survival, effector function, CD8+ T cell cytotoxic capacity and development of T cell memory.[329] It also has a role in immune tolerance, maintaining CD4+CD25+ Treg cells, and, as a consequence, $IL2^{-}/^{-}$ knockout mice succumb early to autoimmune disease, limiting our ability to study the effects of *M.tb* infection.[330] In an immune fatigue mouse model with chronic *M.tb* exposure, IL-2 treatment resulted in

increased CD8+ T cell memory cells, reduced PD-1 expression, increased IFN-γ and IL-2 production and improved protection against *M.tb* infection.[331] Further, IL-2 therapy as an adjunct to antibiotic treatment in TB patients improves outcome by enhancing CD4+ T cell and NK cell effector function.[332]

1.6.3 Th17 cytokines

The Th17 cytokine profile has recently been implicated in early protection. Th17 CD4+ T cells (as well as DURTs, NK cells and to a lesser extent RORγt+ ILC3s), produce IL-17, IL-21, TNFα or IL-22 in response to IL-6, TGFβ and IL-21.[333] These cytokines activate macrophages, recruit neutrophils, through the action of IL-8, and induce antimicrobial responses in the epithelium.[333] IL-17 knockout mice are unable to form mature granulomas and have increased susceptibility to *M.tb* due to impaired inflammatory cell recruitment, while protection to *M.tb* intranasal challenge was correlated with IL-17-producing cells.[334,335] PBMCs from TB patients produce low levels of IL-17 compared to healthy controls which may be mediated by PD-1 expression.[336] Interestingly, TB cases had lower levels of IL-17, IL-13 and IL-10 from *M.tb* stimulated PBMCs, but no difference in IFN-γ or IL-12 compared with healthy and LTBI household contacts.[337]

1.6.4 Polyfunctionality

Recent studies have shown a promising protective signature for polyfunctional CD4+ T cells (producing more than one cytokine).[338-340] However, other studies report mixed effects and in a recent review, there was no definitive correlation in Th1 CD4+ T cells with protection in humans.[330] Importantly there were lower levels of polyfunctional Th1 CD4+ T cells in household contacts (TST+&TST-) than in adults with TB.[337]

In the macaque model, polyfunctional Th17+ T cells in the BAL have been correlated with the protection seen from a low dose BCG aerosol, but not after IV BCG, which is protective in these animals.[91,341] BCG revaccination in Indian adults, which may be protective in some communities, led to expansion of antigen-specific polyfunctional CD4+ T cells, particularly Th17 T cells secreting IL-17A, IL-17F, IL-22 and IL-10.[342] In this study, antigen-specific IFN-γ+ NK, NKT, and γδ T cells were also increased compared to controls.

1.6.5 IL-10

IL-10, along with TGF-β1, is an inflammatory suppressor produced by Tregs. Mycobacterial antigen (*M.tb* or BCG) activating TLR2, TLR3, TLR4, results in secretion of IL-10 by AMs, neutrophils, and epithelial cells.[343] Neutrophils can also interact with DCs to induce IL-10 production. NK cells and CD8+ T cells are also sources of IL-10. IL-10 production is controlled by IFN-γ, while PD-1 ligation of PD-L1 on DCs induces Treg expansion.[344] The role of IL-10 in protection is unclear. In *in-vitro* studies, IL-10 reduces antigen presentation and therefore enhances *M.tb* intracellular survival by inhibiting apoptosis, IL-12 enhanced phagolysosome maturation, and reducing assembly of MHC-II in the endoplasmic reticulum.[345] IL-10 deficient mice have a lower bacterial load early in *M.tb* aerosol infection, but ultimately succumb to an overwhelming infection characterised by Th1/17 responses and extensive tissue damage.[346-348] In another mouse model, early IL-10 derived from monocytes was protective whereas CD4+ and CD8+ T-cell-derived IL-10 21 days post infection, increased susceptibility.[347] Taken together this data suggest that early immune defence and *M.tb* control may be a delicate balance between pro-inflammatory Th1/Th17 responses and anti-inflammatory responses mediated through IL-10.

1.6.6 IL-1 and the inflammasome

Monocyte-derived macrophages from patients who overexpress the pro-inflammatory cytokine IL-1β show improved killing on *M.tb* co-culture.[349] However, while these patients exhibit well-characterised inflammatory disorders, they have no clear resistance against mycobacterial infections on epidemiological studies.[349] In knockout mice, lack of either IL-1α or IL-1β leads to overwhelming infection to either *M.tb* or BCG.[350] Mouse macrophages translocate *M.tb* infection to the interstitium in an IL-1 dependent manner which may aid antigen presentation in LNs but may also increase dissemination.[103]

In a TB case control study in Vietnam, protection from TB was associated with heterozygosity for the proinflammatory leukotriene LTA4H.[351] In a separate cohort study in Nepal, these LTA4H polymorphisms also protected against multibacillary leprosy.[351] The putative mechanism of action is that heterozygosity at this locus enables optimal balance of pro-inflammatory and anti-inflammatory processes within the macrophage. LTA4H mutations that favour inflammation lead to susceptibility to *M.tb* infection in the zebrafish model.[351] However two larger studies in European populations show no association.[352,353]

1.6.7 Type 1 interferons

The main Type 1 interferons, IFN-α and IFN-β, are thought to be detrimental in *M.tb* infection. They are induced when the ESX-1 secretion system from *M.tb* perforates the vacuole membrane and have also been shown to be induced by NOD2 and mitochondrial DNA and TLRs.[354] In Berry and colleagues' study of active TB, LTBI and healthy UK and South African adults, there was a positive correlation in the blood transcriptional profile of patients with active TB disease and a type 1 IFN signature.[218] Further, latently infected patients treated with IFN-α for viral hepatitis are susceptible to reactivation.[355] The source of this Type

1 IFN in *ex-vivo* analyses was neutrophils and monocytes and not CD4+ and CD8+ T cells.[356] In mice, the primary mechanism of action is believed to be via blocking IL-1.[357] Type-1 interferons may also suppress Th1 cytokines and promote early macrophage death and necrosis, increasing *M.tb* spread.[358]

1.7 Adaptive immunity

Vaccines traditionally harness the unique qualities of the adaptive system, memory and specificity, to establish lasting protection. It is likely that early immune responses modulate long-term cellular responses, and determine disease outcome.[52] Robust Th1 CD4+ T cell immune response seems critical in controlling the infection, and understanding how these and other adaptive processes develop early in infection at the mucosa could enable targeted vaccine development.

1.7.1 CD4+ T cells

CD4+ T cells are believed to be key to *M.tb* control, independent of cytokine production.[316,317] CD4+ T cells are phenotypically diverse as outlined in Table 1.1. CD4+ T cell cytokine-independent effector mechanisms include induction of apoptosis through perforin and granzymes, FAS-L, or TNFα; and stimulating CD8+ T cells, B cells and innate cells through contact dependent mechanisms.[317,359]

In their mouse model, Mogues and colleagues demonstrated that in the absence of a classical T cell response (αβ- mice), death following an *M.tb* aerosol infection was rapid (48 days) and dominated by neutrophils.[360] Mice with α/β T cells, but an inability to express MHC class II and hence limited CD4+ T cell mediated immunity, survived longer but still succumbed faster than wildtype mice.[360] CD4+ T cells are critical for protection in NHPs, as depleting

them increases bacterial load following *M.tb* challenge.[361] HIV infection in humans, which classically infects CD4+ T cells, increases susceptibility to active TB disease 26 fold,[362,363] though interestingly, risk is also increased in these patients when CD4+ T cell levels are normal.[364,365]

However neither Th1 cytokines, nor *M.tb*-specific CD4+ T cells are sufficient to protect against *M.tb*.[366] αβ- mice are still able to produce IFN-γ and yet succumb to infection.[360,367] In adoptive transfer mouse studies, early transfer of *M.tb*-specific CD4+ T cells did not control *M.tb* growth, suggesting that there are other key early immune mechanisms required for growth control.[367,368]

It is unclear if improving on natural T cell immunity results in sterilising protection. The T cell response which appears systemically 6-8 weeks following TB exposure is used as a marker of ongoing infection.[369-371] A randomised, placebo-controlled phase 2b trial boosting BCG with the viral vector candidate MVA-85A vaccine in infants resulted in robust CD4+ Th1 cellular immune responses and yet failed to improve protection conferred over BCG alone.[25] In fact activated HLA-DR+ CD4+ T cells have been correlated with risk of progression to TB disease in these infants.[372] These cells were also increased in the BAL and blood of active TB patients, and in the blood they displayed a TNFα+ cytotoxic phenotype and were a marker of recent antigen-induced expansion.[373,374] Abrogation of PD-1 in mice resulted in strong CD4+ T cell responses which mediated increased bacterial burden and early mortality.[375] Human T cell epitopes are largely directed at genes essential for *M.tb* survival, suggesting that evasion of T cell recognition is not a major driving factor for *M.tb*.[376] Hence *M.tb* may manipulate CD4+ responses early in infection to allow for a permissive environment and avoid clearance.

1.7.2 CD8+ T cells

Class I MHC-restricted αIβ CD8+ T cells detect intracellular pathogens through surveillance of MHC-I. They should therefore play a critical role in *M.tb* defence, particularly by overcoming *M.tb* immune evasion strategies by identifying *M.tb* antigen presented via MHC-I on infected MPs which have dysregulated MHC-II expression, or by detecting infection in cells which don't express MHC-II.[137,377] In mice, CD8+ T cells appear at the same time as CD4+ T cells after aerosol *M.tb* infection, and directly kill *M.tb* –infected cells through lysis or apoptosis *in-vitro*.[378,379] *M.tb* activated human and mouse CD8+ T cells on *M.tb* culture are polarised towards a cytotoxic phenotype, but they can also produce Th1 and Th17 cytokines.[380-382] However, the evidence for protection is lacking. In mice, CD8+ T cell depletion does render the animal more susceptible to *M.tb* infection than wild type mice, however antigen-specific CD8+ T cell responses are not sufficient for protection in CD4-/- models.[383] In one mouse model CD8+ T cells were important in maintaining control in chronic infection only.[384]
In humans CD8+ T cell deficiency is associated with autoimmunity and not overwhelming mycobacterial infection.[385] Antigen-specific CD8+ T cells are detected in the blood of LTBI and active TB patients and in granulomas,[377] however the extent of their contribution to the overall immune response is unclear. In addition, antigen-specific cytokine producing CD8+ cells are insignificant compared with the CD4+ T cell response following viral vector *M.tb* candidate vaccination.[386,387] It may be that CD8+ T cells are poorly activated, as the precise mechanism for loading *M.tb* antigen onto MHC-I in APC-infected cells is still debated and may rely on *M.tb* entering the cytosol or cross priming through DCs.[388]

1.7.3 Memory

This is the hallmark of adaptive immunity, enabling a rapid clearing response to secondary exposure and it is the primary immune endpoint in vaccine design. As discussed, the ability to develop a persistent effective memory response to natural TB infection is controversial as up to 20% of immunocompetent people with previous antibiotic-cleared TB are reinfected.[389,390] However developing *M.tb* protective memory responses can be induced by vaccination, as ID BCG prevents *M.tb* disease in certain populations,[6,7,10,74] and *M.tb* memory cells associated with protection have been created following vaccination in animal studies.[391,392] CD4+ and CD8+ T effector memory cells (TEM) circulate between the periphery and tissues, tissue resident memory T cells (TRM) reside permanently in peripheral tissues, and T central memory cells (TCM) are located in the bone marrow or lymph nodes and putatively act as a second line of memory defence.[393] Memory cells are characterised by lack of the naïve T cell marker CD45RA, expression of the memory isoform CD45RO, and tissue homing markers CD69 and CD103 (TRM) or LN homing marker CCR7+ (TCM; also see Table 1.1).[394]
In animal models, vaccines delivered by the lung mucosal route were more likely to induce local TEMs than systemic vaccination, while CD4+ TCMs have been associated with early clearance in a human MGIA.[395] Defining these T cell subsets in the human mucosa following aerosol mycobacterial infection may yield sought-after modifiable vaccine targets.

1.7.4 Humoral immunity and Antibodies

Due to the clear requirement for a T cell-mediated response against *M.tb,*[316,317] the humoral arm of the immune system in *M.tb* defense has historically been neglected.[396] New evidence from animal and human studies has led to renewed interest in the field.[372,396,397] Nonetheless,

to limit the scope of my thesis, early humoral immune responses were not investigated here and rather, form part of ongoing work.

1.8 Summary of literature

Figure 1.1 outlines the hypothesized human innate and adaptive airway immune mechanisms that lead to control of an aerosol *M.tb* infection, or immune escape, as discussed in this chapter.

Table 1.2 summarises the known innate and adaptive cellular responses to mycobacterial airway infection in animal models and human TB infection studies, outlined in this introduction.

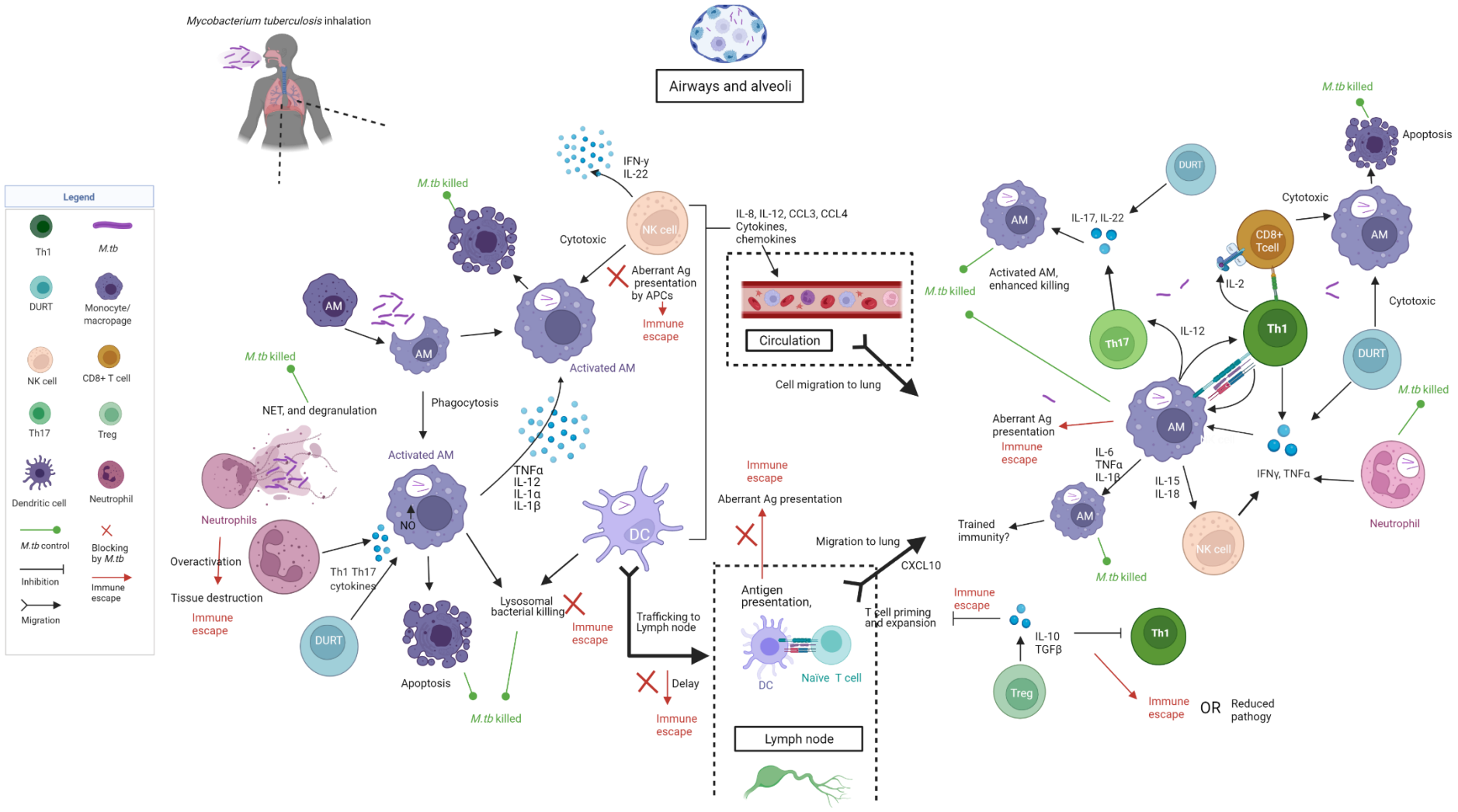


Figure 1.1. Hypothesised initial innate and adaptive response following human aerosol *M.tb* aerosol infection; outlining protective mechanisms (green) or immune escape (red). AM: Alveolar macrophage, DC: Dendritic cell, DURT: Donor unrestricted T cell, *M.tb*: *Mycobacterium tuberculosis*, NO: Nitric oxide, Th: T helper (CD4+ T cell). Created with BioRender.com

Table 1.2. Summary of innate and adaptive cellular responses to mycobacterial airway infection in animal models and human TB infection studies

Cell	Animal studies: mice and other small animal models	Animal studies: non-human primates (NHPs)	Human studies: peripheral blood and genome-wide association	Human studies: lung
Alveolar macrophages (AMs) M1/Classically activated ($CD86^{hi}$ HLA-DR+) or M2/alternatively activated ($CD206^{hi}$ CD14+)	AMs are the first to encounter *M.tb.* and they are the primary infection target of *M.tb* in mouse infection models.[48,103] On mouse *in-vitro* co-culture experiments, AMs recognise *M.tb* PAMPs and ingest the bacteria by phagocytosis. *M.tb* evades killing by inhibiting phagolysosomal fusion and preventing autophagy and reduces antigen presentation.[128,129,137,138] *M.tb* triggers cell necrosis allowing dissemination of bacteria. [129,130,134] Mouse AMs do not upregulate effector function until 10 days after *M.tb* aerosol infection.[54,146] Autophagy of *M.tb* by AMs in *in-vitro* mouse models upregulates MHC-II antigen presentation, stimulating CD4+ T cell responses; also upregulates MHC-I and CD1 antigen presentation inducing CD8+ T cell and CD1-restricted DURTs.[135,136] Mycobacteria-infected mouse AMs aid CD4+ T cell activation and recruitment and are associated with protection on *M.tb* challenge,[398] and yet AM-depleted	Depletion of total AMs increases animal survival, but depletion of M1 AMs reduces survival and/or limits the granuloma's ability to contain the organism.[150] These M1 AMs are overcome *M.tb* inhibition of lysosome fusion and kill the bacteria through the combined effect of NO, exposure to the acidic environment in the lysosome and autophagy.[121]	CD206 (upregulated in M2 AMs) has is associated with poor TB prognosis.[153] Polymorphisms in the Vitamin D Receptor ApaI Gene reduces risk of TB disease. Activation of the receptor leads to transcription of genes involved in macrophage activation and killing of intracellular bacteria.[152] Human mannose receptor activation by *M.tb* on *in-vitro* co-culture inhibits phagolysosome fusion and reduces antigen presentation.[400] *In-vitro* models have also demonstrated BCG phagocytosis, phagolysosome fusion, MHC-II processing, apoptosis, antigen presentation and Th1 polarisation by human AMs on mycobacterial co-culture.[57,121,125-127]	*M.tb* induces expression of Matrix metalloproteinases (MMPs) by infected macrophages *in-vivo* and are detected next to areas of tissue destruction in patients with active TB.[155,156] MMPs degrade the pulmonary extracellular matrix allowing *M.tb* to disseminate. AMs decrease in BAL at D3 following BCG lobar instillation in mycobacteria-exposed patients.[206] ➔ Early AM effector response to mycobacterial infection in the human airway is unknown

Cell	Animal studies: mice and other small animal models	Animal studies: non-human primates (NHPs)	Human studies: peripheral blood and genome-wide association	Human studies: lung
	mice demonstrate improved recruitment of cytotoxic cells leading to *M.tb* clearance.[145,399]			
Monocytes	Mouse monocytes are less able to kill intracellular *M.tb,* and hence infection of monocytes by *M.tb* promotes *M.tb* dissemination in mouse models.[131] In mouse and zebra fish *M.tb* infection models, mycobacteria selectively recruit permissive monocytes to the infection site.[131]	ML ratio increased in the periphery of susceptible NHP species by 12 weeks following *M.tb* infection. This was not seen in the more TB disease resistant Chinese cynomolgus macaque.[401]	Increased monocyte lymphocyte (ML) ratio in the peripheral blood is associated with risk of developing TB disease.[168-171] Monocyte primary immunodeficiency results in disseminated BCG infection.[190]	
Dendritic cells (DCs) The classical DC subtype (cDC) is identified on flow cytometry by CD141/CLEC9 (cDC1); or, CD1c/CCL17 (classical; cDC2); and plasmacytoid DCs (pDC) by CD123. All subtypes are found in the lung, though pDCs are more common in the blood and secondary lymphoid tissue.	Mice depleted of DCs are unable to mount an effective CD4+ T cell response against *M.tb* with subsequent overwhelming *M.tb* infection.[189] The delay in the development of a robust adaptive immune response in mouse models, a key *M.tb* evasion strategy, is likely due to aberrant DC-mediated antigen presentation and delayed migration to the draining LNs.[48,54,148,192] *M.tb in-vitro* co-infection of mouse PBMC-derived DCs, leads to IL-1β-induced Th2 polarisation, and worse bacterial control.[402]	DCs are required to establish an antigen-specific T cell response and control infection on *M.tb* challenge.[192] Delayed DC migration worsens outcome in an NHP model.[192,403]	Human DCs are activated and matured by co-culture with *M.tb* or BCG.[174,187] DC primary immunodeficiency or abrogation results in disseminated BCG infection on intradermal vaccination.[190] Human peripheral DCs co-cultured with *M.tb in-vitro*, phagocytose the bacteria, mature, upregulate HLA-DR expression and induce a Th1 phenotype in CD4+ T cells.[181,183] However, *M.tb* has also been shown to inhibit autophagosome-lysosome fusion in DCs derived from the human circulation, with resultant reduction in IL-12 secretion and poor CD4+ T cell activation on co-culture.[133]	There is limited information on the phenotype and function of lung DCs in humans as they are in low frequency in BAL fluid, and peripheral blood DCs may not represent what occurs in the lungs, especially as DCs reside more in tissues than the periphery.[50] No change in BAL DC proportion at D3 following BCG lobar instillation in mycobacteria-exposed patients.[206] ➔ Early DC effector response to mycobacterial infection in the humans airways is unknown

Cell	Animal studies: mice and other small animal models	Animal studies: non-human primates (NHPs)	Human studies: peripheral blood and genome-wide association	Human studies: lung
			pDCs from human PBMCs cultured with BCG show upregulation of maturation markers and granzyme B in response to cDC activation.[404] pDCs in turn promote cDC IL-1β production, which improves growth control.[404]	
Neutrophils	After BCG intradermal or aerosol instillation in mice, neutrophils rapidly track to the site of infection and are a major early source of IFN-γ and chemokines.[143,204,205] Neutrophil depletion in mice leads to prolonged tracking of dendritic cells to the lymph nodes, delayed initiation of the adaptive immune response[207] and increased *M.tb* and/or BCG bacteria load.[208,209] Enhanced neutrophil recruitment decreases BCG bacterial burden.[209] However, lung neutrophilia is a hallmark of *M.tb* infection in susceptible mice.[213-215]	Higher neutrophil counts within granulomas are associated with greater *M.tb* burden and poor outcome in the NHP model.[405,406] However neutrophil-derived TNFα is associated with protection both early and late in infection and influences adaptive immune responses.[406]	On *in-vitro M.tb* co-culture, human peripherally derived neutrophils kill *M.tb* within one hour and promote apoptosis.[198-200,211,212] Human whole blood depleted of granulocytes, poorly control *M.tb* and BCG, compared with whole blood depleted of CD4+, CD8+ or CD14+ cells. This effect is reversed by adding live granulocytes.[210] A higher initial neutrophil count is protective against risk of developing LTBI in healthy contacts of TB patients.[211] Healthy contacts then demonstrate a fall in neutrophil count by 2- 6 weeks post putative exposure.[89,211] However, IL-8, a potent neutrophil recruiter, is associated with poor outcome in TB disease[219,220] and high neutrophil count is associated with worse prognosis in patients with established TB disease.[221]	Neutrophils increase in the airway by D3 post BCG lobar instillation in historically *M.tb*-exposed volunteers.[206] Active TB disease is defined by neutrophilia in the BAL and sputum, which reverts on treatment.[218] ➔ Information about early neutrophil responses in the *M.tb* naïve human airways is lacking.
Eosinophils	Mice depleted of eosinophils have a higher *M.tb* bacterial load at 2	Eosinophils are present in TB granulomas in NHPs.[225]	Patients with active TB disease have depleted peripheral eosinophils compared with LTBI patients, and	Eosinophils are present in human TB granulomas.[47,225]

Cell	Animal studies: mice and other small animal models	Animal studies: non-human primates (NHPs)	Human studies: peripheral blood and genome-wide association	Human studies: lung
	months post *M.tb* infection than wildtype mice.[225] *M.tb* infection leads to influx of eosinophils into the airways and lungs of guinea pigs from 11 days.[226] Eosinophils are found in zebra fish *M.marinum* granulomas.[225]		levels increase within 2 weeks of commencement of TB treatment.[225] Low peripheral eosinophil count correlates with TB disease severity.[407]	➔ Information about early eosinophil responses in the human airways is lacking.
NK cells $CD56^{dim}CD16^{+}$ are more cytotoxic against *M.tb* while $CD56^{bright}CD16^{-}$ cells are robust cytokine producers.[228,229]	IFN-γ+ NK cells appear in the lung 10-15 days following primary infection with *Mycobacteria* spp. and 7 days in mice primed with BCG.[143,239] In a C57BL/6 mice *M.tb* challenge model, depletion of NK cells alone did not increase susceptibility, however depletion of both NK cells and T cells increased susceptibility to TB compared with depletion of T cells alone.[239] Adoptive transfer of NK cells reduced bacterial load.[239]	No change in total NK cell frequency in BAL following aerosol or IV BCG infection in NHPs, (earliest timepoint investigated (2 weeks).[91] Increase in total NK cells and CD16+CD27+NK cell subset in the lungs of LTBI NHPs following *M.tb* aerosol challenge.[408]	Human NK cells recognise *M.tb* infected cells *in-vitro* and induce apoptosis within 24 hours, using perforin and granzyme proteases.[230,231] *In-vitro M.tb* co-culture NK cells secrete Th1 cytokines IFN-γ and TNFα, which activate AM effector function; and NK cells produce IL-22 which controls *M.tb* growth by facilitating phagolysosomal fusion.[232,235] Risk of progression to active TB disease is correlated with preceding low circulating NK cells and dysfunctional peripheral NK cells (low levels of $CD56^{bright}$ NK cells, reduced IFN-γ production), which improves on treatment.[240-243] Healthy persistently IGRA- contacts have more cytotoxic NK cells than IGRA+ contacts.[244]	NK cells are increased in the lung of active TB patients[240,245] and NK cells in pleural fluid are the major source of IFN-γ and IL-22 production on *ex-vivo M.tb* stimulation.[235] In human lobar instillation studies, BAL NK cell count do not change after PPD instillation at D2 or D3 in mycobacteria-exposed or naive patients.[206,409,410] However, there is a trend for increased NK cells post-BCG instillation at D3 (mycobacteria-exposed patients).[206] ➔ Early NK cell effector response to mycobacterial infection in the human airway is unknown
DURTs: MAITs	In mice there is a transient accumulation of MAITs in the	MAITs increase in the airway by 10-15 days after *M.tb* or BCG airway infection.[253,254]	In *in-vitro* experiments, human MAIT cells inhibit the growth of BCG within AMs in an MR1 and Th1 cytokine	MAITs are enriched in the lung in active TB but may have blunted effector function.[257,259]

Cell	Animal studies: mice and other small animal models	Animal studies: non-human primates (NHPs)	Human studies: peripheral blood and genome-wide association	Human studies: lung
	airway by 10-15 days after *M.tb* or BCG airway infection.[253] MAITs reduced mouse survival early in infection, by contributing to delayed CD4+ T cell priming. However, MAITs may reduce bacterial load in chronic disease, in an IL-17 dependent manner.[255]	GranzymeB+IFN-γ+IL-17+TNFα+ MAITs in the airway at 3 weeks post-*M.tb* infection are associated with control to latency.[254] There is no difference in circulating MAIT frequencies following aerosol BCG infection.[91,411]	dependent manner and lyse *M.tb* infected cells via perforin and granzyme.[105] Variable reports about the frequency of MAIT cells in active TB and LTBI patients with some studies finding an inverse correlation with TB risk and others reporting no association.[256-258] Exposed but uninfected household contacts of active TB patients are variably reported as having decreased circulating IL-17+CD8+ MAIT cells or increased Granzyme B and IFN-γ+ effector MAITs compared to contacts who convert.[262,263]	➔ Information about early MAIT cell responses in the human airways is lacking.
DURTs: iNKTs	CD4+ and CD4- iNKTs, derived from mouse splenocytes, recognise *M.tb*-infected macrophages and restrict mycobacterial growth, which may be through CD1d dependent GM-CSF production.[272,273] There is no difference in survival against *M.tb* challenge in CD1d-/- mice compared with wildtype, however purified iNKT adoptive transfer, or iNKTs activated by the super glycolipid antigen α-galactosylceramide, confers protection.[272,274]	Circulating CD8+ iNKTs correlate with protection against *M.tb* challenge.[276] No change in the frequency of total circulating iNKTs in the blood 4 weeks post aerosol or IV BCG, though however the frequency of T_{EM} and T_{RM} circulating iNKTs increases.[91,411]	iNKTs are decreased in the blood of active TB patients compared to healthy contacts or LTBI patients[170,277] Active TB patients have less Double Negative iNKTs and more CD4+ iNKTs in the blood compared with controls.[271] Early in infection there is no difference seen in peripheral blood iNKT frequency or activation in contacts (IGRA- or IGRA converters) compared with healthy controls.[263]	iNKT levels are present at low frequencies in the pleura of active TB patients compared with the periphery.[278] ➔ Information about early iNKT responses in the human airways is lacking.

Cell	Animal studies: mice and other small animal models	Animal studies: non-human primates (NHPs)	Human studies: peripheral blood and genome-wide association	Human studies: lung
	iNKT cells are enriched in early granuloma formation in mice.[275]			
DURTs: γδ T cells Vδ2Vγ9 cells are the most common type in human peripheral blood, have an innate-like and T-effector memory (TEM) phenotype, and can recognise phosphoantigen from *M.tb*.[285,286] The non-Vδ2s, Vδ1s and Vδ3s, display a more adaptive phenotype.[287]	Th1 and Th17 γδ T cells track to the lung and draining lymph nodes following aerosol *M.tb* infection.[289] Mice depleted of γδ T cells have an initial high *M.tb* load but control infection better at later timepoints than wildtype mice.[290]	There is an increase of Vδ2Vγ9 T cells in lung and peripheral blood following IV BCG in NHPs at 4 weeks.[291] This is associated with protection to subsequent *M.tb* challenge.[291] *M.tb* exposed NHP Vγ2Vδ2 T cells produce Th1 cytokines, perforin and Granulysin in the lung.[292]	γδ T cells recognise a range of *M.tb* and BCG antigens and the TCR-γ and TCR-δ repertoire expands in patients with LTBI or active TB compared to healthy controls.[288] In infants following BCG intradermal vaccination, NK cells and DN γδ T cells were the highest producers of BCG-specific IFN-γ in blood at 10 weeks.[293] Peripheral cytotoxic γδ T cell response is associated with TB disease risk and progression in healthcare workers compared with healthy contacts; while IL-17+ γδ T cells are increased in active TB patients compared with controls.[294,295] CD4+ γδ T cells expand in latency compared with IGRA- contacts and controls and these cell types have reduced activation on *ex-vivo* stimulation.[263] In this study, there was no difference in γδ T cells between IGRA- contacts and controls.	➔ Information about early γδ T cell responses in the human airways is lacking.
CD4+ T cells and Th1 cytokines	CD4+ T cell deficiency in mice increases *M.tb* burden and mortality.[316,360,412] Adoptive transfer of CD4+ T cells from immunised mice protects	NHPs deficient in CD4+ T cells have worse *M.tb* disease outcome and increased bacterial burden compared with controls.[420]	HIV co-infection which decreases CD4+ T cell number and function increases risk of progression from LTBI to TB disease and CD4+ T cell	Total BAL cells contained 19% T cells D2 post-PPD (compared with 10% post-saline), and 40-60% T cells at D3 post-PPD or BCG

Cell	Animal studies: mice and other small animal models	Animal studies: non-human primates (NHPs)	Human studies: peripheral blood and genome-wide association	Human studies: lung
	naive mice against *M.tb* infection.[413] IFN-γ knock-out mice rapidly succumb to TB disease.[414,415] However antigen-specific IFN-γ production post-BCG vaccination does not correlate with protection against *M.tb* challenge in mice.[416,417] In mice the development of the adaptive Th1 CD4+ T cell response is delayed compared with other pathogens, with influx into the lungs seen 10 days to 3 weeks post infection, during which time bacterial replication continue unabated.[54,91,341,418] Following aerosol *M.tb* challenge, IFN-γ+ CD4+ T cells increase in the BAL at the same time as the blood.[419]	SIV infection in NHPs which causes CD4+ T cell depletion and abrogation is associated with increased risk of TB disease.[421] IFN-γ+ CD4+ T cell response after *M.tb* aerosol infection is not detected systemically in NHPs until 3-4 weeks.[91,341,418] Influx into the airways can be as late as 5-13 weeks post *M.tb* aerosol infection.[422,423] Intra-tracheal BCG results in lower systemic ELISpot responses than intradermal BCG.[424] Following aerosol BCG infection in NHPs, total circulating T cells fall at 2 weeks and rise at 6 weeks.[425] Following aerosol *M.tb* infection, IFN-γ+ CD4+ T cells responses in the BAL mirror the blood (increase at the same timepoint).[426]	count is associated with worse disease outcome.[365,427-429] IFN-γ deficiency leads to disseminated *M.tb* and BCG infection.[430,431] TB vaccine candidates induce potent antigen-specific IFN-γ+ CD4+ T cells, but this doesn't improve protection over BCG alone.[432,433] No association with TB risk and IFN-γ+ CD4+ T cells or any of the other T cell properties in a study of 5,662 BCG-vaccinated infants with a 2 year follow-up.[434] And yet, BCG antigen-specific IFN-γ ELISpot response is associated with reduced risk of TB disease in the first 6-12 months of infancy.[372] Studies of healthy contacts or LTBI patients report conflicting results with *M.tb*-specific Th1 cells increased[435,436] or reduced[437,438] compared with active TB disease patients. Systemic TST and IGRA conversion appears as early as 2 weeks, with a peak between 4-8 weeks.[369-371]	instillation.[206,409] CD4+ T cells expanded in the airways D2 following PPD bronchoscopic airway instillation in M.tb exposed patients to > 40% BAL cells.[409] Increase in IFN-γ+ CD4+ T cells at D2 and D3 in the airway following PPD or BCG lobar instillation in *M.tb* exposed volunteers. No change following PPD exposure in *M.tb* naïve subjects. BAL from *M.tb* infected patients are enriched for IFN-γ+ antigen specific CD4+ T cells though most of these also produced IL-10, suggesting a negative correlation with protection.[439] However in another study, TB patients switched to a higher IFN-γ producing, Th1 CD4+ T cell signature in the BAL following treatment.[219] ➔ No known description of CD4+ T cell expansion in the airways following mycobacterial aerosol infection in *M.tb* naïve humans.

Cell	Animal studies: mice and other small animal models	Animal studies: non-human primates (NHPs)	Human studies: peripheral blood and genome-wide association	Human studies: lung
CD8+ T cells	Following aerosol *M.tb* infection in mice, CD8+ T cells appear from D10 in the lung (the same time as CD4+ T cells) and directly kill *M.tb* – infected cells through lysis or apoptosis *in-vitro*.[378,379] CD8+ T cell depletion increases susceptibility to *M.tb* infection in mice, however antigen-specific CD8+ T cell responses are not sufficient for protection.[383] Variable evidence from murine models of CD8+ T cell role in *M.tb* protection.[440-442]	CD8+ T cell depletion increases risk of *M.tb* disease in previously infected or BCG vaccinated NHPs.[443] Post aerosol BCG, antigen-specific IFN-y+ CD8+ T cells increase in the BAL, but not until up to 13 weeks post-BCG.[426]	*M.tb* activated human CD8+ T cells on *M.tb* culture are polarised towards a cytotoxic phenotype, but they can also produce Th1 and Th17 cytokines.[381,382] CD8+ T cell deficiency is associated with autoimmunity and not overwhelming mycobacterial infection.[385] Antigen-specific CD8+ T cells are detected in the blood of LTBI and active TB patients[377] Antigen-specific cytokine producing CD8+ cells are insignificant compared with the CD4+ T cell response following viral vector *M.tb* candidate vaccination.[386,387]	Antigen-specific CD8+ T cells are detected in the granulomas of LTBI and active TB patients[377] ➔ Information about early CD8+ T cell responses in the human airways is lacking.

1.9 The elusive search for an immune correlate of protection against TB

A correlate of protection is defined as a laboratory marker associated with protection against infection or clinical disease occurrence.[444] This biomarker could be causally related or merely associated with protection.[444] Such a marker could be used in pre-clinical or clinical studies to rapidly identify vaccine candidates.[445] As described, CD4+ T cells and IFN-y are necessary but not sufficient to prevent infection or disease. Other possible correlates discussed here include ML ratio, neutrophils, NK cells, DURTs, polyfunctional T cells, activation markers, or cytokines.[316,317,446] It is likely a combination of biomarkers, such as a transcription signature is needed.[395,399,446,447] However, due to the complexity of the disease and the lack of a definitive marker of infection or clearance, it is difficult to identify a validated protective immune signature using epidemiological studies of TB alone. Therefore, despite decades of research a correlate of protection remains elusive.

1.10 Mycobacterial Growth Inhibition Assay (MGIA)

In the absence of an immune correlate, the direct MGIA is a useful *ex-vivo* assay which measures functional immune Mycobacterial control. MGIA co-cultures *M.tb* or BCG with whole blood, peripheral blood mononuclear cells (PBMCs) or other sources of host immune cells (e.g. splenocytes), and quantifies mycobacterial units at the end of the culture using a Bactec™ Mycobacterial Growth Indictor Tube.[448-452] Tubes fluoresce on oxygen depletion, once a threshold of mycobacterial growth has been reached (75 Growth Units, approximately $1x10^5$ – $1x10^6$cfu/mL).[453,454] Time to positivity (TTP) is then used as an inverse marker of *ex-vivo* growth inhibition using a standard curve.[454,455] The MGIA system provides an unbiased functional and reproducible assessment of bacterial growth inhibition that is a sum-of-parts

analysis.[448] Ideally whole blood, which includes granulocytes, would be used to ensure the inclusion of a complete peripheral immune profile, however the iron-rich haemoglobin has been shown to increase variability and reduce sensitivity, and hence PBMC MGIA is more reproducible.[448,456,457]

The MGIA has been validated in the preclinical setting as a correlate of protection. In mice and NHPs, *ex-vivo* MGIA correlated with *in-vivo* protective efficacy of *M.tb* challenge in the lung and spleen.[449-452] In a human UK adult study using PBMCs, MGIA improved following primary BCG vaccination but not after revaccination.[456] This reflects the protective efficacy of primary BCG vaccination against *M.tb* disease in this population which confers up to 80% efficacy in UK adolescent populations for up to 10 years.[8,28] Further, in a recent study of HIV negative UK adults, BCG MGIA was able to discriminate active TB, latently infected and uninfected patients.[447] Hence, BCG MGIA demonstrates promise as a potential correlate of mycobacterial protection in UK adult populations. In the absence of a validated biomarker of protection, the MGIA is an unbiased functional assay recently standardised by a WHO supported working group.[448]

1.11 BCG as an aerosol challenge model

1.11.1 The importance of studying local mucosal immune responses

The airway is the natural route of *M.tb* infection and local mucosal responses differ from systemic responses in TB patients and in animal models.[91,278,458,459] In addition, aerosol immunisation in cattle, mice, NHPs and humans of candidate *M.tb* vaccines or BCG, results in an immune response that is qualitatively and quantitatively different from parenteral

administration.[386,426,460-462] Thus the immune response is compartment-specific and impacted by route of infection or vaccination.

Local immune responses may be more protective against *M.tb* infection than systemic mechanisms. BCG delivered by aerosol but not systemically, protects the tuberculosis-susceptible DBA/2 mouse strain and some NHP models,[391,463-465] and in both mouse and NHP studies, lung localised T_{RM} cells were better able to control TB disease.[91,466,467]

Alternatively, the inherent anti-inflammatory lung environment may contribute to attenuation of any anti-M.tb immune response in humans.[468,469] Therefore defining the immune response at the site of initial host-pathogen interaction is critical.

1.11.2 Defining lung responses in humans

In human studies, detecting lung immune responses can be challenging due to the invasive procedures required to collect samples from draining lymph nodes and lung parenchyma.[470,471] Work has largely been limited to samples from pleural effusion taps and BAL, as lymph node and bone marrow biopsies are painful and confer additional risk.[472,473] Cells and fluid from the BAL is derived from alveolar spaces and the airways and can provide invaluable information about these compartments,[474,475] especially as these are where the first line of defence against an aerosol *M.tb* infection occurs. Further discussion on the relevance of BAL cells in defining local immune responses is found in Chapter 4.

1.11.3 Mycobacterial aerosol challenge models

While aerosol challenge models in mice and NHPs are well established, few studies have investigated early mucosal immune responses, with the majority of studies starting 2-4 weeks after *M.tb* challenge. This is likely due to the focus on adaptive immune responses, with a peak in CD4+ T cell infiltration seen in the lungs of mice at around 4 weeks.[476] In mice, innate immune responses such as AMs don't upregulate pro-inflammatory markers until 10 days after infection and DC tracking and subsequent T cell lung infiltration is also delayed by at least 10 days post *M.tb* infection.[146]From human epidemiological studies, mycobacteria-specific adaptive responses only appear in the periphery from 2 weeks as measured by TST responses and IGRA conversion.[369-371] Recently an aerosol mouse challenge model of CD4+ or CD8+ T cell knockout mice, reported innate-specific protective changes at 7 days, though these mice were BCG primed 7 days prior to aerosol challenge.[143]

In humans, two groups have investigated localised mucosal mycobacterial immune responses. Both studies were at early time points, day 2 or day 3, investigating bronchoscopic lobar instillation of PPD or BCG.[206,409,410] The first, by Silver and colleagues, described cellular responses 2 days after 0.5TU PPD instillation between TST positive and negative subjects.[409] They noted segment specific lymphocytic infiltration, predominantly of CXCR3+ IFN-γ CD4+ T effector memory cells in PPD instilled lobes of TST positive subjects only.[410] They saw minimal macrophage response, and no significant responses at this timepoint in naïve volunteers, however their study was limited to *in-vivo* antigen stimulation only. The second group, in Dheda's lab, instilled BCG (10^2, 10^3, or 10^4 cfu), 0.5 TU PPD and saline in different segments in *M.tb*-exposed BCG vaccinated HIV negative subjects and investigated cellular and humoral immune responses 3 days later.[206] Again they found an enrichment of Th1 CD4+ T cells, specific only to the antigen stimulated segments. They reported significant differences

between BAL and blood responses and between *in-vivo* PPD stimulated and *in-vitro* PPD stimulated cells. Importantly BCG and PPD instilled segments produced significantly different immune responses to each other, both in cellular composition and on differential gene expression (DGE) analysis.

This highlights the need for a live mycobacterial inhaled challenge model which investigates mucosal immune responses at the infection site.

1.11.4 BCG as a model of *M.tb* infection

Controlled human infection models for other pathogens (malaria, typhoid) have been successful in facilitating both vaccine development and characterising pathophysiology,[477,478] however a similar model using virulent *M.tb* would be unethical due to potential treatment toxicity and unclear curative endpoint.[479] The replicating *Mycobacterium*, BCG, licensed for ID vaccination against TB in humans, elicits a similar $CD4^+$ T-cell mediated immune response to that of *M.tb*[480-482] and could be used as a surrogate replicating mycobacterial agent. ID BCG challenge models developed in our group have successfully detected differences in vaccine-induced anti-mycobacterial immunity,[480,483,484] however, the aerosol route would more closely mimic natural infection and enable assessment of the mucosal immune response.

1.11.5 Differences between *M.tb* and BCG

One limitation of a BCG model is that in robust immune systems, BCG is cleared before causing disease or latency.[16] Why BCG is less virulent than *M.tb* in humans has not been fully elucidated, however, BCG lacks the region of difference 1 (RD1) locus and subsequently the virulent ESX-1 secretion system.[485,486] Evidence from mouse *in-vitro* experiments show that *M.tb*-infected DCs are polarised to a more permissive or aberrant Th2 response compared to

BCG or RD-1/ESAT-6-deleted *M.tb,* with increased production of IL-4 and IL-1β.[402] By contrast, all three strains induced equal amounts of IFN-y and IL-12. Further, *M.tb,* but not BCG*,* inhibits autophagy in human DCs by inhibiting maturation of the autophagosome *in-vitro.*[133] Recombinant BCG with ESAT-6 secretion restored, inhibits autophagy in macrophages.[133] Other virulence factors linked to ESAT-6 include the migration of infected mouse AMs into the interstitium where infection is established[103] and induction of Type 1 interferons IFN-α/IFN-β, associated with TB disease risk.[354] Aerosol *M.tb* in mice preferentially disseminate to neutrophils and non-resident macrophages, while BCG remain within AMs.[305]

However, BCG retains a number of *M.tb* virulence and immune evasion strategies. BCG can reduce detection by downregulating PAMPs like TLR2, or through dormancy, as it retains the DosS/DosR system.[45,139] Further, vaccination with ALF-exposed BCG improves resistance to subsequent *M.tb* challenge in mice compared to standard BCG (in a CD8+ T cell dependent manner), indicating ALF exposure reduces BCG immune-evasion mechanisms.[487] Furthermore, BCG downregulates MPs ability to present antigen on MHC-II in mice.[139] Importantly, using a less virulent *Mycobacterium,* such as BCG, in an aerosol challenge model allows us to elucidate what mechanisms are associated with an effective clearing immune response. These biomarkers could then be validated in TB disease and animal models.

1.11.6 Safety considerations

BCG is widely used in open clinic environments, and in immunocompetent humans, ID BCG usually results in a self-limiting infection, with rare vaccination site ulceration or regional lymphadenitis.[480,488,489] In immunocompromised individuals, ID BCG can lead to disseminated or life-threatening BCG infection.[490] The two strains used in our challenge trials, BCG Bulgaria and BCG Danish, are sensitive to the anti-tuberculosis medications isoniazid, rifampicin, rifabutin and ethambutol.[491] Volunteers are challenged under ethically approved controlled

conditions with 24 hour medical cover. If BCG disease occurred, volunteers would be referred to an Infectious Disease consultant and treated with the appropriate course of antibiotics, which would clear the infection.

Three small studies in the 1960s and 1970s delivered intrapulmonary BCG to either healthy volunteers or lung cancer patients and showed good safety profiles,[492-494] as did NHP models using the same strain and dose to that planned here.[392,426] Further, Dheda's group delivered 10^4 cfu BCG by local bronchoscopic instillation to mycobacteria-sensitised volunteers without any clinically significant AEs.[206]

We have therefore designed an initial dose-finding BCG aerosol human challenge model which starts at a low delivered dose of $1x10^3$cfu, with planned dose escalations, after appropriate periods of observation, and clearly defined safety stopping rules.

1.12 Aims and Hypotheses

Primary aims:

- To develop a safe and well tolerated aerosol BCG challenge model in healthy, Mycobacteria-naïve UK adult volunteers.
- To use this model to characterise the early immune responses in the airway, to better understand protective mechanisms to further vaccine development.

Secondary aim: To define the early immune response in the systemic circulation.

Tertiary aim: To correlate immune responses with protective immunity as defined by *ex-vivo* mycobacterial growth inhibition in the blood.

Main hypothesis:

That a BCG aerosol challenge model can be developed which is safe and feasible in healthy *M.tb* and BCG naïve UK adults; and that this model can be used to assess the early local and systemic immune responses, in order to better understand protective mechanisms, to aid rational vaccine design.

My specific hypotheses are:

- BCG challenge by the aerosol inhaled route in healthy, Mycobacteria-naïve UK adult volunteers will be safe and well tolerated.
- Neutrophils will increase in the BAL fluid at D2 and decrease systemically at D2 compared with baseline.
- NK cells will increase in the BAL fluid at D7 and decrease in systemically at D7 compared with baseline.

- Mycobacteria-specific IFN-γ+ and TNFα+ CD4+ T cells will be detectable in the airway at D7.
- DCs will upregulate gene expression associated with impaired function and delayed LN tracking in the airway.
- High innate and adaptive responders in the mucosa will be better at controlling systemic MGIA growth at D56 compared with low responders.
- Early gene expression of IL-10 in the airway will negatively correlate with systemic MGA growth control at D56.

Chapter 2.

Materials and Methods

2 Materials and Methods

Table 2.1. Reagents

Reagent	Supplier	Catalogue No.
0.4% Trypan blue	Merck Life Science	T8154
10XFACS lysing solution	BD Biosciences	349202
16% Paraformaldehyde (for PFA FACS)	Thermo Fisher	43368
anti-CD28	BD Biosciences	340975
anti-CD49d	BD Biosciences	340976
BBL MGIT supplement: OADC and PANTA (polymyxin B, amphotericin B [AMB], nalidixic acid, trimethoprim [TMP], and azlocillin)	BD Biosciences	245124
BBL® MycoPrep™ Specimen Digestion/ Decontamination Kit	BD Biosciences	240862, 240863
BCIP/NBT(Plus) solution	Solution Europa Bioproducts	MO711A-1000
Benzonase	Novagen	70664-3
Brefeldin-A	Sigma Aldrich	B7651
Brilliant Stain Buffer	BD Biosciences	563794
Carbonate Buffer Capsules	Sigma Aldrich	C-3041
Casyton counting buffer	Sedna Scientific	43003
CD3 microbeads	Miltenyi Biotec	130-050-101
Dimethylsulphoxide (DMSO)	Sigma Aldrich	D2650
Ethylenediaminetetraacetic acid solution (EDTA)	Merck Chemicals	4055
Fixation/Permeabilisation Solution Kit ("Fix/Perm")	BD Biosciences	554714
Foetal Calf Serum (FCS)	BioSera Ltd.	S1810
Genotype Mycobacterium CM VER 2.0	HAIN Lifescience	299A
GenoType MTBC VER 1.X	HAIN Lifescience	301
Glycerol	Merck Life Science	49767
HBSS (no Ca^{2+}, no Mg^{2+})	Life Technologies	14170088
Human IFN-γ ELISpot kit (ALP)	Mabtech	3420-2A
Human TruStain FcX™ (Fc Receptor Blocking Solution)	Biolegend	422302
L-Glutamine	Gibco	G7513
Lymphoprep	Stemcell Technologies	7811
Middlebrook 7H11 agar powder	Sigma Aldrich	M0428-500G
Multi Tissue Dissociation Kit 1	Miltenyi Biotec	130-110-201

Reagent	Supplier	Catalogue No.
OADC supplement (oleic acid, albumin, dextrose, and catalase)	Sigma Aldrich	M0678-1VL
OneComp eBeads Compensation Beads-100 tests	Life Technologies	01-1111-42
PBS with Tween-20	Sigma Aldrich	P3563
Penicillin G 1650units/mg	Sigma Aldrich	PENNA-100MU
Pen-strep: 10,000u/ml penicillin and 10,000µg/mL streptomycin sulphate	Gibco	PO781
Perm/Wash™ Buffer	BD Biosciences	51-2091KZ
Phosphate buffered saline (PBS) tablets	Sigma Aldrich	3813
PPD (Tuberculin purified protein derivative)	Staten Serum Institute	2391
Qiagen kit for DNA extraction	Qiagen	69504
RBC Lysis solution	Qiagen	158904
RPMI 1640	Sigma Aldrich	R0883
RPMI 1640 media with 25mM HEPES	Thermofisher	42401018
Sodium Azide	Sigma Aldrich	S2002
Sodium Pyruvate	Gibco	11360-039

Table 2.2. Antibodies used in Flow Cytometry

Marker	Colour	Panel	Supplier	Catalogue No.
CD103	PE	Homing	Biolegend	350206
CD11c	BV785	Innate	Biolegend	301644
CD14	BV421	Innate	ebioscience	48-0149-42
CD14	PE dazzle	Homing	Biolegend	325634
CD14	PerCpCy5.5	MAIT/NKT, Polycytokine	Biolegend	301824
CD153	AF647	Homing	R&D Systems	FAB1028R-100
CD16	AF488	Innate	Biolegend	302019
CD16	APC-Cy7	Polycytokine	Biolegend	360710
CD161	BV785	MAIT/NKT	Biolegend	339930
CD19	ECD	Innate, Homing	Biolegend	302252
CD19	BV510	MAIT/NKT, Polycytokine	BD Biosciences	562947
CD206	APC-CY7	Innate	Biolegend	321120
CD26	PE	MAIT/NKT	Biolegend	302706
CD27	BV605	MAIT/NKT	Biolegend	302830
CD29	APC-Cy7	Homing	Biolegend	303014
CD3	BUV496	Innate	BD Biosciences	564809
CD3	AF700	Homing, MAIT/NKT, Polycytokine	ebioscience	56-0038-42
CD326	PercpCy5.5	Innate	Biolegend	369804
CD4	Pacific Blue	Homing	Biolegend	300521
CD4	BUV395	MAIT/NKT	BD Biosciences	564724

Marker	Colour	Panel	Supplier	Catalogue No.
CD4	Pacific Blue	Polycytokine	Biolegend	300521
CD45	BV650	Innate	Biolegend	304044
CD49D	BV605	Homing	Biolegend	304324
CD56	PECY5	Innate	Invitrogen	15-0567-42
CD56	BV785	Homing	Biolegend	362550
CD66b	AF700	Innate	Biolegend	305114
CD86	APC	Innate	Biolegend	305412
CD8a	APC-H7	MAIT/NKT	BD Biosciences	641400
CD8a	BV650	Homing, Polycytokine	Biolegend	301042
CXCR3	PerCpCy5.5	Homing	Biolegend	353714
HLADR	BV650	MAIT/NKT	Biolegend	307650
HLA-DR	PE	Innate	Biolegend	307606
IFN-γ	PE-CY7	Homing, Polycytokine	Life Technologies	25-7319-82
IL-17	AF488	Polycytokine	Biolegend	512308
IL-2	PE	Polycytokine	Beckman coulter	PNIM2718U
KLRG-1	AF488	Homing	Biolegend	367714
Live/Dead	Aqua	MAIT/NKT, Polycytokine	Invitrogen	L34957
Live/Dead	Red	Innate/Homing	Invitrogen	L23102
MR1 CTR: 6-FP	AF488	MAIT/NKT	NIH Tetramer Core facility	n/a
MR1 TEM: 5-OP-RU	APC	MAIT/NKT	NIH Tetramer Core facility	n/a
PD-1	BUV395	Homing	BD Biosciences	745619
Siglec8	PE-Cy7	Innate	Biolegend	347112
TNF-α	AF647	Polycytokine	Biolegend	502916
Vα24	PercpCy5.5	MAIT/NKT	Biolegend	360004
Vα7.2	BV421	MAIT/NKT	Biolegend	351716
Vβ 11	PE-vio770	MAIT/NKT	Miltenyi	130-108-734
γδ pan: B1 (11F2)	BV711	Homing	BD Biosciences	745505
γδ2: (B6)	BV480	Homing	BD Biosciences	746567

Table 2.3. Consumables

Consumable	Supplier	Catalogue No.
0.22µm filters	Millipore	GSWP01300
70µl cell strainer	Fisher Scientific	22363548
BACTEC MGIT tube	BD Life Sciences	245122
Casyton tubes	Sedna Scientific	43001
C-Chip Disposable Hemocytometer	Neubauer Improved	DHC-N01
Cell strainers (70µm)	Corning Life Sciences	352350
Cool Cell Cryocontainers	Biocision	BCS-136
CountessTM Cell counting chamber slides	Invitrogen	C10228
Cryobabies labels	Web Scientific Ltd	LCRY-1700
Cryovials (2ml)	Sigma Aldrich	BR114832
ELISpot plates (MultiScreen-IP; 96 well)	Millipore	MAIPS4510
Eppendorf tubes (2ml)	Sigma Aldrich	Z628034
Falcon tubes (15/50ml)	Falcon	352097/98
Flat-bottom 48-well plates	Costar	3548
GentleMACs dissociator with no incubator	Miltenyi	130-093-235)
gentleMACS™ C Tube	Miltenyi	130-093-237
Leucosep tubes (50ml)	Greiner	G227290
L-shaped spreader (sterile)	Starlab	E1410-1025
MACS magnet separator	Miltenyi Biotech	130-091-051
Microtubes (0.2ml)	Sigma Aldrich	Z374962
MS magnetic separation columns	Miltenyi Biotech	130-042-201
Multimix tube rotator plates	VWR	11807
Pasteur pipettes	Fisher Scientific	12827625
Petri dishes (60mm)	Sigma Aldrich	P5481
Pipette tips and filter tips (10, 20, 200 and 1000µl)	Starlab	Various
Polypropylene round-bottom tubes	Fisher Scientific	10314791
Polystyrene round-bottom tubes	Fisher Scientific	14-959-48
Sarstedt screw-cap tubes (2ml)	Sarstedt	72.694.007
Stripettes (2, 5, 10, 25, 50ml)	Corning	4487
360° Tube rotator	VWR	13916-822
Vaccutainer tubes (green, heparin)	BD Life Sciences	368987
Vaccutainer tubes (red, serum)	BD Life Sciences	368815
V-bottom 96 well FACS staining plates	VWR	734-1798

Table 2.4. Solutions

Solution	Recipe
Cryopreservation media	FCS with 10% DMSO
Diluted Perm Wash buffer (P/W buffer)	1:10 dilution of perm/wash buffer with dH2O
Enriched buffer	HBSS media (no Ca2+, no Mg2+) + 10% FCS
FACS Buffer	Sterile PBS supplemented with 0.1% bovine serum albumin and 0.01% sodium azide. Stored in the dark.
FACS Lysing Solution	1 part 10xFACS lysing solution in 9 parts sterile water. Stored at RT in the dark.
MACS buffer	HBSS media (noCa2+, no Mg2+) + 0.5% FCS + 2mM EDTA.
MGIT media	RPMI 1640 media with 25mM HEPES supplemented with 2mM L-glutamine and 10% FCS
Middlebrook 7H11 agar	900mL dH2O + 19.7g Middlebrook 7H11 agar powder + 5mL Glycerol. After autoclaving and cooling 100mL OADC added.
PBS	0.01M: NaCl 0.138M, KCl 0.0027M, pH 7.4; made by dissolving tablets in dH2O
R0 media	RPMI 1640 supplemented with 2mM L-glutamine, 5ml "pen-strep"
R10 media	RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine, 5ml "pen-strep" (10,000u/ml penicillin, 10,000µg/mL streptomycin sulphate) and 1.7mM sodium glutamate
R10 no antibiotics	RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine, and 1.7mM sodium glutamate
R10NS media	RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine, 10,000units penicillin G and 1.7mM sodium glutamate. Penicillin powder reconstituted in PBS (30mg/ml) and filtered using 0.22um filter.
Resuspension media for 10x	RPMI 1640 + 10% FCS

2.1 Clinical trials

TB041 was a randomised controlled clinical trial of healthy, BCG and *M.tb* naïve UK adults. Phase 1 was a dose escalation delivering aerosolised BCG. In phase 2, blinded volunteers were randomised to receive either aerosol or intradermal (ID) BCG. All volunteers had a bronchoscopy at day 14 (D14), and ID BCG volunteers also had a skin biopsy of the BCG injection site (Figure 2.1). The study opened using BCG Danish 1331 produced by Serums Statens Institute (SSI) as this was the BCG licenced for use as a vaccine in the UK. 13 volunteers were enrolled into the study before recruitment was stopped due to a global shortage of BCG SSI. After regulatory approval, Arm 2 was added to the study design, using BCG Bulgaria manufactured by Intervax (RD Russia). This was the BCG strain recommended for routine clinical use by Public Health England during the BCG SSI shortage. The clinical results from the final safety comparator groups using BCG Bulgaria, Groups 2E and 2F, are presented in this thesis. The full study protocol of which I am primary author, is in the appendices. The protocol outlines the full study design and inclusion/exclusion criteria. The schedule of procedures is shown in Table 2.5.

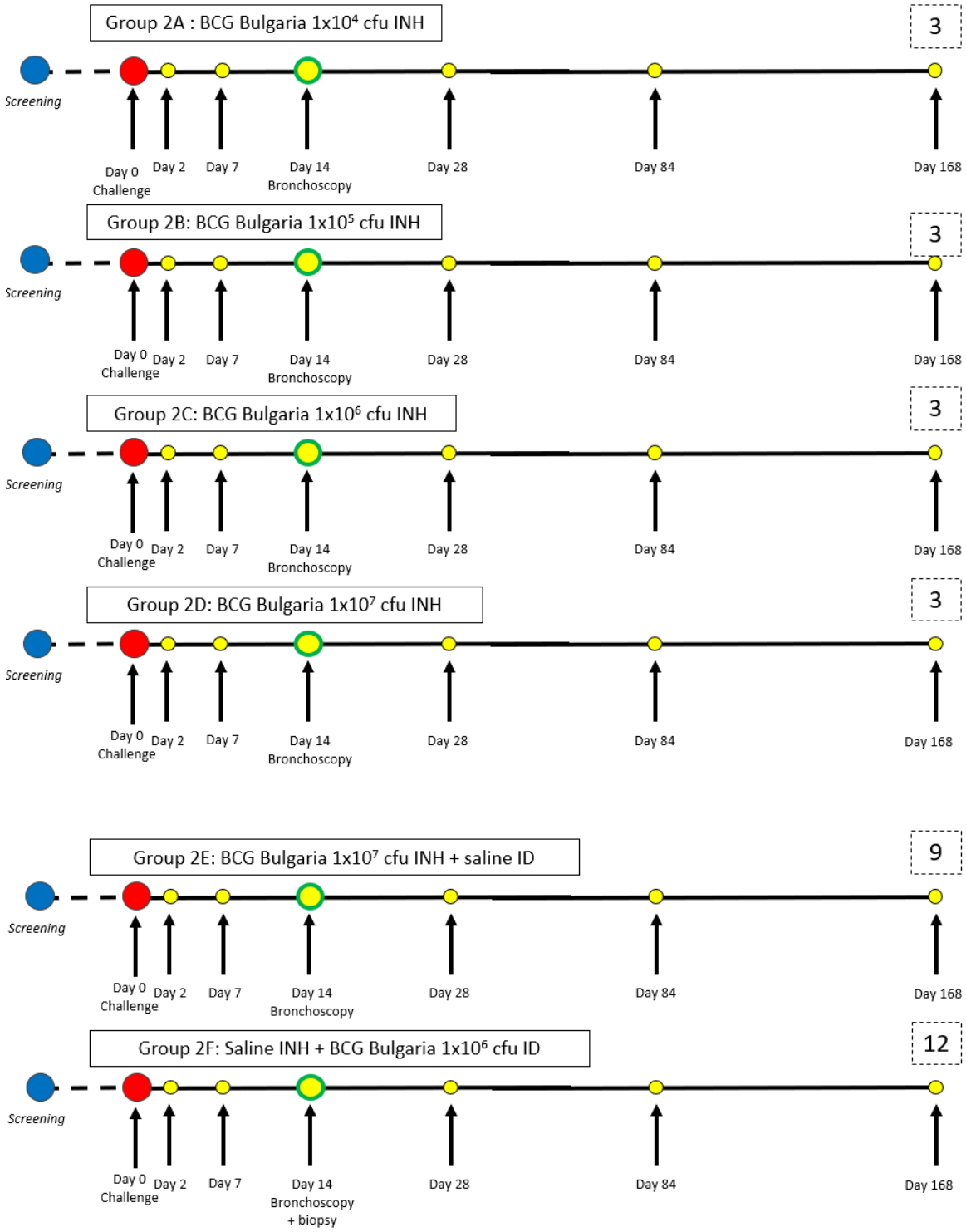


Figure 2.1. TB041 schedule of visits. (INH: Inhaled, ID: Intradermal)

Table 2.5. TB041 Schedule of procedures

Visit number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Timeline (days)*	Screening	0 (Challenge)	2	7	14	28	84	168
Timeline (weeks)*	Screening	0		1	2	4	12	24
Time windows (days)			±1	±2	±21	±7	±14	±21
Inclusion/exclusion criteria	X							
Review contra-indications	X	X			X			
Informed consent	X							
Medical history	X	(X)	(X)	(X)	(X)	(X)	(X)	(X)
Physical examination	X	(X)	(X)	(X)	(X)	(X)	(X)	(X)
Vital signs	X	X	X	X	X	X	X	X
Urinalysis	X							
PFTs	X	X	X	X	X	X		
β-HCG urine test	X	X			X			
BCG Challenge		X						
Biopsy (Groups 1D and 2F)					X			
Bronchoscopy					X			
Chest radiograph	X							
Induced sputum (Groups 2A, 2B, 2C, 2D, 2E)						X	X	X
Local & systemic events/reactions		X	X	X	X	X	X	X
Ediary setup		X						
Ediary final review						X		
Biochemistry (4mL)**/***	X		X	X	X	X		
Haematology (2mL)***	X			X	X	X		
Coagulation (4mL)***	X							
HBV, HCV, HIV (10mL)	X							
HLA typing (4mL)		X						
Exploratory immunology incl ELISpot (10-60mL****)	X	X	X	X	X	X	X	X
Blood vol (mL)	30	64	20	56	66	66	60	60
Cumulative blood vol (mL)***	30	94	114	170	236	302	362	422

X Event scheduled to occur

(X) If considered necessary, emphasising any complaint or change in medications

* Timeline is approximate only, as exact timings (± windows periods) of visits relate to the actual (not intended) date of the previous visit

** U&Es, LFTs, CRP (except day 2 when CRP only)

*** Blood volumes stated for Oxford. The exact volumes of blood taken will depend on the local site – cumulative blood volumes for volunteers outside Oxford may be slightly lower or higher than those of Oxford volunteers, due to the use of different volume vacutainers for biochemistry, haematology and serology samples as per local Trust standard procedures

**** 60ml at all visits except screening (10ml), visit 3 (16ml) and visit 4 (50ml)

TB043 was a randomised controlled clinical study in the same population as TB041. Blinded volunteers were randomised to receive either aerosol BCG or aerosol saline, followed by a bronchoscopy at either 2 days (D2; Group 1) or 7 days (D7; Group 2) post aerosol inhalation

(Figure 2.2). In this study we used BCG Danish 1331, now manufactured by AJ Vaccines (AJV), as this is now the BCG licenced for use as a vaccine in the UK. The dose, $1x10^7$cfu, is also the maximum aerosol dose delivered in TB041, in Group 2D/E of the randomised phase. The full TB043 study design has five groups, which differ by the timing of bronchoscopy (Group 3 D14, Group 4 D28, Group 5 D56). The study is still ongoing. However, my thesis, investigating early responses, encompasses Group 1 and 2 only, which are complete. The study protocol, of which I am primary author, is in the appendices. TB043 samples include peripheral blood, bronchoalveolar lavage fluid, endobronchial biopsies and induced sputum. Time points for these collected samples are outlined in Figure 2.2 and the schedule of procedures in Table 2.6.

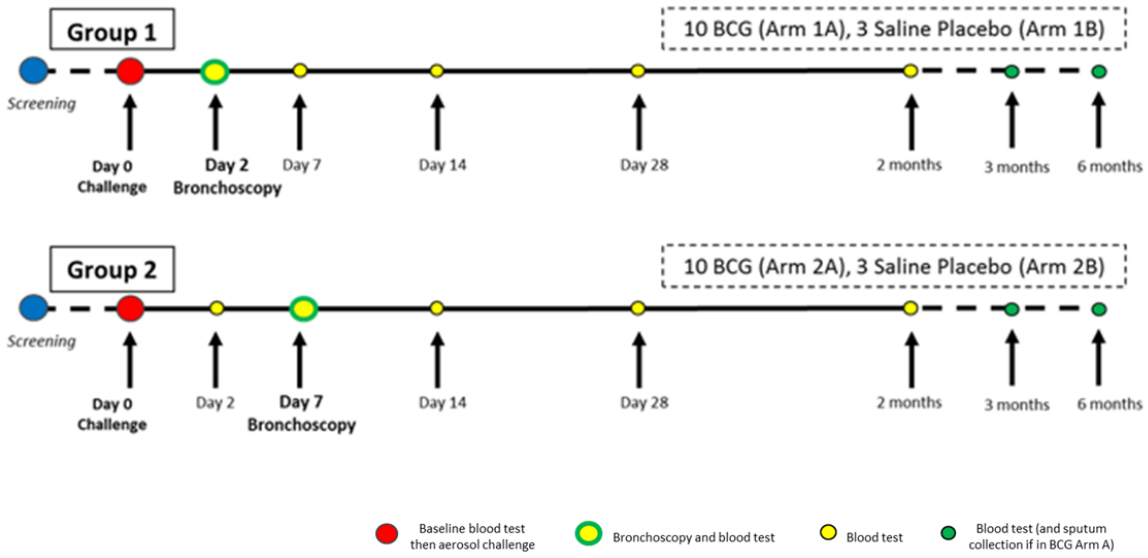


Figure 2.2. TB043 schedule of visits

Table 2.6. TB043 Schedule of procedures

Visit number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Timeline (days)	Screening	0	2	7	14	28	56	84	168
Timeline (weeks)	Screening	0		1	2	4	8	12	24
Time windows (days)			±1	±2	±5	±7	±14	±21	±28
Inclusion/exclusion criteria	X	X							
Review contra-indications	X	X	G1	G2	G3	G4	G5		
Informed consent	X	X	G1	G2	G3	G4	G5		
Medical history	X	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)
Physical examination	X	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)
Vital signs	X	X	X	X	X	X	X	X	X
Urinalysis	X								
Pulmonary Function Tests[+]	X	X	X	X	X	X	X	X[^]	X[^]
β-HCG urine test (females)	X	X	G1	G2	G3	G4	G5		
BCG challenge		X							
Bronchoscopy			G1	G2	G3	G4	G5		
Chest radiograph	X								
Induced sputum*								X	X
Local & systemic events/reactions		X	X	X	X	X	X	X	X
E-diary #1 setup		X							
E-diary #1 final review					X				
7-day E-diary #2 for bronchoscopy symptoms					G3	G4	G5		
Biochemistry (mL)	4					4			
Haematology (mL)	2					2			
Coagulation (mL)	4								
HBV, HCV, HIV (mL)	10								
HLA typing (mL)		4							
Exploratory immunology +/- IGRA (mL)	10	60	60	60	60	60	60	60	60
Blood vol per visit (mL)	30	64	60	60	60	66	60	60	60
Cumulative blood vol (mL)	30	94	154	214	274	340	400	460	520

X Event scheduled to occur
(X) If considered necessary, emphasising any complaint or change in medications
G1-G5 Designates Group number
+ Allowable window for formal pulmonary function tests is +/- 7 days on top of specified time-point windows
^ If clinically indicated
* No induced sputum for controls (Arm 2 volunteers)

2.1.1 Safety

This was the first time that BCG was inhaled via nebuliser by humans in a controlled modern human trial, and so BCG was administered carefully under full MHRA regulation following first-in-man dose escalation guidelines. As the aim was to use aerosol BCG as an infectious agent in research studies, any conclusions on safety and tolerability are limited to data gleaned from the small volunteer numbers in the trial, and by no means aims to establish a comprehensive set of safety and tolerability data which might be needed for an aerosol vaccine indication. For this, large scale trials powered to detect rare safety events following inhaled BCG would be required.

Tolerability was assessed by comparing local respiratory and systemic AEs after aerosol BCG with those following ID BCG delivery, as an alternative delivery route for a human infection model.[484] Aerosol BCG delivery would be deemed tolerable, if the frequency, duration and severity of adverse events (AEs) was not significantly higher than following ID BCG, a licenced route of delivery. Aerosol BCG infection might result in more respiratory AEs than saline inhalation and hence in this case, overall assessment of AEs following either route could be compared.

In TB041, local intradermal related BCG adverse events were not formally solicited beyond monitoring injection site healing as these AEs are fully documented in the relevant BCG SmPC and were not directly related to the primary aim of the trial. Solicited respiratory adverse events were selected based on known adverse events following aerosol vaccine delivery, such as from our MVA85A vaccine aerosol studies.[386,387]

Unlike TB041, TB043 control volunteers only received saline aerosol with no intradermal BCG comparator. Hence, for tolerability assessment, while AEs following BCG Danish were compared with saline inhalation, it was expected there might be increased adverse event reporting following BCG infection. Hence tolerability in TB043 is also assessed by comparing

adverse event data from the matched dose of BCG Bulgaria aerosol infection ($1x10^7$ cfu) in TB041.

Severity grading is outlined in Table 2.7. Laboratory and vital signs AEs were graded as outlined in the protocol (Section 11 TB041 and TB043). Causality was assessed as per Table 2.8. For definitions of serious adverse events (SAEs) and suspected unexpected serious adverse reactions (SUSARs) refer to the relevant sections in the protocols.

We did not have dose escalation groups in TB043, but the safety data was monitored at planned intervals by an independent Safety Monitoring Committee (SMC).

Table 2.7. Severity grading for physical adverse events

GRADE 0	None
GRADE 1	Mild: Transient or mild discomfort (< 48 hours); no medical intervention/therapy required
GRADE 2	Moderate: Mild to moderate limitation in activity – some assistance may be needed; no or minimal medical intervention/therapy required
GRADE 3	Severe: Marked limitation in activity, some assistance usually required; medical intervention/therapy required, hospitalisation possible

Table 2.8. Causality assignment criteria for TB041 and TB043

0	**No Relationship**	No temporal relationship to trial product; ***and*** Alternate aetiology (clinical state, environment or other interventions); ***and*** Does not follow known pattern of response to challenge
1	**Unlikely**	Unlikely temporal relationship to trial product; ***and*** Alternate aetiology likely (clinical state, environment or other interventions); ***and*** Does not follow known typical or plausible pattern of response to challenge
2	**Possible**	Reasonable temporal relationship to trial product; ***or*** Event not readily produced by clinical state, environment or other interventions; ***or*** Similar pattern of response to that seen with other challenge agents
3	**Probable**	Reasonable temporal relationship to trial product; ***and*** Event not readily produced by clinical state, environment or other interventions; ***and*** Known pattern of response seen with other challenge agents
4	**Definite**	Reasonable temporal relationship to trial product; ***and*** Event not readily produced by clinical state, environment or other interventions; ***and*** Known pattern of response seen with the challenge agent

2.1.2 BCG concentration/dilutions preparation

The doses of BCG for aerosol or ID delivery were prepared as per Table 2.9.

TB041 BCG Intervax (Bulgaria) contained 1.5-6 x 10^6 per vial, hence an approximate median of 4x10^6 cfu was used for calculations. BCG AJV (Danish) contained 2-8 x 10^6 per vial, and a median of 5.5 x 10^6cfu was used for calculations. BCG Bulgaria diluent was sterile saline and this was used for the dilution steps (Table 2.9). BCG AJV diluent was not saline alone but contained Magnesium sulphate heptahydrate, Dipotassium phosphate, Citric acid monohydrate, L-asparagine monohydrate, Ferric ammonium citrate, Glycerol 85% and sterile water. Each vial was reconstituted with only 100ml diluent, then 450ml saline, to reduce the theoretical risk of airway irritation from inhaling the diluent contents.

Table 2.9. TB041 BCG Bulgaria or TB043 BCG Danish preparation for delivery by nebuliser or intradermal injection

Target loaded dose	Reconstitution step BCG Bulgaria: 4 x 10^8cfu/vial BCG Danish: 5.5 x 10^6cfu /vial	1:10 dilution	1:4 dilution	Delivered dose: 1:10 (100ul) of final concentration	Delivered dose: No dilution step. 1ml of final concentration
TB041 Group 2A 1x10^4 cfu	1 vial BCG Bulgaria Intervax reconstituted as per SmPC with 1ml of diluent	100µl of reconstituted BCG added to 900µl diluent (saline).	250µl of the 1:10 dilution added to 750µl diluent (saline).	1:40 dilution (1x10^5cfu/ml) 100µl loaded into nebuliser with 900µl sterile saline	
Group 2B 1x10^5 cfu	1 vial BCG Bulgaria Intervax reconstituted as per SmPC with 1ml of diluent	No	250µl of reconstituted vial added to 750µl of diluent	1:4 dilution (1x10^6cfu/ml) 100µl loaded into nebuliser with 900µl sterile saline	
Group 2C or 2F 1x10^6 cfu	1 vial BCG Bulgaria Intervax reconstituted with 400µl of diluent	No	No	2.5 x concentration (1x10^7cfu/ml) Group 2C: 100µl loaded into nebuliser with 900µl sterile saline OR Group 2F; 100µl loaded into syringe for intradermal injection	
Group 2D/E 1x10^7 cfu	3 vials BCG Bulgaria Intervax reconstituted, each with 400µl of diluent. Contents combined.	No	No	No	2.5 x concentration (1x10^7cfu/ml) 1ml loaded into nebuliser
TB043 All groups 1x10^7 cfu	2 vials BCG Danish AJV reconstituted, each with 550ml (100ml diluent and 450ml saline). Contents combined	No	No	No	1.82 x concentration (1x10^7cfu/ml) 1ml loaded into nebuliser

2.1.3 Enumerating reconstituted BCG

For Group 2A-2D and 2F; a second or third 100µl of the final solution was used as required, if more than one volunteer was enrolled on the day, and if within 2 hours of reconstitution. Reconstituted vials and diluted vials were kept in the dark between 2-8°C and the remaining contents plated within 2 hours of reconstitution. 1:10 serial dilutions were prepared from each vial and 100µl of the minus 3 and minus 4 dilutions were plated onto M7H11 agar plates in triplicate, then incubated at 37°C for 3 weeks before colonies were counted.
In addition, to ensure my method of reconstitution did not alter BCG viability or increase risk of clumping due to limited use of the recommended diluent for BCG AJV, I plated three vials reconstituted as per the BCG AJV SmPC and three vials reconstituted as outlined above.

2.1.4 BCG recovery after nebulisation

To determine how much BCG was lost through nebulisation, duplicate samples of 1ml phosphate-buffered saline (PBS) containing 1 x 10^6 cfu BCG was added to an Omron nebuliser and collected in a glass impinger using a Vacusafe pump (Integra Biosciences). 10ml PBS (minus the volume collected) was used to wash the impinger. Serial dilutions were then plated onto Middlebrook 7H11 agar to calculate the recovered BCG. A separate aliquot of the BCG was plated out to verify the number of cfu added to the nebuliser. Results are presented in Chapter 3.

2.2 Sample collection

Table 2.10 outlines the laboratory analysis plan for TB043 samples.

Table 2.10. TB043 Laboratory analysis plan

Visit number	1	2	3	4	5	6	7	8	9	
Timeline (days)	Screening	0 (Challenge)	2	7	14	28	56	84	168	Bronchoscopy
Timeline (weeks)	Screening	0		1	2	4	8	12	24	
Time windows (days)			±1	±2	±5	±7	±14	±21	±28	
Ex-vivo ELISpot		X	X (G1 only)	X	X	X	X	X	X	
WB ICS		X	X (G1 only)	X (G2 only)	X	X	X	X	X	X
Freeze PBMC, plasma and serum		X	X	X	X	X	X	X	X	
Flow Cytometry same day - phenotype panels (BAL, whole blood and biopsy)										X
Flow Cytometry next day - after overnight stimulation (BAL)										X
Endobronchial biopsies										X
Single cell RNA-seq BAL										X
MGIT (BAL fluid)										X
MGIT (Induced Sputum)								X (BCG vol only)	X (BCG vol only)	
BCG plating (Vial)		X								
No. 10 ml green tubes	0	5	5	5	5	5	5	5	5	
No. 10 ml red tubes	0	1	1	1	1	1	1	1	1	
Immunology blood vol (mL)		60	60	60	60	60	60	60	60	
Safety blood volume	30	4				6				
Cumulative blood vol (mL)	30	94	154	214	274	340	400	460	520	

2.3 Preparation of BCG as a stimulatory antigen for WB or BAL ICS

BCG Pasteur (Aeras) vials containing $1.2x10^7$ cfu in 20 µl PBS were stored at -80°C, and thawed at RT prior to use. As vials lose 1 log on average on thawing, one 20µl vial was used to provide $1.2x10^6$ cfu BCG (unpublished data from McShane lab).

2.4 Culture media

As WB and BAL ICS samples were stimulated with live BCG, standard R10 media could not be used due to the presence of streptomycin, to which BCG is susceptible.[491] In addition, I wanted to be certain that the presence of Penicillin in the R10 did not exert a bacteriostatic effect (see solutions Table 2.4). I therefore experimented with four different media to allow for preservation of BCG while minimising contamination; R10, R10 with no streptomycin (R10NS), R10 with no antibiotics; or R10 with PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin) supplied with Mycobacterial Growth Indicator Tubes (MGIT)).

BCG Bulgaria was added to culture media for a final concentration of $2x10^6$ cfu/ml and incubated overnight at 37°C with 5% CO_2. The next day 100µl of the -3 and -4 dilutions were plated in triplicate onto Middlebrook 7H11 agar plates. Bacterial counts at 3 weeks yielded no growth from the R10 or "PANTA" media. "No antibiotics media" yielded 300+ colonies while R10NS yielded an average of 70 colonies. ELISpot and flow cytometry (overnight stimulation followed by staining for the Polycytokine panel) were then performed on sterile PBMC and PBMC contaminated with respiratory flora, by adding 5ml saliva (to mimic BAL contamination). PBMCs from healthy donors were thawed and divided into two tubes (sterile or contaminated) and rested for 2 hours at 37°C in "no antibiotics" media prior to counting. Cell death was approximately 23%, with no difference between conditions. Cells were then

resuspended in the four different media and the ELISpot plated. The remaining cells were further rested for 2 hours and recounted prior to stimulation for ELISpot or flow cytometry. Cell counts are shown in Figure 2.3. Both “PANTA” and “no antibiotics” produced poor responses, however R10NS produced similar responses to R10 (see Figure 2.4 for ELISPOT response to PPD and BCG). I therefore used R10NS media for all ICS overnight stimulations.

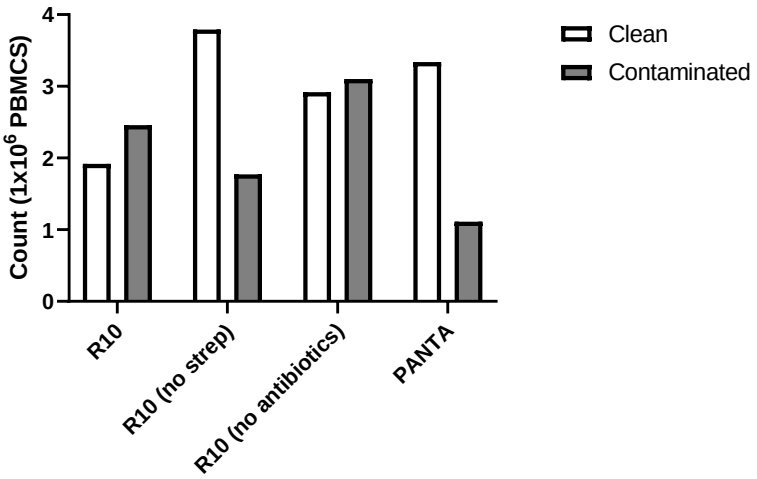


Figure 2.3. Cell counts following 2 hour incubation with four different media

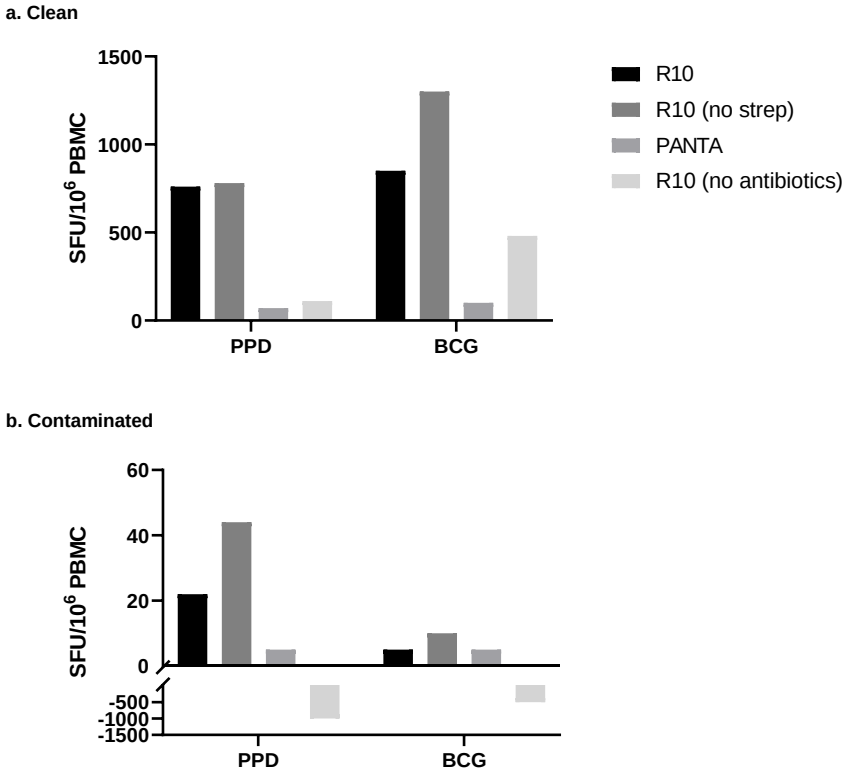


Figure 2.4. IFN-y+ Spot forming units (SFU) on ELISpot following overnight stimulation of PBMC with PPD or BCG. High background levels in the contaminated no antibiotics condition resulted in negative results.

2.5 Counting

Cells were counted using a Countess II FL (Thermofisher) and 10µl of a 1:1 cell sample with filtered 0.4% Trypan blue following manufacturer's instructions. For all PBMC and BAL cells used for flow cytometry, live cells 5-15µM diameter were counted. (BAL cell flow cytometry panels were standardised by adding $1x10^6$ live lymphocytes per condition). For 10x, live and dead BAL cells 5-30µM were counted, to include granulocytes and macrophages.[495,496] BAL cell counting was cross checked against manual microscopy using 10µl of 1:1 cell sample and 0.4% Trypan blue into the C-chip haemocytometer, counted in duplicate.

2.6 Blood processing

2.6.1 PBMC processing

Blood samples were drawn into lithium heparin tubes and processed within 4 hours of collection. 3ml of whole blood was set aside for processing. The remaining whole blood was added to Leucosep tubes, pre-filled with 15ml of Lymphoprep and spun (1000rpm; 13 min; at room temperature (RT), without brake). Once separation was complete, 4ml of plasma was collected into 2 x 2ml sarstedt tubes for freezing at -80°C for future batch MGIA analysis. PBMCs were collected from the interface layer and cells were washed twice with R0 media (1800rpm 5 min, maximum brake). Cells were then re-suspended in R10 media for counting. PBMCs was allocated for ELISpot and the remaining cells were frozen in cryopreservation media in cool cell cryocontainers at -80°C for 24 hours, before storing in liquid nitrogen. Batched thawed PBMCs were used in the "MAIT/NKT" flow cytometry panel or for the MGIA assay. Cells were thawed in a 37°C water bath, and transferred into 9ml R10. Tubes were spun (1400rpm; 7 min), re-suspended in 2µl Benzonase/ml R10, and rested for 2 hours at

37°C, 5% CO_2 before counting. ("R10 no antibiotics" was used instead of R10, throughout thawing for MGIA).

2.6.2 Serum

Blood samples were drawn into red serum vacutainers and processed within 4 hours of collection. Tubes were spun (1800rpm; 5 min) and up to 4ml of serum was harvested into 2 x 2ml Sarstedt tubes for freezing at -80°C. Serum was used for ELISA (results not presented in this thesis).

2.6.3 Whole blood (WB)

Fresh WB samples were collected at all timepoints for flow cytometry. Analysis included unstimulated phenotype and stimulated Intracellular Cytokine Staining (ICS).

For unstimulated WB, I developed a protocol based on our WB ICS assay. 200µl of 20mM EDTA was added to 2ml of WB and gently vortexed for 10 seconds. Samples were incubated (15 min; RT), then vortexed again. 18ml of FACS lysing solution was added and the cells were incubated (10 min; RT, in the dark). Samples were washed in 20ml PBS (1500 rpm; 7 min) before freezing by adding 1ml PBS with 10% DMSO and dividing the sample between 2 x 2ml Sarstedt tubes. Samples were frozen at -80°C in a Cool Cell for at least 24 hours before storage in liquid nitrogen.

1ml WB was also set aside for overnight stimulation as previously described.[497] Samples were stimulated overnight with co-stimulatory antibodies, anti-CD28 and anti-CD49d, and either; BCG ($1.2x10^6$ cfu/ml); *Staphylococcus* enterotoxin B (SEB; 5 ug/ml) as a positive control; or left unstimulated. Cells were then lysed, fixed, and cryopreserved.

Once all samples were collected and batched, samples were thawed at RT prior to "innate panel" staining (unstimulated WB); or staining using the "Homing" or " Polycytokine" panel (WB ICS).

2.6.4 *Ex-vivo* ELISpot

An IFN-γ ELISpot assay was performed on freshly isolated PBMC as previously described.[498] As responses were low at D2 in Group 1, D2 ELISpot was not performed on Group 2 (D7) volunteers.

Mycobacterium-specific responses were measured in triplicate using Purified Protein Derivative from *M.tb* (PPD) at a final concentration 20 μg/ml. SEB (10 μg/ml) was used as a positive control and R10 as a negative control. Results are reported as spot-forming cells (SFC) per million PBMC, calculated by subtracting the mean of the unstimulated wells from the mean of the antigen-stimulated wells and correcting for number of PBMC in the well (1 or 3 x 10^5/ well).

2.7 Induced sputum

I adapted this procedure from two NHS Trust standard operating procedures (SOPs) in consultation with the Respiratory consultant Dr Henry Bettinson.[499,500] Volunteer FEV1 was measured using the CareFusion Micro 1^{TM} spirometer and volunteers were then dosed with 200mcg Salbutamol MDI using an AeroChamber PlusNote spacer. After 10 minutes, volunteers inhaled 20ml 3% sterile hypertonic saline nebulised via the Omron NE-U780 Ultrasonic nebuliser over 20 minutes. At 5 minute intervals the nebuliser was stopped and volunteers encouraged to expectorate sputum into a collection pot. FEV1 was measured and the nebuliser restarted only if no clinically significant drop in baseline (>20%). Sputum was

processed within 2 hours of collection (see Section 2.10 BCG qualification and quantification from BAL and sputum).

2.8 Bronchoscopy sample collection

Fibreoptic bronchoscopy was performed on all volunteers by the respiratory consultant. Intravenous sedation (Midazolam and Fentanyl) was offered and local anaesthetic spray above and below the cords was administered to reduce discomfort and facilitate the bronchoscopy. Macroscopic airway appearance was recorded and photographs taken before bronchoalveolar lavage (BAL) was obtained from the right middle lobe (medial segment) using 100ml of 0.9% sodium chloride. Up to six endobronchial biopsies were then collected, depending on tolerance. BAL was collected into sterile 50ml falcon tubes and placed on ice. The first two biopsies were placed in formalin and sent to the Oxford Centre for Histopathology Research laboratory (OCHRE) for fixing in paraffin prior to cutting and immunohistochemistry staining. (Analysis is still in process, so further methods are not presented here). Any additional biopsies were then collected into pre-chilled RPMI 1640 and taken to our laboratory for digestion. Samples processing commenced within 2 hours of collection.

2.9 Bronchoscopy sample processing

On receipt into the lab, BAL volume was recorded and tubes were pooled from the same volunteer before being centrifuged (400g, 10min) to isolate human BAL cells. The supernatant BAL fluid (BALF) was decanted and set aside. The pellet of human BAL cells was resuspended in 5ml red blood cell (RBC) lysis solution and incubated at RT for 5 minutes, swirling occasionally. The falcon tube was then topped up to 45ml with R10NS (see Section 2.3.), centrifuged (400g, 10min) and supernatant discarded. The pellet was resuspended in

3ml R10NS before counting, then resuspended to achieve a concentration of $1x10^6$ live lymphocytes/ml. Cells divided for planned assays as per protocol depending on cell numbers (Table 2.11). For flow cytometry, $1x10^6$ lymphocytes were allocated for each condition, 0.5 $x10^6$ cells were for unstimulated and Fluorescence Minus One (FMO) controls.

Table 2.11. Allocation of BAL cell assays

1. Innate Panel (Stained+ FMO) [$1.5x10^6$ cells]
2. 10x RNAseq, Unstimulated [$1.0x10^6$ cells] *n=3 BCG + saline volunteers each group
3. Homing ICS Panel (Unstimulated, BCG, SEB + FMOs) [$3.5x10^6$ cells]
   OR if insufficient cells
4. Homing Panel (Stained + FMO) [$1.5x10^6$ cells]
5. MAIT iNKT Cell Panel (Stained + FMO) [$1.5x10^6$ cells]
6. B Cell Panel (Stained + FMO) [$1.5x10^6$ cells]
7. Polycytokine ICS Panel (Unstim., BCG, SEB + FMOs) [$3.5 x10^6$ cells]

2.9.1 BAL cell stimulation (Polycytokine and Homing ICS panels)

1×10^6 BAL cells were stimulated with BCG ($1.2x10^6$ cfu/ml), SEB (5 µg/ml) or left unstimulated as a negative control. Co-stimulatory antibodies, anti-CD28 and anti-CD49d at 1 µg/ml each, were added to all samples. Cells were incubated in 5% CO_2 at 37°C for 2 hours, then 3 µg/ml of Brefeldin A was added. The samples were incubated overnight and stained the following morning (see 2.12 Flow Cytometry). Polypropylene tubes were used for the Polycytokine panel to discourage macrophage adhesion, and, following overnight stimulation, samples were incubated for 15min in 2mM EDTA before washing. Homing ICS samples were incubated overnight in polystyrene tubes to promote adhesion, as macrophages were co-stained with live/dead stain and excluded on flow cytometry gating.

2.9.2 BALF (supernatant from first BAL spin)

BALF was centrifuged (3000g; 17min) to collect a BCG pellet. The BALF was then decanted and frozen for future analysis (ELISA, ALF oxidative status).[99]

The BCG pellet was resuspended in 1ml sterile PBS and processed for BCG detection by MGIT (see section 2.10 BCG qualification and quantification from BAL and sputum).

2.9.3 Endobronchial biopsy digestion

I used the Miltenyi lung dissociation Multi Tissue Dissociation Kit 1 as it yielded the best proportion of cells (lymphocyte, granulocyte, epithelial cell) from a similar mouse digestion protocol.[501]

Enzymes were prepared as per manufacturer instructions; 2.35ml RPMI 1640, 100µl Enzyme D, 50µl Enzyme R and 12.5µl Enzyme A were added to a gentleMACS^{TM} C Tube containing the biopsies. The C tubes were incubated (20min, 37 °C) on a multimix tube rotator (VMR), before being run on the gentleMACS Dissociator (Miltenyi), program Multi_A. The incubation and dissociation steps were then repeated. A short spin collected the sample at the bottom of the tube (300g; 1 min). The cells were strained using a pre-moistened (RPMI) 70µm cell strainer, and washed with a further 10ml RPMI 1640. The suspension was centrifuged (300g; 10min) and supernatant discarded. The cells were resuspended in 5ml RBC lysis Solution, incubated for 5 minutes then washed with RPMI (1800rpm; 5min). Cells were counted and resuspended at $1x10^6$/ml for flow cytometry.

2.10 BCG qualification and quantification from BAL and sputum

2.10.1 Detection of BCG in BAL and induced sputum samples

800µl of PANTA/OADC MGIT supplement was added to the BACTEC Mycobacterial Growth Indicator Tube (MGIT) containing Middlebrook 7H9, as per manufacturer instructions. BAL and sputum were decontaminated by adding 1:1 of sample with BBL MycoPrep™ Specimen Digestion/ Decontamination Kit (NALC-NaOH solution) and incubating at RT for 15 minutes with occasional swirling. The solution was then topped up with sterile PBS to 40ml and centrifuged (3000g, 17min). The supernatant was discarded and the pellet transferred to the MGIT. Tubes were incubated at 37°C in the BACTEC 960 (BD) until time-to-positivity (TTP) was detected by fluorescence. If there was no TTP by D42 a negative result was recorded. All tubes were then centrifuged (3000g; 17min) to pellet the BCG. The pellet was resuspended in residual volume and topped up with PBS to 200µl. Cells were frozen at -80°C for batched DNA extraction, using the Qiagen kit as previously,[502] and HAIN genotyping.

2.10.2 HAIN sequencing

Genotyping was performed using the HAIN lifescience GenoType MTBC VER 1.X, Mycobacterium CM VER 2.0 and Mycobacterium AS VER 1.0 as per the manufacturer's instructions. The Hain MTBC kit detects *M. tuberculosis*, *M. bovis* (including BCG), *M. canettii, M. africanum, and M. microti*. The Hain CM and AS kits detect *M. tuberculosis* complex (but does not differentiate between species) and 46 NTM spp. DNA extracted from BAL and induced sputum was amplified using kit-specific primers and PCR conditions. 20µl of the PCR product was chemically denatured and hybridised to the kit DNA•STRIP, containing specific probes complementary to the amplified PCR products. The bound amplicon was detected by adding streptavidin-alkaline phosphatase, detected by a colourimetric reaction. The

resultant strip banding pattern was matched to the mycobacterium spp., using the kit-specific key.

2.11 Mycobacterial growth inhibition assay

500cfu BCG Pasteur and $3x10^6$ PBMC in a total volume of 480µl MGIT media with 120µl autologous plasma (matched to time-point) per well were added to a 48-well plate. These were incubated for 96 hours at 37°C, then transferred to 2ml screw-cap tubes and centrifuged (12000rpm; 10min). 500µl sterile water was added to each well to lyse adherent monocytes and release intracellular mycobacteria. Supernatant was discarded from the 2ml tubes, and the pellet resuspended with the water from the corresponding well. After vortexing, the lysate was transferred to MGIT tubes supplemented with 800µl of BBL MGIT OADC/PANTA, as recommended by the manufacturer. Tubes were incubated at 37°C in the BACTEC 960 machine until positivity was detected by fluorescence. Duplicate supplemented BACTEC MGIT tubes were inoculated with the same volume of mycobacteria as the samples and incubated on the BACTEC 960 machine from day 0, to create direct-to-MGIT viability control tubes. TTP was converted to log_{10}cfu by creating standard curves against TTP, inoculum volume and cfu. Normalised mycobacterial growth results are calculated as log_{10}cfu of sample – log_{10}cfu of control tubes.

2.12 Flow cytometry

I developed these protocols based on current procedures in our group, adjusted to allow for the additional of Fc-receptor blocker and the MAIT tetramers (TEM). TEMs were donated by the NIH Tetramer Core facility. Flow panels were designed with the help of my supervisor Dr Iman Satti. Antibody titration experiments to determine volumes were performed using PBMCs from BCG-naïve donors (data not shown). In order to allow for flexibility with

processing, I performed a comparator experiment using PBMCs from PPD-reactive donors stained with the Polycytokine panel which confirmed there was no significant difference in detection of cytokine+ T cell and monocyte populations between immediate and overnight (up to 24 hours) acquisition once cells were stained and fixed in 1% paraformaldehyde (results not shown).

2.12.1 BAL Staining

Following stimulation (day 2, ICS panels), or immediately after counting the cells (day 1, phenotype surface stain panels) cells were washed twice in PBS (1800rpm; 5min; RT) and transferred to V-bottom flow cytometry (FACS) staining plates. L/D stain was diluted in PBS as per Table 2.12.

- For panels analysing mononuclear phagocytes (MPs; Innate and Polycytokine flow cytometry; Table 2.12), 5µl of Fc-receptor blocker was then added with 95µl FACS buffer and incubated at RT, before centrifuging (1800rpm; 3min; RT) and discarding supernatant. The pellet was then resuspended in L/D, antibody mix, additional 5µl Fc Blocker and FACS buffer to a total volume of 100µl.
- For the homing flow cytometry panel (Table 2.12) after the two initial washes, the cell pellet was resuspended with the L/D stain for 10min at 4°C (in fridge) in the dark. FACS buffer and antibody mix were then added directly into wells (total volume 100µl).
- For MAIT/iNKT flow cytometry panel using tetramers (Table 2.12), TEM stock was diluted 1:100 in PBS. The pellet resuspended in 10µl of each diluted TEM with 5µl FACS buffer, to a total volume 25µl/well (FMO wells had 25µl FACS buffer only), and incubated (45min, RT, dark). L/D and antibody mix were then added to make up a total volume of 106µl.

Cells were mixed, then incubated for 30min at 4°C in the dark. Cells were then washed twice with 200µl FACS buffer (1800rpm; 3min; 4°C). At this point, cells for surface staining only were resuspended in 100µl FACS buffer with 1% paraformaldehyde (PFA) and set aside for acquisition.

For ICS panels (homing and polycytokine flow cytometry panels), cells were permeabilised for ICS staining as follows. The pellet was resuspended in 200µl fixation/permeabilisation solution (Fix/Perm) and cells incubated (20min, 4°C, dark). Cells were then washed twice using 200µl diluted Perm/Wash™ (P/W buffer) (1800rpm; 3min; 4°C). The pellet was resuspended in pre-prepared ICS antibody mix with P/W buffer in a total volume of 100µl. Cells were incubated (30min, 4°C, dark), washed twice (P/W buffer; 1800rpm; 3min; 4°C), then resuspended in 100µl FACS buffer with 1% paraformaldehyde and transferred to cluster tubes for acquisition.

All stained cells were acquired immediately or overnight on an LSR Fortessa v.2 Std X20 flow cytometer, laser configuration - 2-Blue 3-Red 6-Violet 5-Yel Gr 2-UV, using BD FACSDiva 8.0.2 and analysed on FlowJo (BD) v10.

2.12.2 Blood staining

For PBMC and WB ICS staining, cells were thawed as outlined in section 2.6. Blood processing. Staining was as outlined above with the exclusion of the L/D staining step for WB (innate) and WB ICS (Homing and Polycytokine) panels. Volumes for antibody incubation were made up with FACS buffer or PW buffer as appropriate.

2.12.3 Flow cytometry gating

Gating strategies are outlined in the below figures Figure 2.5Figure 2.8. Cells were gated on FMOs. Dead cells were excluded from all BAL samples and from PBMCs.

Table 2.12. Volumes used in Innate, Homing, MAIT and Polycytokine BAL panels

INNATE STAINED PANEL

Antibody	Colour	Fortessa laser	µl/well
L/D (diluted to 1:1000)	Red	YG610/20	5

Antibody mix

CD19	ECD	YG610/20	2
CD3	BUV496	UV525/50	1
CD56	PE-CY5	YG670/30	2
CD14	efluor 450 (PB/BV421)	V450/40	2
CD16	AF488	B530/30	2
CD45	BV650	V670/30	0.1
CD66b	AF700	R730/45	4
CD86	APC	R670/30	2
CD206	APC-CY7	R780/60	2
CD326	Percp-Cy5.5	B710/50	4
Siglec8	PE-Cy7	YG780/60	2
HLA-DR	PE	YG586/15	5
CD11c	BV785	V780/60	4
Brilliant stain buffer			50

Total volume added to wells

Antibody mix			82.10
Additional Fc blocker			5
L/D			5
FACS			7.9

MAIT/NKT STAINED PANEL

Antibody	Colour	Fortessa laser	µl/well
MR TEM	APC	R670/30	10
CTR TEM	FITC	B530/30	10

Antibody mix

CD19	BV510	V525/40	2.5
CD14	BV510	V525/40	2.5
CD3	AF700	R730/45	1
CD4	BUV395	UV379/28	0.5
CD8	APC-H7	R780/60	3
CD26	PE	YG586/15	2.5
CD27	BV605	V610/20	2
Vα7.2	BV421	V450/50	2
CD161	BV785	V780/60	2
Vα24	PercpCy5.5	B710/50	3
HLADR	BV650	V670/30	2.5
Vβ11	PE-vio770	YG780/60	5
Brilliant stain buffer			50

Total volume added to wells

Antibody mix			78.5
TEM+FACs mix			25
L/D (diluted 1:10)	AQUA	V525/40	2.5
Additional FACS			0

HOMING SURFACE STAINED PANEL

Antibody	Colour	Fortessa laser	µl/well
L/D (diluted to 1:1000)	Red	YG610/20	5

Antibody mix

CD19	ECD	YG610/20	5
CD14	ECD	YG610/20	5
CD4	Pacific Blue	V450/40	1
PD-1	BUV395	UV379/28	2
CD56	BV785	V780/60	1
CXCR3	PerCpCy5.5	B710/50	5
γδ pan (B1 (11F2))	BV711	V710/50	1.5
γδ2 (B6)	BV480	V525/40	1.5
CD103	PE	YG586/1	2.5
KLRG-1	AF488	B530/30	3
CD29	APC-Cy7	R780/60	2
CD49D	BV605	V610/20	2
CD153	AF647	R660/20	3
CD3	AF700	R730/45	1
CD8a	BV650	V660/20	0.5
Brilliant stain buffer			50

Total volume added to wells

Antibody mix			86
L/D			5
FACS			9

HOMING ICS STAINED PANEL

Antibody	Colour	Fortessa laser	µl/well
L/D (diluted to	Red	YG610/20	5

SURFACE 1			
CD19	ECD	YG610/20	5
CD14	ECD	YG610/20	5
CD4	Pacific Blue	V450/40	1
PD-1	BUV395	UV379/28	2
CD56	BV785	V780/60	1
CXCR3	PerCpCy5.5	B710/50	5
γδ pan (B1 (11F2))	BV711	V710/50	1.5
γδ2 (B6)	BV480	V525/40	1.5
CD103	PE	YG586/1	2.5
KLRG-1	AF488	B530/30	3
CD29	APC-Cy7	R780/60	2
CD49D	BV605	V610/20	2
CD153	AF647	R660/20	3
Brilliant stain buffer			50

Surface antibody mix			84.5
L/D			5
FACS			10.5

ICS 1			
CD3	AF700	R730/45	1
CD8a	BV650	V660/20	0.5
IFN-γ	PE-CY7	YG780/6	0.5

TOTAL ICS antibody mix			2
P/W Buffer			98

POLYCYTOKINE ICS STAINED PANEL

Antibody	Colour	Fortessa laser	µl/well

SURFACE 1			
CD19	BV510	V525/40	2.5
CD4	Pacific Blue	V450/40	1
CD14	PerCpCy5.5	B710/50	2
CD16	APC-Cy7	R780/60	2

Surface antibody mix			7.5
L/D (diluted 1:10)	Aqua	V525/40	2.5
Fc Blocker			5
FACS			85

ICS 1			
CD3	AF700	R730/45	1
CD8a	BV650	V670/30	0.5
IFNg	PE-CY7	YG780/60	0.25
IL-2	PE	YG586/15	2
IL-17	AF488	B530/30	4
TNF-a	AF647	R670/30	1

TOTAL ICS antibody mix		8.75
P/W Buffer		91.25

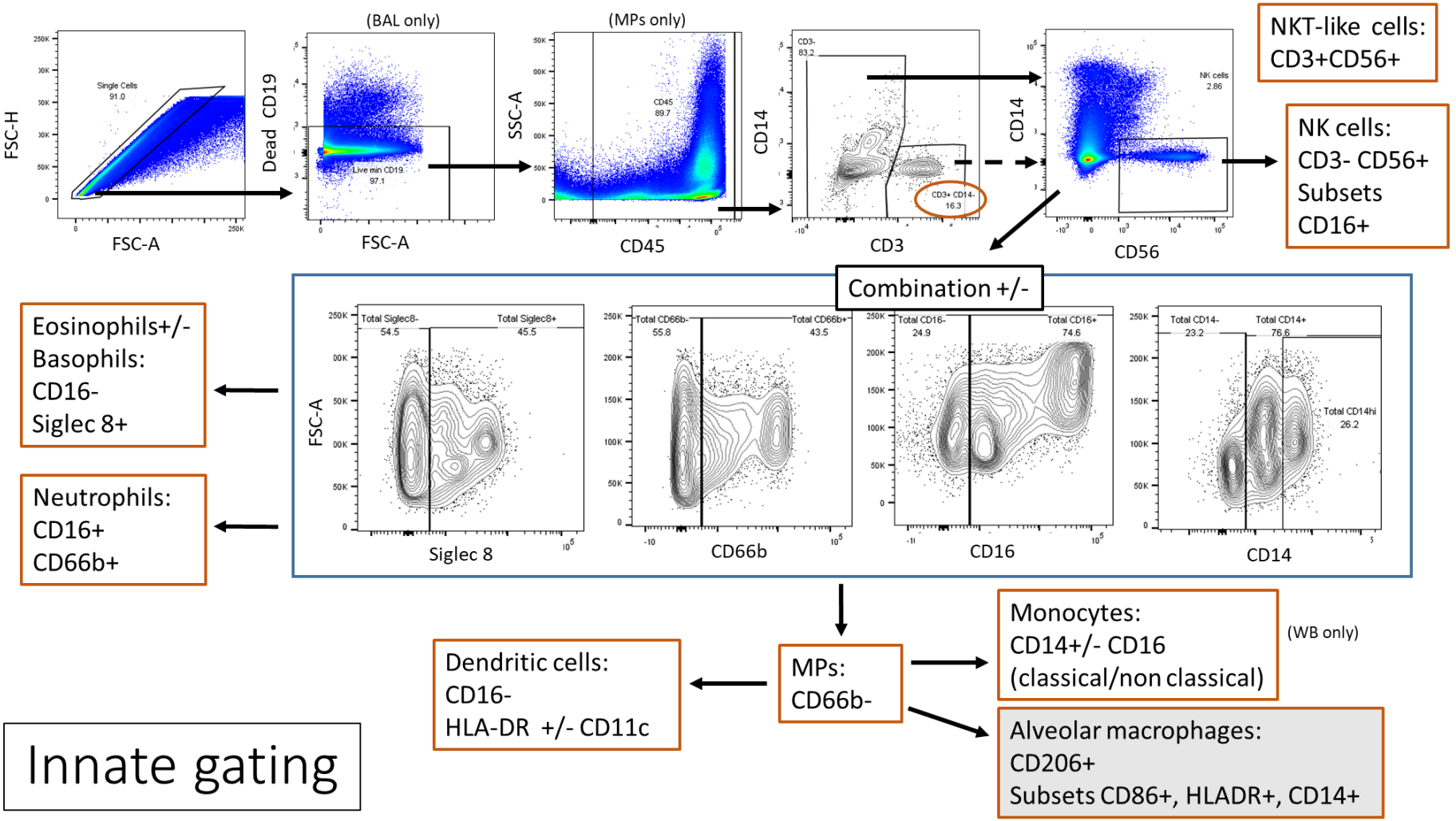


Figure 2.5. Innate gating strategy. Bronchoalveolar lavage (BAL) and whole blood (WB).
Orange boxes and circles indicate cell endpoints. Greyed box indicates where gating was not completed.

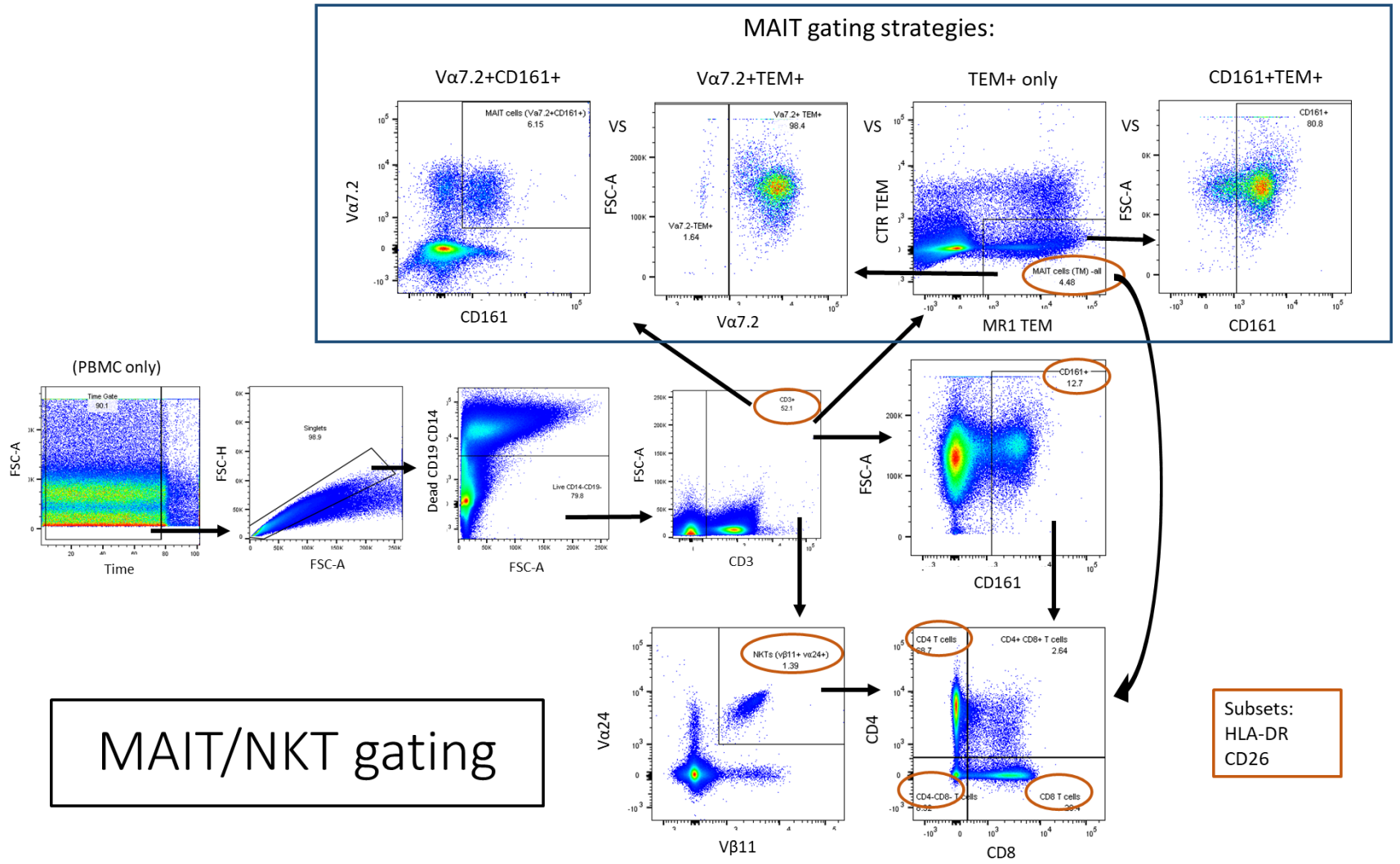


Figure 2.6. MAIT/NKT gating strategy. Bronchoalveolar lavage (BAL) and peripheral blood mononuclear cells (PBMC). Orange boxes and circles indicate cell endpoints. TEM: Tetramer. CTR: Control.

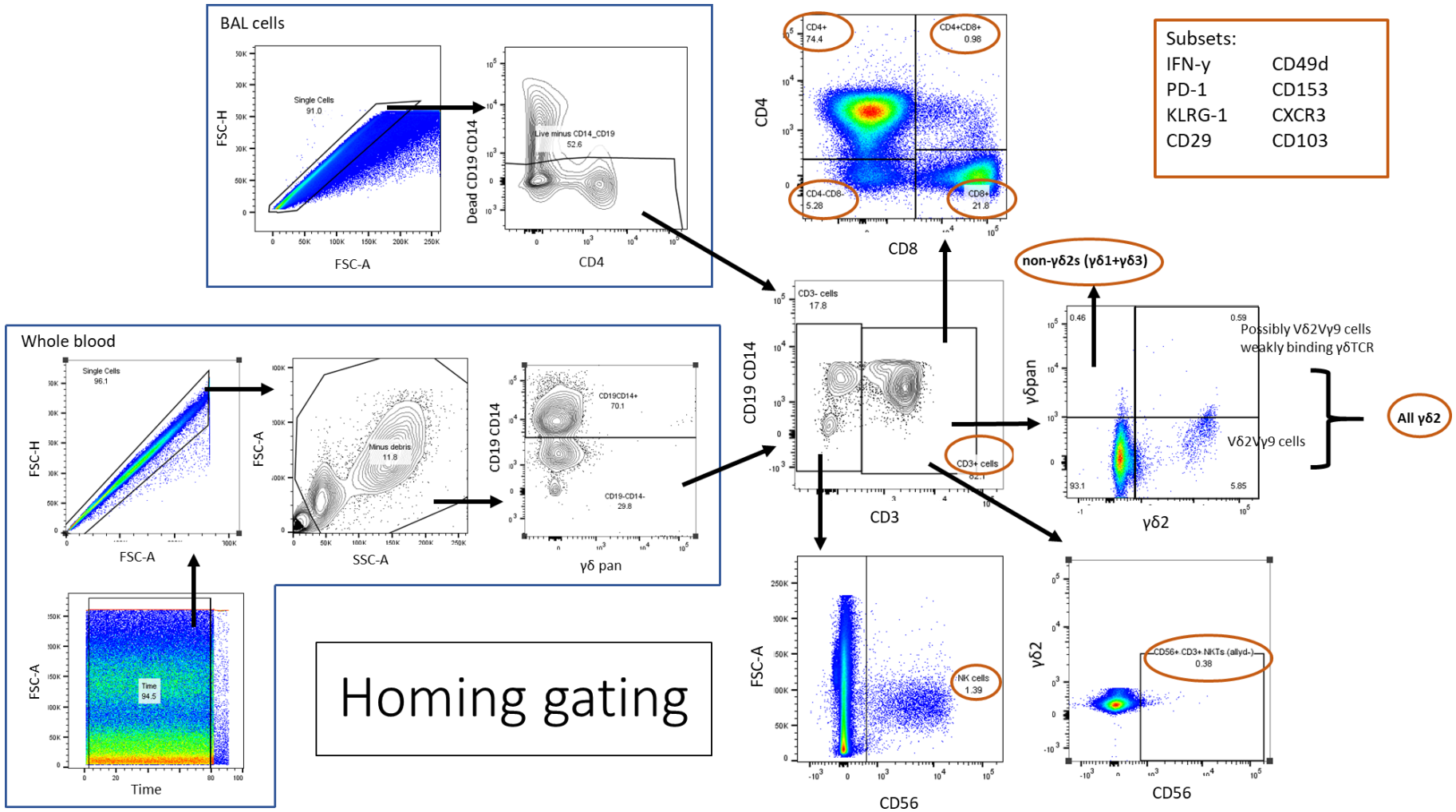


Figure 2.7. Homing gating strategy. Bronchoalveolar lavage (BAL) and whole blood (WB). Orange boxes and circles indicate cell endpoints.

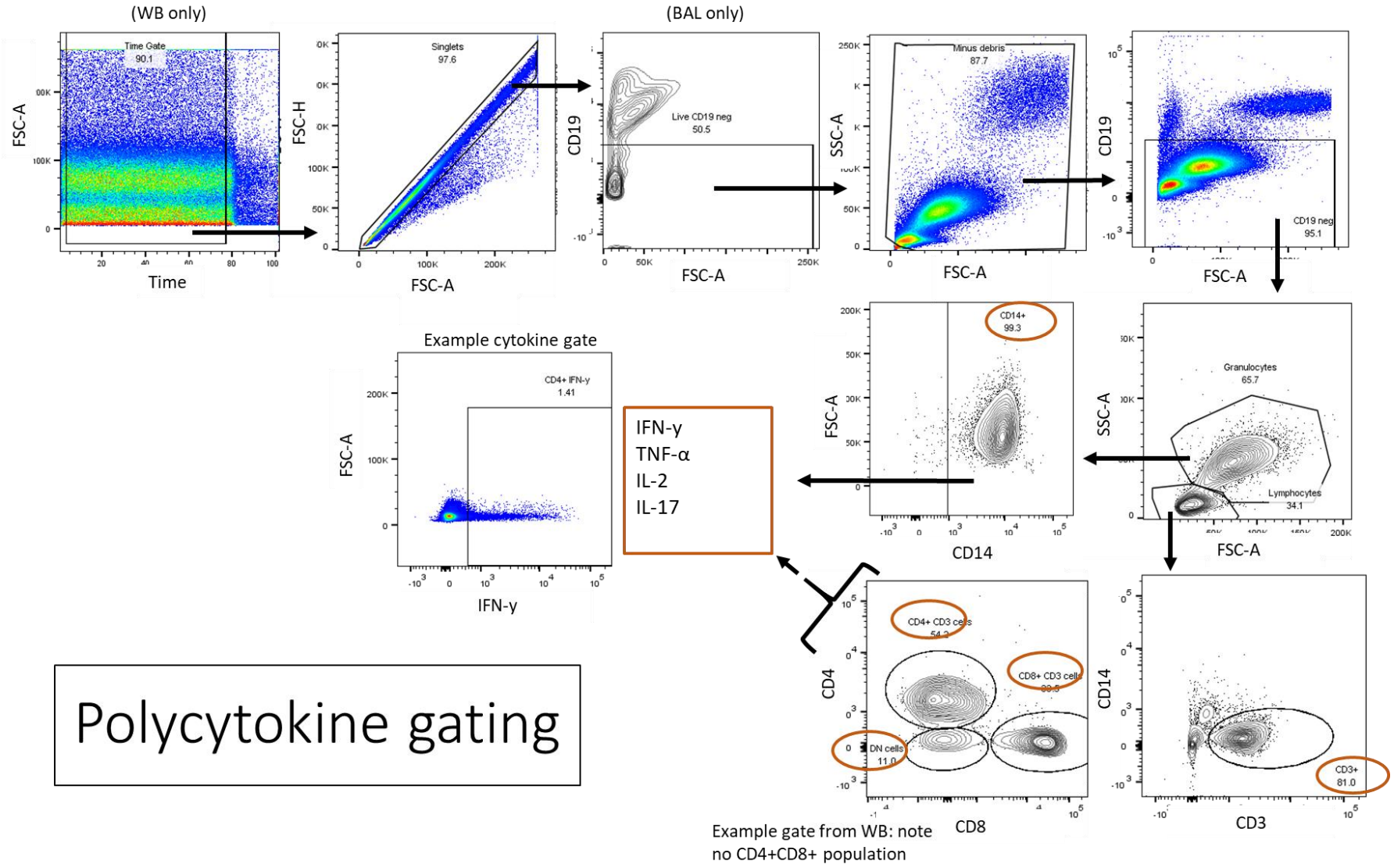


Figure 2.8. Polycytokine gating strategy. Bronchoalveolar lavage (BAL) and whole blood (WB). Orange boxes and circles indicate cell endpoints.

2.13 Single cell sequencing

This was performed on three BCG and three control volunteers per group on BAL cells only.

2.13.1 MACs bead separation

T cells from healthy individuals are as low as 3% total BAL cells and macrophages up to 95% total BAL cells.[503] Single cell sequencing utilises between 5000-10,000 cells for sequencing, which could mean as few as 250 T cells could be sequenced. In order to be able to capture T cell subset populations I decided to enrich the cell population for CD3+ cells while being aware we would therefore be unable to analyse cell proportions by 10x.

Following BAL cell counting, Group 1 (D2 bronchoscopy) cells for 10x single cell sequencing were filtered with a 70µl cell strainer into a 50ml pre-cooled falcon tube and then positively selected for CD3+ cells using MACs™ CD3 microbeads and an MS column as per manufacturer's instructions. This protocol results in 30% epitope "light-touch" attachment per cell, allowing ongoing functionality and availability for TCR sequencing.[504] MACs buffer was used for bead incubation and the first column wash, and Enriched buffer for the last two column washes, to collect CD3-, and flush through to collect CD3+ cells (see Table 2.4. Solutions). Cells were centrifuged (1800rpm, 5min, 4°C) and re-suspended in 1ml pre-chilled Resuspension media for counting. The plan was to send $0.5x10^6$ cells in a 1:1 CD3-:CD3+ ratio for sequencing however at the time the Casycounter was giving high background reads and our Countess was faulty (and was replaced by the manufacturer). As I was in the process of gaining skills in haemocytometer counting, I made the decision to recombine the separated suspensions without further manipulation of cell ratios. Cells were then sent to the Wellcome Trust for library preparation. To ensure consistency across the group, all Group 1 cells sent for sequencing were processed this way. However, for Group 2, I made the decision to send

the cells directly without MACs manipulation. This was because the two groups were part of a larger trial and I wanted to ensure Group 2 sequencing could be compared with subsequent Grp3-5, with as little cell manipulation as possible. I sequenced two volunteer samples (saline D14 bronchoscopy) with and without MACs bead processing. One sample was mislabelled during library preparation and so was not usable. The results from the second volunteer are discussed in Chapter 4.

2.13.2 scRNA-seq

The 10x Chromium platform was used and libraries prepared at the Wellcome Trust Genomics Facility. Cells were re-suspended in Resuspension media and sent on ice to the Wellcome Trust facility in a 1.5ml Eppendorf at a concentration of 1000 cells/µl (live and dead; viability >90%; 5-30µm) in a volume of at least 100µl. The libraries were created as per manufacturer's instructions using 10x single cell RNA 5 prime kits (v1.1), reference genome GRCh38 (human38 (Genome Reference Consortium). Target number of cells per channel was 5000 and two channels were used per sample (with the pooled count of 10,000 cells sequenced). Libraries were sequenced on a Novaseq 6000 S4 flowcell (150 paired-end configuration). Accepted minimum sequencing depth was 30,000 mean reads per cell and 50% sequencing saturation.

2.13.2.1 scRNA-seq data alignment and sample aggregating

All analysis was performed on R version 4.0.2 (see Appendix C for packages used). The Cell Ranger Software Suite (v.3.1.0) was used to perform sample de-multiplexing, barcode processing and single-cell 5' unique molecular identifier (UMI) counting. FASTQs were aligned to the human reference genome GRCh38-3.0.0. The following criteria were then applied to each cell in all samples; gene number between 250 and 7,000, UMI count > 500 and

mitochondrial gene percentage < 0.1, log10 Genes Per UMI > 0.80. Doublets were removed using the "scDblFinder" library. Genes with 10 read counts or less were removed. After filtering, a total of 81898 cells were left for the following analysis.

2.13.2.2 Dimensionality reduction and clustering

The filtered gene-barcode matrix were normalized using the SCTransform method with Seurat v.3.[505] Variables including S.Score, G2M.Score, mitochondria Ratio, nUMI and nGene were regressed out and the top 2000 variable genes were used. To remove batch effects across different participants, filtered gene-barcode matrix of all samples was integrated with Seurat v.3 using the reference-based method in which one sample from each group was set as a reference. Then UMAP was performed on the top 40 principal components for visualizing the cells. Graph-based clustering was performed on the PCA-reduced data for clustering analysis and the resolution was set to 2 to obtain a finer result.

2.13.2.3 Marker identification

Gene markers that identified each cluster relative to all other clusters were determined using FindAllMarkers function in Seurat using a Wilcoxon test. The log fold change threshold was set to 0.10. Initial cell prediction was performed using the SingleR package and Blueprint, Monaco, Human cell atlas, Immune and Cord blood data packages.[506] However these packages were not designed for BAL RNA prediction[507,508] and were not helpful in establishing cluster identities for this BAL cell population. The final cell labelling was performed manually, after inspecting the top markers genes for each cluster.[408,509-513]

2.13.2.4 Differential gene expression analysis

Differential gene expression analysis for each cluster against 2 different groups was performed using MAST test[514] with FindAllMarkers function in Seurat v.3. Differentially expressed genes were selected based on a log fold change threshold of at least 0.25, where the gene was expressed in at least 25% cells, in one or both of the two groups.

2.14 Statistical analyses

Statistical analysis was performed using GraphPad Prism Software 8 and SPSSv25. Comparisons between timepoints which were made against baseline only unless otherwise stated. A Shapiro-Wilk test was performed for normality testing. Comparisons were made using a mixed-effects analysis corrected with Dunnett's for parametric data, or Wilcoxon corrected with Holm-Bonferroni for non-parametric data. Comparisons between groups were made using a Mann-Whitney or unpaired t-test for normally or non-normally distributed data respectively. Single cell sequencing analysis was performed as outlined under section 2.13. Sc RNA-seq. Correlations were performed using IBM SPSS v25, using Spearman Rho and splitting comparisons by Group (D2 or D7) for variables with only D2 or D7 datapoints. Missing values at D2 and D7 (half volunteers each group) for WB ICS were not random and hence results at these timepoints may be underestimating (due to reduced power) or over estimating significance (due to non-randomness). The missing values at D56 for three volunteers are considered to be random.

2.15 Statement of authorship

This thesis is an original body of work written by J.Marshall, who led on every aspect of the clinical trial and sample processing, with the help of a team. The specific contribution of each individual to the project is outlined in each results chapter. J.Marshall performed the data analysis for all variables in this thesis, and produced each figure and table, unless otherwise stated. Help and advice for data analysis was received from N. Williams, M.Surakhy, R. Tanner, S.Harris, I. Satti and H. McShane.

Chapter 3.

BCG Aerosol Challenge Studies

3 BCG Aerosol Challenge Studies

3.1 Introduction

This chapter presents the BCG aerosol human challenge model developed in our group, which was designed to mimic the natural infection route of *M.tb*, to understand the initial Mycobacterial immune responses. BCG was delivered to healthy UK adults using the Omron nebuliser which generates particles up to 4µm.[515,516] This mimics the hypothesised optimum *M.tb* infective particle size of 0.5-5µm, which enables deposition into the bronchial tree and alveoli.[517,518] As lead clinician, I ran the pilot phase 1 clinical trial, TB041, evaluating the safety and optimal dose of aerosol inhaled BCG challenge in healthy, BCG-naïve adults (ClinicalTrials.gov NCT02709278). The trial completed enrolment in October 2018, with the target dose of BCG Bulgaria (Intervax) successfully delivered. The safety results and assessment of dose delivery are presented here.

With optimum dose and safety established, a second study, TB043, commenced in 2019 where I delivered aerosol BCG Danish (AJVaccines) to healthy UK adults (ClinicalTrials.gov NCT03912207). While the primary aim of this second study was to define the early innate immune events and subsequent adaptive immune response after an aerosol mycobacterial infection, we continued to analyse safety parameters. BCG Danish safety data, delivered dose, and BCG Danish recovery from the lung are presented in this chapter.

3.2 Statement of Authorship

The original TB041 documents for Arm 1 were written by M.Riste. Subsequent amendments to the trial, including the addition of Arm 2 (BCG Bulgaria) were written by J.Marshall, with support in editing and submission by M Wilke, S.Vermaak, R.Lopez-Ramon, A.Lawrie and

L.Rask. All TB043 study documents in this thesis were written and submitted for ethical review by J.Marshall, with support in editing and submission by S.Vermaak, R.Powell-Doherty, A.Lawrie and R.Lopez Ramon. Volunteer recruitment coordinators over the two studies were N.Lella, M.Fuskova and P.Lane. For studies discussed here (Arm 2 TB041 and Group 1+2 TB043), J.Marshall performed the majority of screening, eligibility assessment, randomisation, volunteer enrolment, administration of BCG, safety assessment and monitoring, medical supervision of volunteers at bronchoscopy and performance of intradermal biopsies for TB041. Assistance with these tasks for TB041 Arm 2 was provided by M.Wilke, and for TB043 Group 2 volunteers by H.Morrison. The majority of bronchoscopies were performed by H.Bettinson, with help from T.Hinks and L.Pei Ho. Nursing support for BCG administration, volunteer supervision at bronchoscopy, follow-up visits and running the clinics was performed by R.Lopez Ramon, F.Ramos Lopez, I.Poulton, M.Baker, C.Mitton, L.Kingham-Page and O.Griffiths. R.Lopez-Ramon was responsible for vaccine control, study monitoring compliance, and management of the clinic for all TB studies. Standard Operating Procedure (SOP) documents for both trials were written by J.Marshall and R.Lopez Ramon. For TB041, the Birmingham staff F.C.Stead, A.Finnegan, A.Williams, T.McCarthy, E.Oates and F Evans, provided support for follow up visits and screening clinics at the NIHR Wellcome Trust Birmingham Clinical Research Facility. Data management support for the E-diary and Openclinica systems was provided by S.Pawluk, D.Marshall-Searson, S.Ziyenge, J.Quaddy and F.Niaz. Randomisation envelopes were provided by G.Reilly and N.Williams. J.Marshall assigned causality in partnership with D.Jenkins, H.Morrison and S.Jackson (including independent and second assessment). S.Harris, J.Marshall, R.Wittenberg and D.Wright plated and counted the BCG from BCG vials. S.Harris, E.Stylianou and J.Marshall performed the impinger experiment. J.Marshall, S.Harris and H.Morrison extracted the DNA and performed the HAIN from the BCG MGIT.

3.3 Ethical and regulatory approval

3.3.1 TB041

The TB041 trial protocol and associated documents were approved by the South Central Oxford A Research Ethics Committee (REC), chaired by Dr Hugh Davies, on 14 January 2016 (REC: 15/SC/0716). These documents were approved by the regulatory authority, the Medicines and Healthcare products Regulatory Agency (MHRA) on 18th January 2016. Health Research Authority (HRA) approval was given to run the trial over two study sites, at Oxford University Hospitals and at Birmingham Queen Elizabeth Hospital attached to the NIHR Wellcome Trust Clinical Research Facility. From 15th April 2016 through 12th October 2017, five substantial amendments were submitted and approved by the REC and three were submitted to and approved by the MHRA. I have outlined the main reasons for these amendments in Chapter 2: Materials and Methods.

3.3.2 TB043

The TB043 study protocol and all documents were approved by the South Central Oxford A REC, chaired by Dr Hugh Davies, on 20 September 2018 (REC: 18/SC/0307). After discussion with the MHRA, it was decided that no regulatory approval was required, as the use of BCG in this study was classed as an infectious agent, not a vaccine. HRA approval to run the study at one site, Oxford University Hospitals NHS Trust, was sought and obtained.
Both studies were run accordingly to Good Clinical Practice (GCP) procedures.

3.4 Volunteer enrolment and demographics

3.4.1 TB041

75 subjects attended a screening visit and were formally assessed for eligibility across the two sites (Figure 3.1). Nine withdrew consent prior to enrolment and 20 were excluded due to the reasons outlined in Table 3.1. 13 volunteers were enrolled into Arm 1 (Oxford n=12 and Birmingham n=1) and 33 volunteers into Arm 2 (Birmingham n=9). The first TB041 volunteer was screened on 7th April 2016, and enrolment commenced into Arm 1 (BCG Danish) on 20th April 2016 and Arm 2 (BCG Bulgaria) on 12th April 2017. The final TB041 volunteer was enrolled on 30th October 2018 and the last volunteer last visit (LVLV) took place on 16th April 2019.

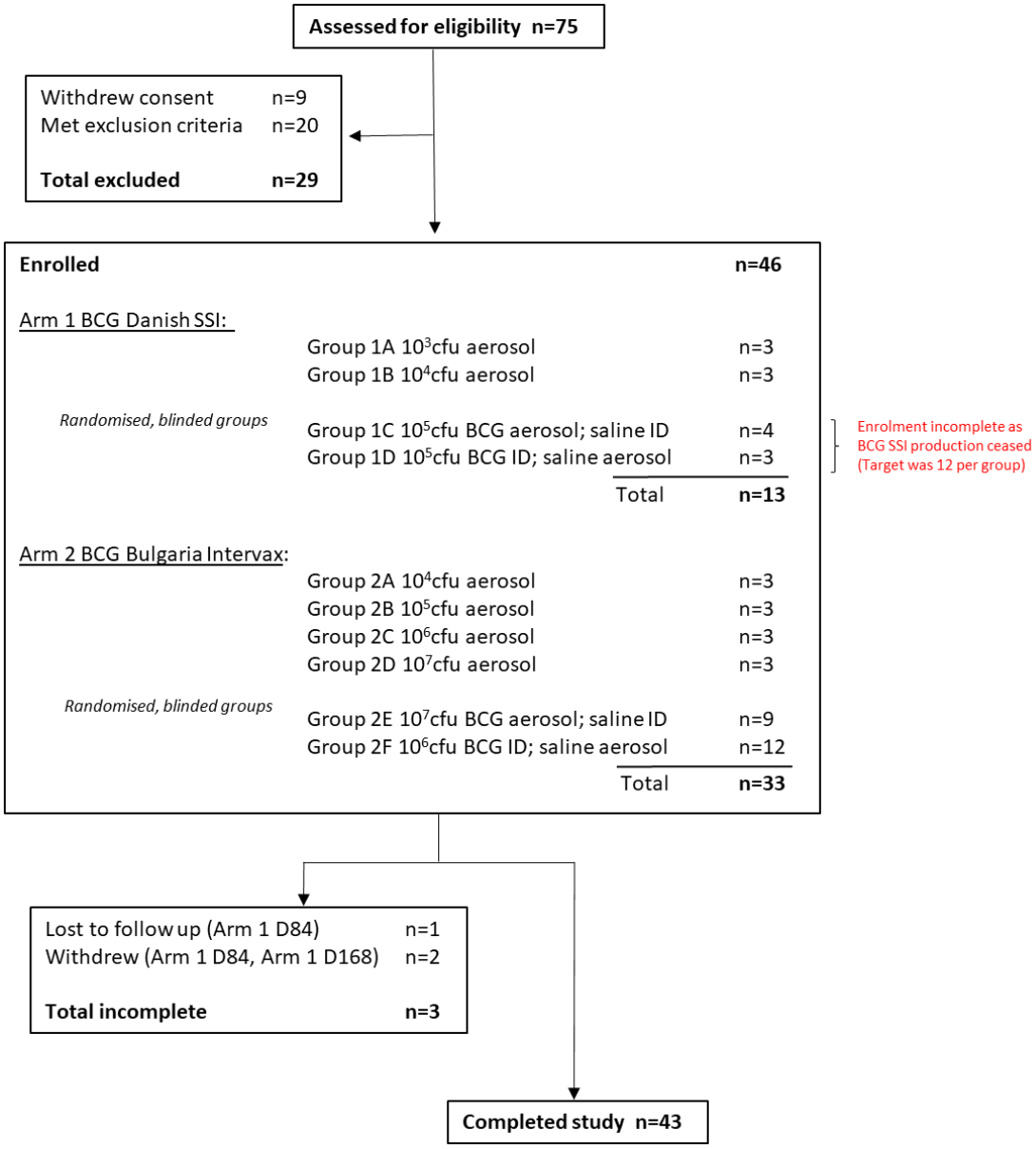


Figure 3.1. TB041 Consort Diagram for Arms 1 and 2

Table 3.1. Reasons for exclusion at screen. TB041 Arm 1 and Arm 2

	Previous BCG vaccination	Respiratory disease or poor lung function	Blood dyscrasias	Other	Total
Oxford	2	5	8	1	16
Birmingham	0	2	2	0	4
Total	2	7	10	1	20

* Blood dyscrasias: eosinophilia, elevated liver enzymes, anaemia, thrombocytopaenia

3.4.2 TB043

For Groups 1 and 2 of TB043, 40 adults attended a screening visit at the CCVTM in Oxford (Figure 3.2). Reasons for exclusion are shown in Table 3.2. The first TB043 volunteers were screened on 19th March 2019 and enrolment commenced on 29th April 2019. The final volunteer was enrolled on 12th February 2020 and LVLV was 30th July 2020.

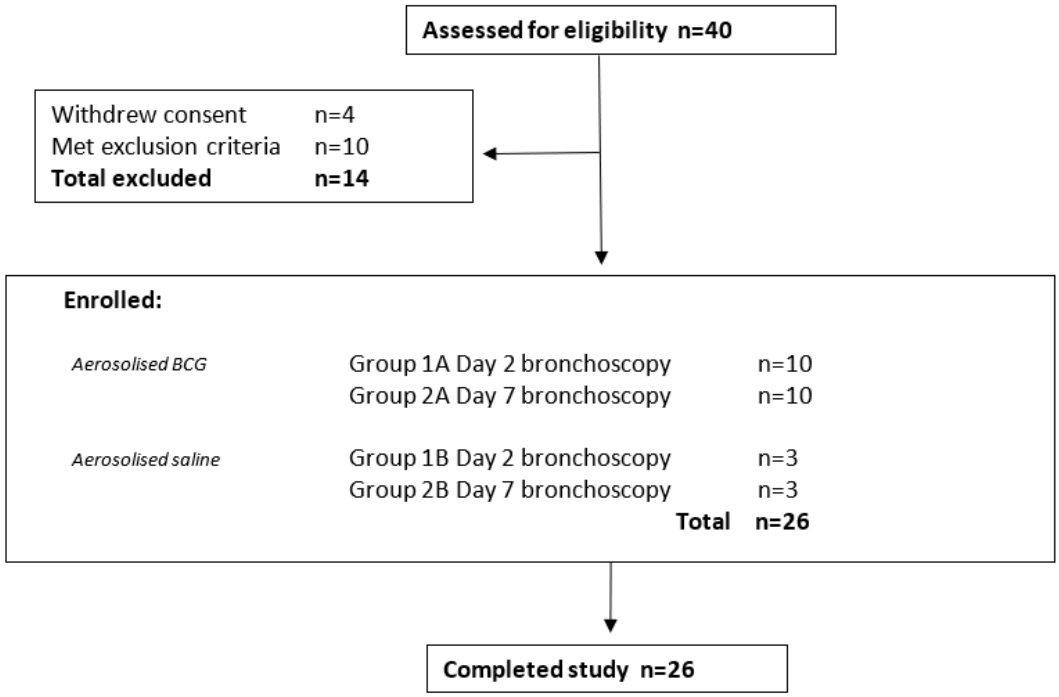


Figure 3.2. Consort diagram TB043

Table 3.2. Reasons for exclusion at screen; TB043.

Latent TB (IGRA positive)	Respiratory disease or poor lung function	Blood dyscrasias	Total
2	6	2	10

3.4.3 Exclusion and withdrawal TB041 and TB043

Prior to attending screening, volunteers completed a pre-screen questionnaire. Volunteers deemed likely ineligible due to this questionnaire were not invited to screening. Top reasons for exclusion on questionnaire were a medical history of asthma, or previous BCG vaccination.

Main reasons for exclusion at screening visit were medical comorbidities. Reasons for withdrawal included change in work or study circumstances affecting availability. Withdrawal rates for TB041 and TB043 are broadly typical of that seen in other trials in our unit.

3.4.4 Impact of COVID-19 pandemic

In TB041, no visits were missed for enrolled volunteers, other than after withdrawal (as outlined in Figure 3.1). In TB043, altered or missed visits are shown in Figure 3.3. Volunteer TBT-04301028's Day 56 visit was missed due to unforeseen volunteer travel. All other changed visits were due to the lockdown of university facilities during the 2020 COVID-19 pandemic.[519] TB043 was paused to all face-to-face visits from 23rd March through to the beginning of July 2020, to comply with UK government policy and regulatory advice. Adverse event (AE) data was recorded via telephone at these time points, and blood was not collected. When follow up visits recommenced, induced sputum procedures were not performed, to reduce the risk of spread of SARS-CoV-2 virus due to aerosol generating procedures. I will discuss the consequences of these missing immune parameters in later chapters.

Group	Vol no.	D0 Challenge	**D2 Bronch**	D7	D14	D28	D56	D84	D168
1A	TBT-04301003	29/04/2019	**01/05/2019**	06/05/2019	13/05/2019	27/05/2019	24/06/2019	22/07/2019	14/10/2019
1A	TBT-04301007	22/05/2019	**24/05/2019**	29/05/2019	05/06/2019	19/06/2019	17/07/2019	22/08/209	06/11/2019
1A	TBT-04301008	11/06/2019	**13/06/2019**	18/06/2019	25/06/2019	09/07/2019	20/08/2019	03/09/2019	26/11/2019
1A	TBT-04301010	15/07/2019	**17/07/2019**	22/07/2019	29/07/2019	14/08/2019	10/09/2019	07/10/2019	30/12/2019
1A	TBT-04301017	15/07/2019	**17/07/2019**	22/07/2019	29/07/2019	12/08/2019	09/09/2019	07/10/2019	30/12/2019
1A	TBT-04301018	15/07/2019	**17/07/2019**	22/07/2019	29/07/2019	12/08/2019	09/09/2019	07/10/2019	30/12/2019
1A	TBT-04301019	12/08/2019	**14/08/2019**	19/08/2019	26/08/2019	09/09/2019	07/10/2019	04/11/2019	27/01/2020
1A	TBT-04301020	12/08/2019	**14/08/2019**	19/08/2019	26/08/2019	09/09/2019	07/10/2019	04/11/2019	27/01/2020
1A	TBT-04301021	12/08/2019	**14/08/2019**	19/08/2019	26/08/2019	09/09/2019	07/10/2019	04/11/2019	27/01/2020
1B	TBT-04301005	29/04/2019	**01/05/2019**	06/05/2019	13/05/2019	27/05/2019	24/06/2019	22/07/2019	14/10/2019
1B	TBT-04301006	22/05/2019	**24/05/2019**	29/05/2019	05/06/2019	19/06/2019	17/07/2019	20/08/2019	06/11/2019
1B	TBT-04301011	22/05/2019	**24/05/2019**	29/05/2019	05/06/2019	19/06/2019	17/07/2019	07/08/2019	06/11/2019
Group	Vol no	D0 Challenge	D2	**D7 Bronch**	D14	D28	D56	D84	D168
2A	TBT-04301024	11/09/2019	13/09/2019	**18/09/2019**	26/09/2019	09/10/2019	06/11/2019	03/12/2019	26/02/2020
2A	TBT-04301026	23/10/2019	25/10/2019	**30/10/2019**	06/11/2019	20/11/2019	18/12/2019	14/01/2020	26/03/2020
2A	TBT-04301027	09/10/2019	11/10/2019	**16/10/2019**	23/10/2019	06/11/2019	04/12/2019	02/01/2019	26/03/2020
2A	TBT-04301028	20/11/2019	22/11/2019	**28/11/2019**	10/12/2019	19/12/2019	~~14/01/2020~~	13/02/2020	07/05/2020
2A	TBT-04301029	23/10/2019	25/10/2019	**30/10/2019**	06/11/2019	20/11/2019	02/01/2020	13/01/2019	30/03/2020
2A	TBT-04301030	23/10/2019	25/10/2019	**30/10/2019**	06/11/2019	20/11/2019	18/12/2019	14/01/2019	27/03/2020
2A	TBT-04301033	20/11/2019	22/11/2019	**28/11/2019**	09/12/2019	18/12/2019	14/01/2019	18/02/2020	07/05/2020
2A	TBT-04301031	21/01/2020	23/01/2020	**29/01/2020**	04/02/2020	18/02/2020	30/03/2020	21/04/2020	30/07/2020
2A	TBT-04301037	21/01/2020	23/01/2020	**29/01/2020**	04/02/2020	18/02/2020	04/03/2020	29/04/2020	16/07/2020
2A	TBT-04301042	12/02/2020	14/02/2020	**19/02/2020**	26/02/2020	11/03/2020	07/04/2020	06/05/2020	16/07/2020
2B	TBT-04301025	15/01/2020	17/01/2020	**22/01/2020**	29/01/2020	13/02/2020	11/03/2020	27/03/2020	16/07/2020
2B	TBT-04301034	15/01/2020	17/01/2020	**22/01/2020**	29/01/2020	12/02/2020	11/03/2020	24/03/2020	16/07/2020
2B	TBT-04301038	15/01/2020	17/01/2020	**22/01/2020**	29/01/2020	12/02/2020	11/03/2020	24/03/2020	16/07/2020

Completed
Telephone visit instead
Visit but no Induced sputum
Visit out of window (10 weeks+1day)
~~Missed~~ Missed visit

Figure 3.3. TB043 schedule outlining missed or changed visits

Demographic data for TB041 (target dose comparator groups in Arm 2) and TB043 (groups 1 and 2) are in Table 3.3 and Table 3.4 respectively. Normality tests were performed and data is presented as mean or median accordingly, with comparisons calculated as two-tailed Mann–Whitney of medians or unpaired two-tailed t-test of means, controlling for multiple comparisons.

For TB041, there was no significant difference between groups for any characteristic outlined. Importantly, baseline respiratory function was similar between the aerosol and ID groups.

For TB043, demographic data is split by condition (BCG or saline) and also by day of bronchoscopy (Table 3.4). Within group and between group comparisons were performed. In the following chapters I will be presenting immunological data based on the day of bronchoscopy and hence it is important to examine any differences in baseline characteristics between the groups. After controlling for multiple comparisons, there were no differences in any characteristics between groups. As there were only three saline volunteers per group, the ability to detect any differences is limited. As for TB041, in three of the four groups, the majority of TB043 volunteers were female. However, the Day 7 (D7) saline group had two males and only one female. For TB043, we collected both country of birth and ethnic origin. All but two volunteers were Caucasian, (D2 BCG group).

Table 3.3. Baseline characteristics, TB041

Characteristic	Group 2D/2E BCG Aerosol (n = 12)	Group 2F BCG ID (n = 12)
Female, n (%)	8 (67%)	9 (75%)
Mean age in years (range)	26.2 (18-46)	24.3 (18-46)
BMI (kg/m^2) median (range)	25.0 (22-34)	26.4 (20-41)
Spirometry		
Mean % predicted FEV_1 (range)	96.6 (85-112)	98.0 (83-116)
Mean % predicted FVC (range)	94.7 (82-111)	100.4 (85-116)
Mean % FEV_1/FVC (range)	86.0 (76-93)	83.7 (78-93)
Median baseline % SaO_2 (range)	98.0 (96-100)	98.0 (97-99)
Country of birth		
UK	10	10
Europe	1	1
Oceania	1	1

Table 3.4. Baseline characteristics, TB043

Characteristic		BCG Aerosol (n = 20)		Saline aerosol (n = 6)	
		Group 1A (bronchoscopy D2)	Group 2A (bronchoscopy D7)	Group 1B (bronchoscopy D2)	Group 2B (bronchoscopy D7)
Female, n (%)		8 (80%)	7 (70%)	3 (100%)	1 (33%)
Median age in years (range)		27.8 (19-45)	23.4 (19-45	29.1 (28-31)	22.2 (19-31)
BMI (kg/m^2) mean (range)		25.5 (20-39)	24.4 (20-35)	25.0 (21-28)	24.5 (24-25)
Lung Function					
Median % predicted FEV_1 (range)		109 (94-120)	95 (85-111)	110 (87-112)	101 (88-116)
Median % predicted FVC (range)		110 (97-123)	98 (84-116)	112 (97-118)	100 (97-111)
Median % FEV_1/FVC (range)		97 (93-104)	99 (88-107)	94 (90-99)	100 (90-105)
Median baseline % SaO_2 (range)		98 (96-100)	98 (97-100)	98 (97-98)	98 (98-99)
Median % predicted TLCO (range)		107 (92-125)	97 (85-119)	108 (100-123)	110 (95-117)
Country of birth	UK	6	10	2	3
	Australia	2			
	Germany			1	
	Canada	2			
Ethnicity	Caucasian	8	10	3	3
	Chinese	1			
	Mixed heritage	1			

3.5 Safety

3.5.1 TB041

There were no serious adverse reactions (SARs) nor suspected unexpected serious adverse reactions (SUSARs). There were no withdrawals due to safety concerns and no pre-defined trial stopping or holding rules were activated.

There was one Serious Adverse Event (SAE). The volunteer was admitted to hospital for pain management for a pre-existing ovarian cyst, 52 days after $1x10^7$ cfu BCG aerosol challenge. The volunteer was discharged on oral analgesia with planned follow up in outpatient clinic. They completed the study with no further complications. The SAE was unrelated to BCG challenge or other trial procedures.

Arm 1 (BCG Danish SSI) dose escalation groups were completed without any safety concerns and enrolment into the target dose comparator arms commenced. In 2016, Arm 1 ceased recruitment permanently prior to full enrolment due to worldwide shortage of BCG Danish as the Serums Statens Institute ceased production. Due to the low numbers recruited, further safety data is not shown here. As outlined in Chapter 2: Materials and Methods, I submitted a protocol amendment and recommenced the trial using BCG Bulgaria.

Arm 2 (BCG Bulgaria) dose escalation groups proceeded without any safety concerns. The target dose was reached and all groups were fully enrolled (Figure 3.1).

One bronchoscopy was delayed by a week as the volunteer was unwell (TBT-04101033). This was within the allowable window as per protocol. All others occurred either on D14 or D15 following BCG infection. All bronchoscopies following BCG aerosol inhalation were reported as normal.

3.5.2 TB043

There were no SAEs nor SUSARs. There were no withdrawals due to safety concerns and no pre-defined study stopping or holding rules were activated.

All bronchoscopies took place on D2 (Group 1) or D7 (Group 2) following aerosol inhalation.

One D2 bronchoscopy was reported as normal but with changes (TBT-04301003) and another D2 bronchoscopy as abnormal (TBT-04301018). The two reports were as follows:

TBT-04301003. Abnormal: Mild erythema throughout, airways friable, otherwise normal.

- This volunteer reported grade 1 shortness of breath, chest tightness and coughing up phlegm on D0. Symptoms resolved by D1 AM, with no further respiratory symptoms.

TBT-04301018. Normal: Mild mucosal erythema RML carina noted.

- This volunteer reported grade 1 cough and sore throat on D1, no other respiratory symptoms.

There was no further action recommended by the respiratory consultant for either bronchoscopy.

All other D2 BCG bronchoscopies, all D7 BCG bronchoscopies, and all bronchoscopies in the control volunteers were reported as normal.

Based on the findings from TB041, in TB043, I made some minor changes to safety data collection. We reduced the electronic diary from 1 month to 2 weeks and added two additional solicited AEs “tickly throat” and “chest tightness”, as these were common unsolicited AEs reported by volunteers who repeatedly distinguished these from the solicited AEs “sore throat” and “chest pain”.

We also altered lung function tests. We reduced the frequency of spirometry, only performing spirometry at clinic visits and not on volunteer daily electronic diary. This was primarily because such frequent readings were no longer required due to the reassuring

spirometry data from TB041 (see below). We were comfortable that delivering BCG by aerosol did not induce hyper-responsive bronchospasm in this patient demographic in this sample size.

In addition, there were data quality and logistical reasons for ceasing home spirometry readings. Spirometry is by nature effort dependent.[520] In clinic, this effect can be minimised with coaching, recording the best of three consistent blows (within 5% variation) and observed good effort.[520,521] Unobserved daily spirometry performed at home is more variable and may not be reflective of true lung function. Further, there was significant logistical burden in home spirometer readings. Best of three daily readings for a full month required a significant amount of time commitment, and volunteers needed to remember to carry the spirometer with them whenever they travelled. Significant staff time was also required to supply cleaned, charged spirometers for each volunteer and to chase returns.

A second change to the lung function recording for TB043 was the addition of routine TLCO measurements (transfer capacity of the lung for carbon monoxide uptake). Spirometry readings record changes to flow rates and therefore detect airway or mechanical changes. Spirometry is unable to detect changes in gas exchange and parenchyma pathology.[520] The addition of TLCO was on the advice of the respiratory physician in our Safety Monitoring Committee (SMC). We also felt it was valuable to have baseline TLCO readings (taken at screening) as in TB041 we had a volunteer with significant respiratory symptoms, for whom we performed full lung function to clinically investigate their symptoms. Interpretation of the results was difficult as we didn't have baseline TLCO readings (see section 3.5.5.6).

3.5.3 Data presentation

Where indicated, data is presented as percent of solicited occurrences, to show duration and frequency of the AE in one read-out. This normalisation of readings allows comparison when groups have different numbers of volunteers, or when follow-up periods are different.

The first two sets of figures show solicited AEs reported by group following BCG infection or bronchoscopy. The first figure (Figure 3.4, Figure 3.12, Figure 3.16 or Figure 3.19) presents the data as frequency and maximum severity for each solicited AE reported at any point over the two week period. The next figure in the series (Figure 3.5, Figure 3.13, Figure 3.17, or Figure 3.20) shows the same data but presented by individual. AEs are presented as percent solicited to show AE duration. I then present tables which document the frequency, duration and maximum severity by volunteer arising immediately post infection or bronchoscopy and within a discrete time period (48 or 72 hours).

In TB041 bronchoscopy occurred at D14 and the e-diary continued for two weeks following bronchoscopy. In order to differentiate AEs possibly due to BCG infection from that of bronchoscopy, AE data is split based on bronchoscopy timing (2 weeks post-infection and then 2 weeks post-bronchoscopy).

TB043 volunteers had their bronchoscopies at earlier time points (D2 and D7), making distinguishing AEs due to BCG infection or bronchoscopy more difficult. To compare TB043 and TB041 data I have presented the solicited AEs in two ways. Left hand figures show a full two weeks of data as with TB041; however, unlike TB041, these volunteers all had a bronchoscopy sometime during that period. The figures on the right therefore show AEs which occurred exclusively following BCG infection (or saline inhalation) but *before* bronchoscopy. For half the TB043 volunteers, this is two days of AEs and for the other half, seven days.

3.5.4 TB041 safety data following BCG Bulgaria infection, before bronchoscopy

3.5.4.1 Systemic AEs

Following infection with BCG Bulgaria, volunteers in both groups reported a range of systemic symptoms, consistent with known AEs from ID BCG vaccination (Figure 3.4a). For both groups the most frequent systemic AEs were headache and fatigue. There was no difference in the incidence of these systemic AEs by route (Figure 3.5b). However, if a volunteer were to experience symptoms, these symptoms were more likely to be reported as severe in those who had inhaled BCG (Table 3.5a).

Table 3.6 shows duration, frequency and severity of each solicited AE reported by each volunteer if arising within the first 72 hours. The majority of symptoms by either route were mild and all were transient. There was no difference between groups when analysing each symptom either by incidence and maximum severity (first column), or by duration of that symptom (second column).

3.5.4.2 Respiratory AEs

Despite the ID volunteers inhaling only saline, both groups reported respiratory AEs (Figure 3.4b). There was no difference in frequency or severity of respiratory symptoms by route, before bronchoscopy (Figure 3.5c and Table 3.5).

For both groups, the most common respiratory AE after BCG infection was cough or sore throat. Three (25%) aerosol BCG volunteers reported chest pain, as did three (25%) ID BCG volunteers. Other volunteers reported the unsolicited symptom, chest “tightness”, with no overt pain. Both chest pain and chest tightness were described as pleuritic in nature and they

were likely part of the same symptom spectrum. Hence, in order to ensure these symptoms were captured fully, I added reports of chest tightness into the solicited symptom analysis presented here.

Volunteers who received BCG by aerosol tended to report more chest pain/tightness than ID BCG volunteers, but this did not reach statistical significance when looking at symptoms arising within the first 72 hours (Table 3.6), nor over the first 2 weeks. (67% versus 25% of volunteers in first 2 weeks, median percent possible occurrences (IQR): 0 (0-0.266) aerosol vs 0 (0-0.047) ID, p=0.190, maximum severity p=0.362).

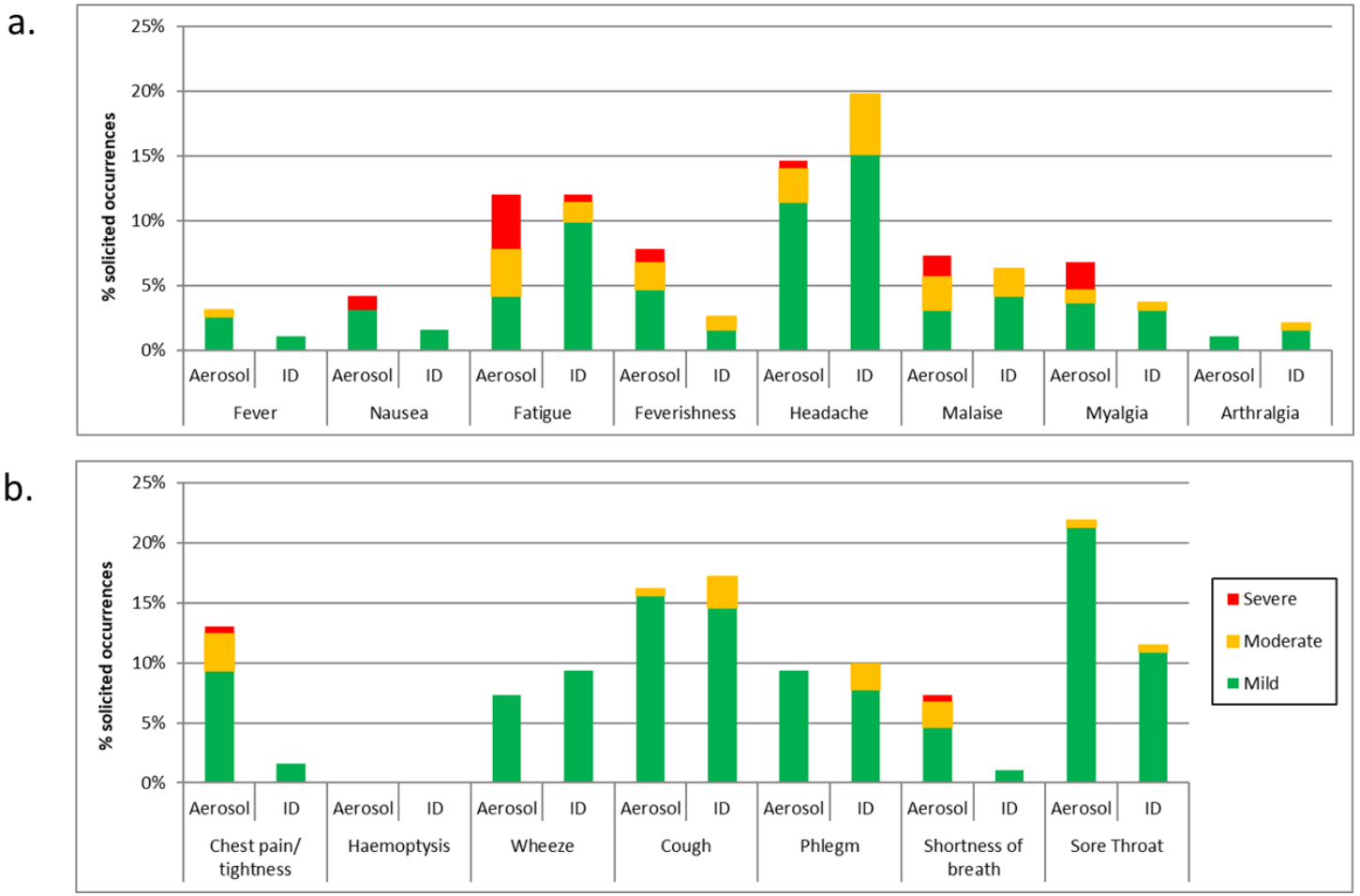


Figure 3.4. TB041: Percent of (a) systemic, or (b) respiratory AEs by symptom, out of total solicited occurrences reported on volunteer electronic diary for two weeks following BCG Bulgaria challenge and prior to bronchoscopy. Arm 2 target dose comparator groups. 12 volunteers per group. ID: Group 2F (Intradermal route). Aerosol: Group 2D (3 un-blinded volunteers aerosol BCG Bulgaria $1x10^7$ cfu), and 2E (randomised blinded volunteers aerosol BCG Bulgaria $1x10^7$ cfu and ID saline). AEs were collected every 12 hours for 2 days following challenge then daily. Total solicited occurrences calculated as 12 volunteers x 16 time points.

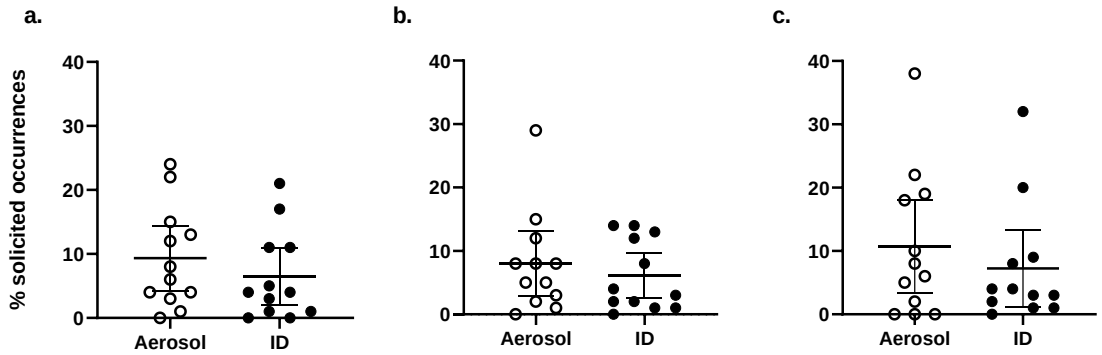


Figure 3.5. TB041 Percent of (a) total AEs, (b) systemic or (c) respiratory AEs by volunteer out of total solicited occurrences in the 2 weeks following BCG Bulgaria infection. All AEs occurred prior to bronchoscopy (performed 14 days following infection). AEs were collected every 12 hours for 2 days following challenge then daily. Total solicited occurrences calculated as; Total AEs: 15 possible AEs x 16 collected time points; Systemic: 8 systemic adverse events x 16 collected time points; Respiratory: 7 respiratory adverse events x 16 collected time points. N=12 per group. Aerosol: Group 2D/2E BCG Bulgaria $1x10^7$cfu aerosol; ID: Group 2F BCG Bulgaria $1x10^6$ cfu ID. No difference between groups by Mann Whitney. Each point represents one volunteer. Median and interquartile range shown.

Table 3.5. TB041 Severity by Group. Volunteers reporting AEs only. Significant results highlighted.

a. Number of Mild & moderate versus Severe adverse events

Time period	Aerosol (Grp 2D/E) vs ID (Grp 2F)	Significant difference?*
28 days post challenge	Total (Respiratory and systemic)	Yes, p=0.0001 Aerosol > ID
	Respiratory only	No
	Systemic only	Yes, p=0.0001 Aerosol > ID
First 2 weeks post challenge	Total (Respiratory and systemic)	Yes, p=0.001
	Respiratory only	No
	Systemic only	Yes p=0.0013 Aerosol > ID
2 weeks following bronchoscopy (Week 3 and 4 post challenge)	Total (Respiratory and systemic)	No
	Respiratory only	No
	Systemic only	No

b. Number of Mild versus Moderate & severe adverse events

Time period	Aerosol (Grp 2D/E) vs ID (Grp 2F)	Significant difference?*
28 days post challenge	Total (Respiratory and systemic)	Yes, p=0.0236 Aerosol > ID
	Respiratory only	No
	Systemic only	Yes, p=0.0207 Aerosol > ID
First 2 weeks post challenge	Total (Respiratory and systemic)	No
	Respiratory only	No
	Systemic only	No
2 weeks following bronchoscopy (Week 3 and 4 post challenge)	Total (Respiratory and systemic)	Yes, 0.0324 Aerosol > ID
	Respiratory only	Yes, 0.0468 Aerosol > ID
	Systemic only	No

*Chi square (Fisher's exact)

Table 3.6. TB041: Adverse events in the first 72 hours following BCG Bulgaria challenge by duration and severity

Adverse Event	Number of volunteers (frequency, max severity)			Median duration of AE Days (range)		
Route	Aerosol	Intradermal	p-value	Aerosol	Intradermal	p-value
Respiratory						
Cough	5 (0.42, 2)	7 (0.58, 2)	0.614	1.0 (0.5-24)	1.0 (0.5-7)	0.558
Sore throat	5 (0.42, 1)	4 (0.33, 2)	>0.999	1.0 (0.5-9)	0.5 (0.5-9)	0.603
Wheeze	2 (0.17, 1)	1 (0.08, 1)	>0.999	4.5 (1-8)	16	*
Dyspnoea	2 (0.17, 2)	1 (0.08, 1)	>0.739	2.75 (0.5-5)	2	*
Phlegm	2 (0.17, 1)	1 (0.08, 2)	>0.999	1.25 (0.5-2)	22	*
Chest pain/ tightness	4 (0.33, 2)	0	0.093	2.5 (0.5-8)	-	*
Systemic						
Temperature	3 (0.25, 2)	0	0.217	0.5 (0.5-1.5)	-	*
Arthralgia	2 (0.17, 1)	1 (0.08, 1)	>0.999	0.5	0.5	*
Myalgia	4 (0.33, 3)	2 (0.17, 1)	0.349	0.5 (0.5 -3)	0.5	*
Feverish	6 (0.50, 3)	2 (0.17, 1)	0.086	1 (0.5-2)	0.5	*
Headache	6 (0.50, 3)	7 (0.58, 2)	0.883	1.5 (1-3)	1 (0.5-3.5)	0.445
Fatigue	8 (0.67, 3)	6 (0.50, 3)	0.386	1.25 (0.5-3)	1.5 (0.5-3)	0.937
Nausea	4 (0.33, 3)	0	0.093	0.5 (0.5-2)	-	*
Malaise	4 (0.33, 3)	2 (0.17, 1)	0.252	1 (0.5-3)	2 (1-3)	*

N=12 per group. Aerosol: Group 2D/2E BCG Bulgaria 1x107cfu aerosol; ID: Group 2F BCG Bulgaria 1x106 cfu ID.
Grading range 1-3. Adverse events presented here are any that occurred within 72 hours following BCG aerosol challenge. No haemoptysis recorded in any volunteers within 72 hours following BCG challenge. Mann Whitney of median of frequency and severity of AEs, or median duration. Exact p-values calculated using tied ranks.
* p-value not calculated due to insufficient numbers

3.5.4.3 Lung Function

Peripheral oxygen saturation at clinic visits remained within the normal range for all volunteers except as anticipated during bronchoscopy, when oxygen was provided as part of routine practice.

Spirometry results in the first two weeks following BCG Bulgaria infection are presented in Figure 3.6a. Data is presented as a percent drop in volunteer baseline reading. Due to the

effort dependent, variable nature of spirometry, the trial's respiratory consultant Dr Bettinson advised we deem a fall in lung function greater than 15% as clinically significant. There was a trend for the ID group to record a slightly lower lung function on spirometry, though this was neither clinically significant nor significant on median area-under-the-curve analysis (FEV_1 median reduction in aerosol -30.4 (IQR -47.8 to -17.3); intradermal -58.7 (IQR -97.0 to -8.6), p=0.225. FVC median reduction in aerosol -41.8 (IQR -68.1 to -7.2); intradermal -14.8 (IQR -51.9 to -4.9), p=0.644 on Mann Whitney). There was also no difference in FEV_1/FVC ratio (data not shown).

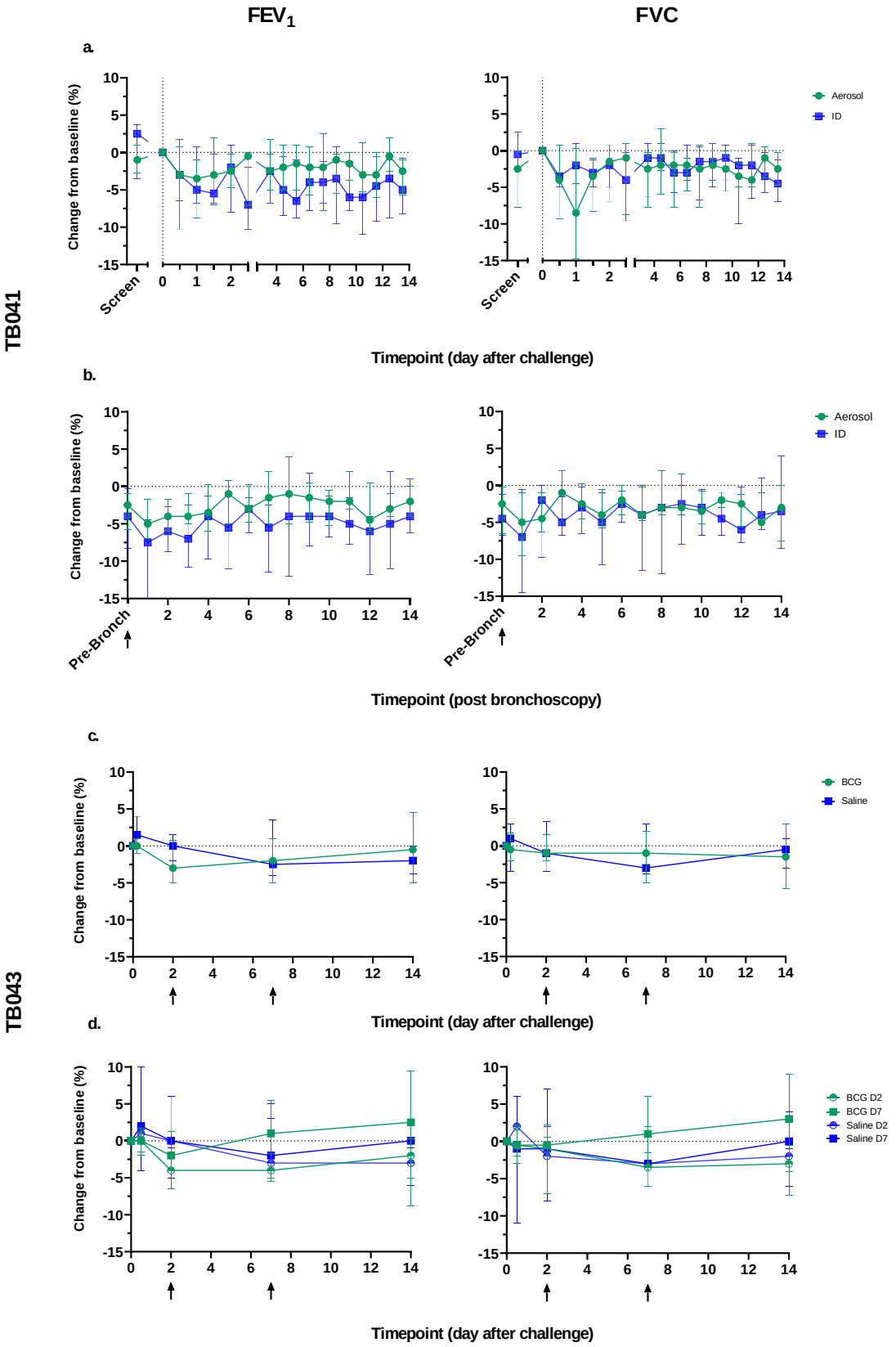


Figure 3.6. Median percent change in FEV_1 and FVC from volunteer's baseline reading. (a) TB041 Aerosol BCG Bulgaria (2D/2E) and ID (2F) groups in the fourteen days following challenge and prior to bronchoscopy; (b) TB041 Aerosol BCG Bulgaria (2D/2E) and ID (2F) groups in the fourteen days following

bronchoscopy; (c) TB043 volunteers grouped by experimental condition (Aerosol BCG Danish or saline); (d) TB043 groups (Aerosol BCG Danish or saline) split by condition and day of bronchoscopy. Arrows: pre-bronchoscopy reading. Median and interquartile range shown. Baseline taken from the volunteer's Day 0 pre-challenge reading. FEV_1: Forced expiratory volume in 1 second; FVC: Forced vital capacity. No significant difference in Area Under the Curve (AUC) between groups in TB041 or TB043 pre or post bronchoscopy. (a) TB041. Taken from volunteer e-diary. Collected every 12 hours for 2 days following challenge then daily hence note different scale Day0-Day3 vs Day4-Day13. (b) TB041. Taken from volunteer e-diary following bronchoscopy (performed 14 days post BCG aerosol or intradermal challenge). (c+d) TB043. Readings taken at clinic visits. Circles: volunteers had bronchoscopy at D2, after "D2" spirometry reading taken; squares: volunteers had bronchoscopy on day 7 after "D7" spirometry reading taken).

3.5.4.4 Blood parameters

Full blood count, liver function tests, kidney function and electrolytes were collected at screening, D7, D14 and D28 following BCG infection. There was one episode of lymphocytopaenia and one of lymphocytosis deemed possibly or probably related to BCG infection (Table 3.14 and Table 3.15).

C-reactive protein (CRP), as a marker of systemic inflammation (acute phase reactant) was performed at D2 and repeated if elevated. Five of 12 volunteers infected with high dose aerosol BCG Bulgaria had an elevated CRP at D2 and one of 15 ID volunteers (Figure 3.7). (Normal CRP <10mg/L in Birmingham lab). Volunteers with CRP greater than 30mg/L also had concurrent "flu-like symptoms".

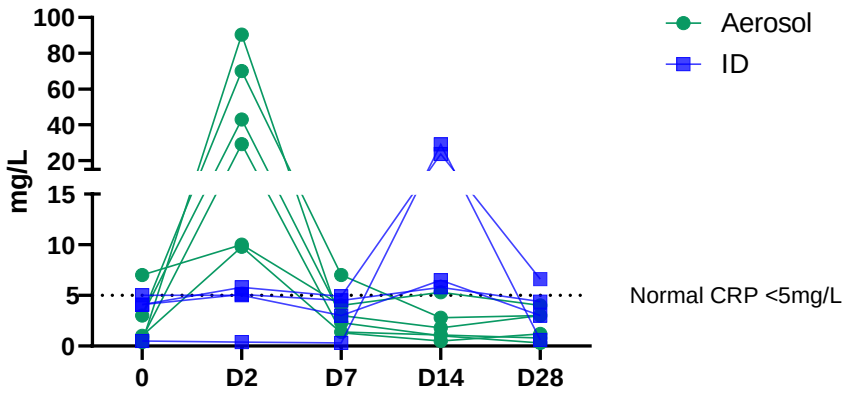


Figure 3.7. TB041: C-reactive protein levels (CRP) in volunteers recording elevated levels in the first month following BCG Bulgaria challenge. D14 blood test taken prior to bronchoscopy. Aerosol: Group2D/2E $1x10^7$ cfu aerosol BCG Bulgaria. ID: Group 2F $1x10^6$ cfu intradermal BCG Bulgaria. "0": screening blood test (up to 4 months prior to challenge). D: Days post challenge. D14: pre-bronchoscopy blood test.

3.5.4.5 Symptoms of note in aerosol BCG Bulgaria volunteers

For two volunteers in the high dose aerosol groups (2D/E), chest pain or tightness was accompanied by dyspnoea (TBT-04101057 and TBT-04101033). Both volunteers maintained their peripheral oxygen saturation.

TBT-04101057 reported systemic flu-like illness, including fever, as well as cough, chest tightness and shortness of breath from the evening of BCG inhalation. Spirometry dropped to 47% baseline (FEV_1 and FVC) at D1. Most symptoms resolved after 3 days and chest tightness had resolved by D9. The volunteer was reviewed at D8 where observations and examination, including spirometry, was normal. TLCO was 66.80% predicted, however we had no baseline. There was a corresponding increase in CRP to 90mg/L and a Grade 1 lymphocytosis. Blood cultures were negative, as was a viral respiratory PCR on nasal swab (performed at D2). Chest x-ray was performed prior to bronchoscopy due to the aberrant PFTs, and this was normal. Repeat TLCO 1 month later was low normal and symptoms had resolved. The volunteer was referred to our respiratory consultant for review and a diagnosis of possible hypersensitivity pneumonitis to the BCG was made. The volunteer was discharged from clinic with no further follow up.

TBT-04101033 developed symptoms of cough, sore throat, fatigue, chest pain and malaise from D7, though wheeze was present intermittently from D1. By D9 they were reporting Grade2 chest pain and shortness of breath and had a vasovagal attack as a result of their dyspnoea and chest tightness on D10 (see Table 3.14). Paramedics assessed the volunteer at the scene but deemed her well and did not commence any treatment. Spirometry was normal throughout. The volunteer was seen on D12 when examination was normal. The volunteer's respiratory viral PCR was negative on nasal swab, blood parameters, including CRP and D-Dimer, were normal, ECG and chest x-ray was unremarkable. Bronchoscopy was deferred by a week to allow symptoms to fully resolve.

Chest symptoms (pain or tightness) were reported by volunteers either immediately post infection, or around 10 days later (Figure 3.8). A similar pattern is evident when looking at all solicited systemic AEs, but not other respiratory AEs (Figure 3.9).

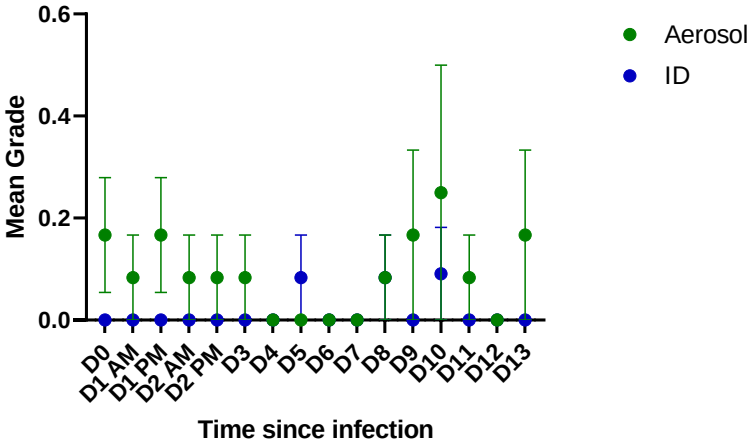


Figure 3.8. TB041: Mean reports of chest pain by Aerosol or ID volunteers following BCG Bulgaria infection and prior to bronchoscopy. Dots represents mean, lines SEM.

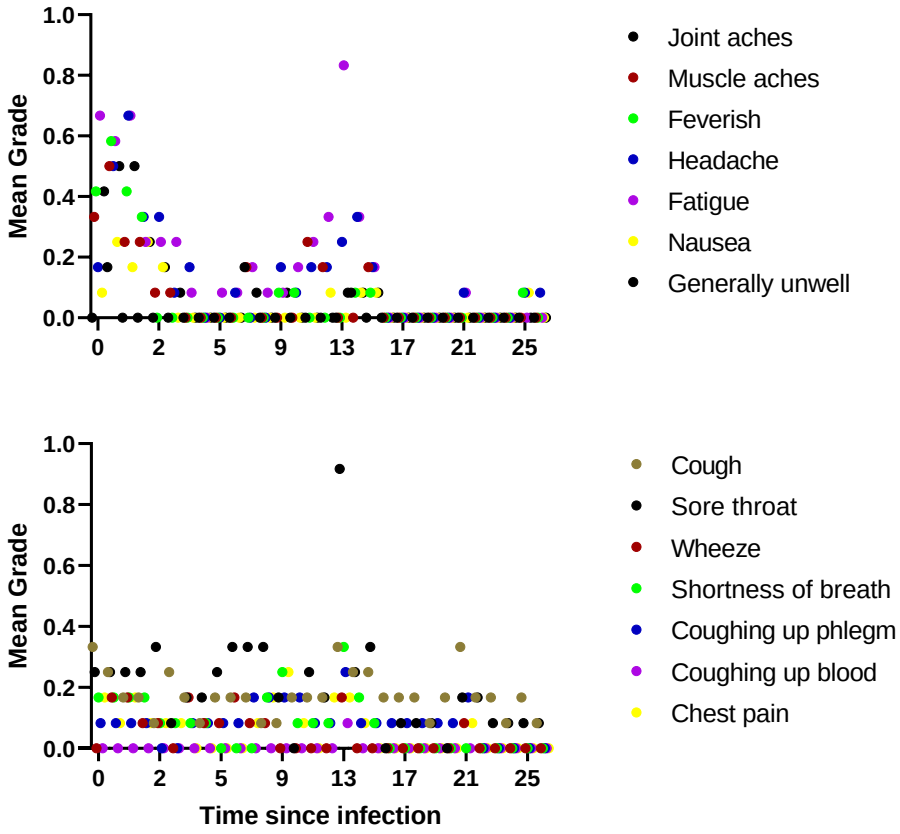


Figure 3.9. TB041: Mean grade of (a) systemic or (b) respiratory solicited adverse events reported on e-diary following BCG Bulgaria aerosol infection. Aerosol BCG volunteers shown only. D13 is the last timepoint prior to bronchoscopy. Mean temperature grade was 0 throughout.

3.5.4.6 Unsolicited AEs

These are listed in Table 3.14 and Table 3.15 at the end of the chapter. Related unsolicited AEs were unremarkable and there were no possible or probable related grade 3 unsolicited AEs other than those discussed above relating to volunteers TBT-04101033 and TBT-04101057. There were no recurring patterns of unsolicited AEs that were probably or definitely related to BCG aerosol infection.

3.5.4.7 Comparing aerosol BCG Bulgaria Groups 2D (unblinded) with 2E (blinded) volunteers

The data above is presented with Groups 2D and 2E combined. However, the three Group 2D volunteers were unblinded to route and did not receive an ID saline injection. This was because Group 2D were the first volunteers to receive $1x10^7$ cfu BCG Bulgaria by aerosol and hence were part of the dose escalation protocol.

Because of the differences between Group 2D and 2E volunteers, I ran an analysis comparing incidence of AEs between Groups 2D and 2E. There was no difference in total AEs or systemic AEs (Figure 3.10), however Group 2D volunteers reported significantly more respiratory AEs than the blinded Group 2E volunteers (Figure 3.11). This difference reached significance despite the low numbers in Group 2D (n=3). Reassuringly, despite more reported AEs in Group 2D as reported above, there was still no significant difference in the incidence of AEs between aerosol volunteers (Groups 2E and 2D combined) and intradermal volunteers (Group 2F); as shown above in Figure 3.5, and again below in Figure 3.11.

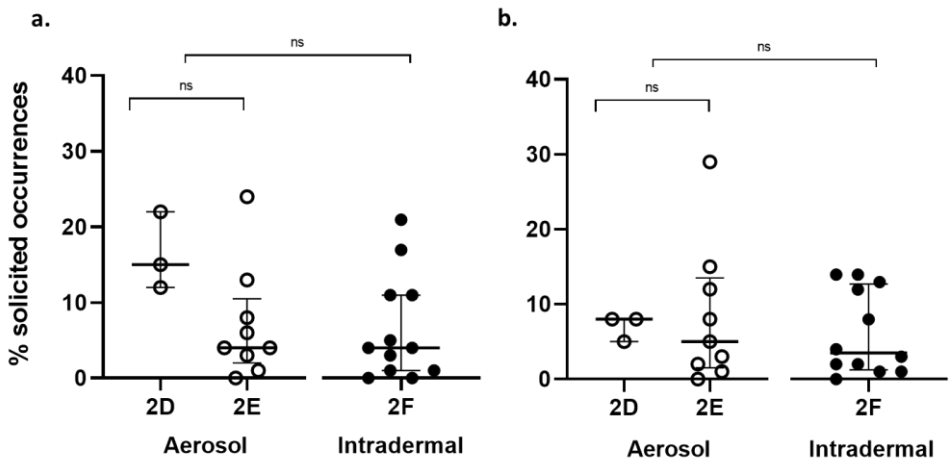


Figure 3.10. TB041: Percent of (a) total and (b) systemic AEs by volunteer out of total solicited event points in the 2 weeks following BCG Bulgaria infection, with Group 2D and 2E split. AEs were collected every 12 hours for 2 days following challenge then daily. Total solicited occurrences: 15 possible AEs x 16 collected time points; 8 systemic AEs x 16 collected time points. Aerosol BCG Bulgaria $1x10^7$cfu: Group 2D n=3 unblinded, Group 2E n=9 blinded. Intradermal BCG Bulgaria $1x10^6$ cfu: Group 2F n=12. No difference between groups by Mann Whitney. Dots represent individual values, lines show median and IQR.

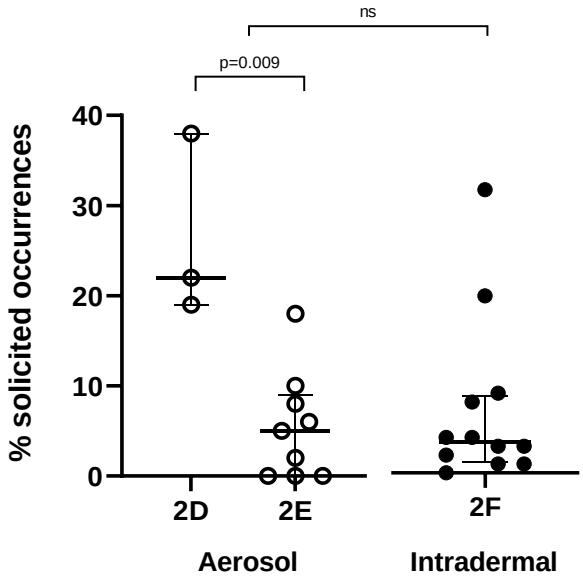


Figure 3.11. TB041: Percent of respiratory AEs by volunteer out of total solicited event points in the 2 weeks following BCG Bulgaria infection with Group 2D and 2E split. AEs were collected every 12 hours for 2 days following challenge then daily. Total solicited occurrences: 7 respiratory AEs x 16 collected time points. Aerosol BCG Bulgaria $1x10^7$cfu: Group 2D n=3 unblinded, Group 2E n=9 blinded to route. Intradermal BCG Bulgaria $1x10^6$ cfu: Group 2F n=12 blinded to route. p values calculated as difference in median by Mann Whitney. Dots represent individual values, lines show median and IQR.

3.5.5 TB043 safety data following BCG Danish infection, before bronchoscopy

3.5.5.1 Systemic AEs

Following BCG Danish aerosol infection, the majority of AEs were mild and Grade 3 AEs were rare. As with BCG Bulgaria, the most frequent systemic AEs reported following BCG Danish infection were headache and fatigue. As expected, there was a trend for a higher frequency of systemic AEs following BCG Danish infection than with saline alone, though this did not reach significance (Figure 3.13b). Saline volunteers did not report fever, feverishness, malaise or arthralgia, unlike volunteers inhaling BCG (Figure 3.12a). Of those volunteers who did report systemic AEs, there was no difference in severity between BCG and saline groups (Table 3.7). In the first 48 hours following inhalation, there was no significant difference in any symptom, though there was a trend for increased fatigue in BCG volunteers (Table 3.8). Here the interval is 48 hours not 72 hours, as in TB041, to show only AEs arising before bronchoscopy.

3.5.5.2 Respiratory AEs

Following aerosol inhalation, the most common reported AEs in both groups was cough, sore or tickly throat. There was no difference in respiratory AEs after BCG inhalation compared with saline (Figure 3.12b, Figure 3.13c, Table 3.7 and Table 3.8).

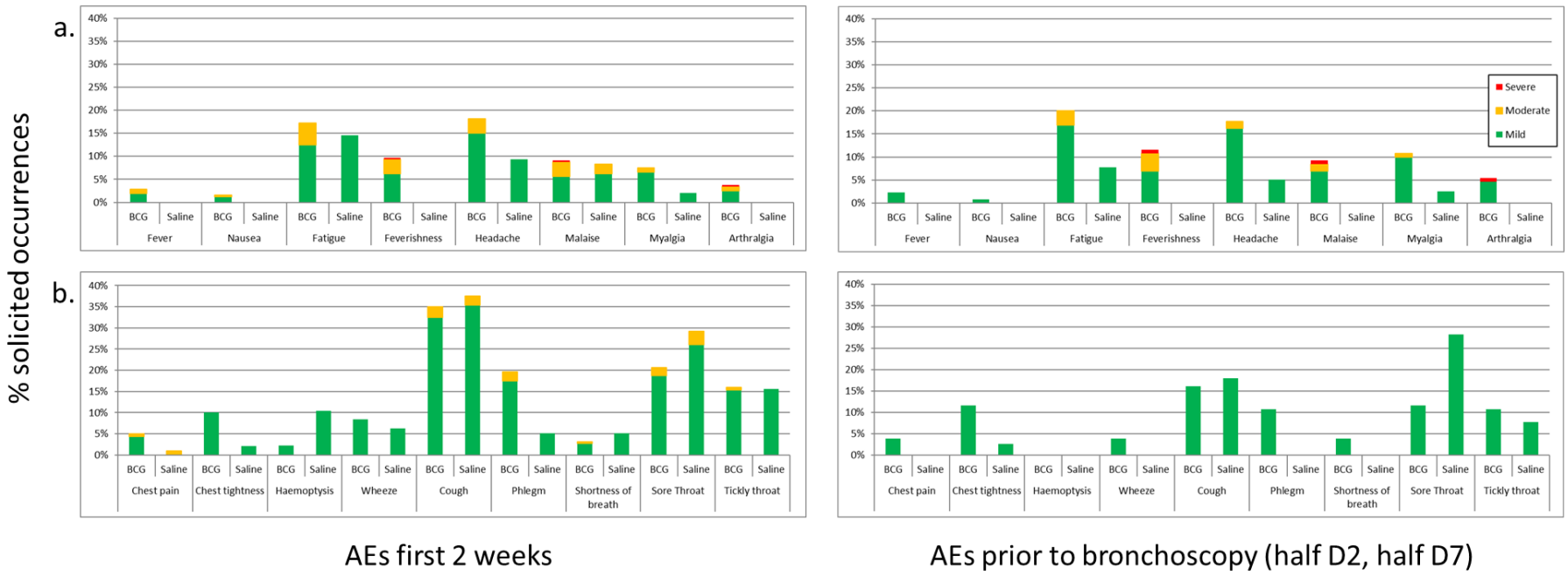


Figure 3.12. TB043: Percent of (a) systemic, or (b) respiratory AEs by symptom, out of total solicited occurrences reported on volunteer electronic diary following BCG Danish 1x10^{7}cfu or saline inhalation. Bronchoscopy occurred 2 days (Group 1) or 7 days (Group 2) following aerosol infection. AEs shown for full 2 weeks following infection or only prior to bronchoscopy (Group 1 volunteers 2 days of AEs, Group 2 volunteers 7 days of AEs). Shown as percent of total solicited occurrences. AEs were collected every 12 hours for 2 days following challenge then daily. N=20 BCG and 6 Saline. For "AEs first 2 weeks", total solicited occurrences calculated as number of volunteers x 16 time points. For "AEs prior to bronchoscopy" total solicited occurrences calculated as number of volunteers x 6.5 time points (average time points between groups: 4 time points for D2 volunteers and 9 time points for D7 volunteers, even numbers per group).

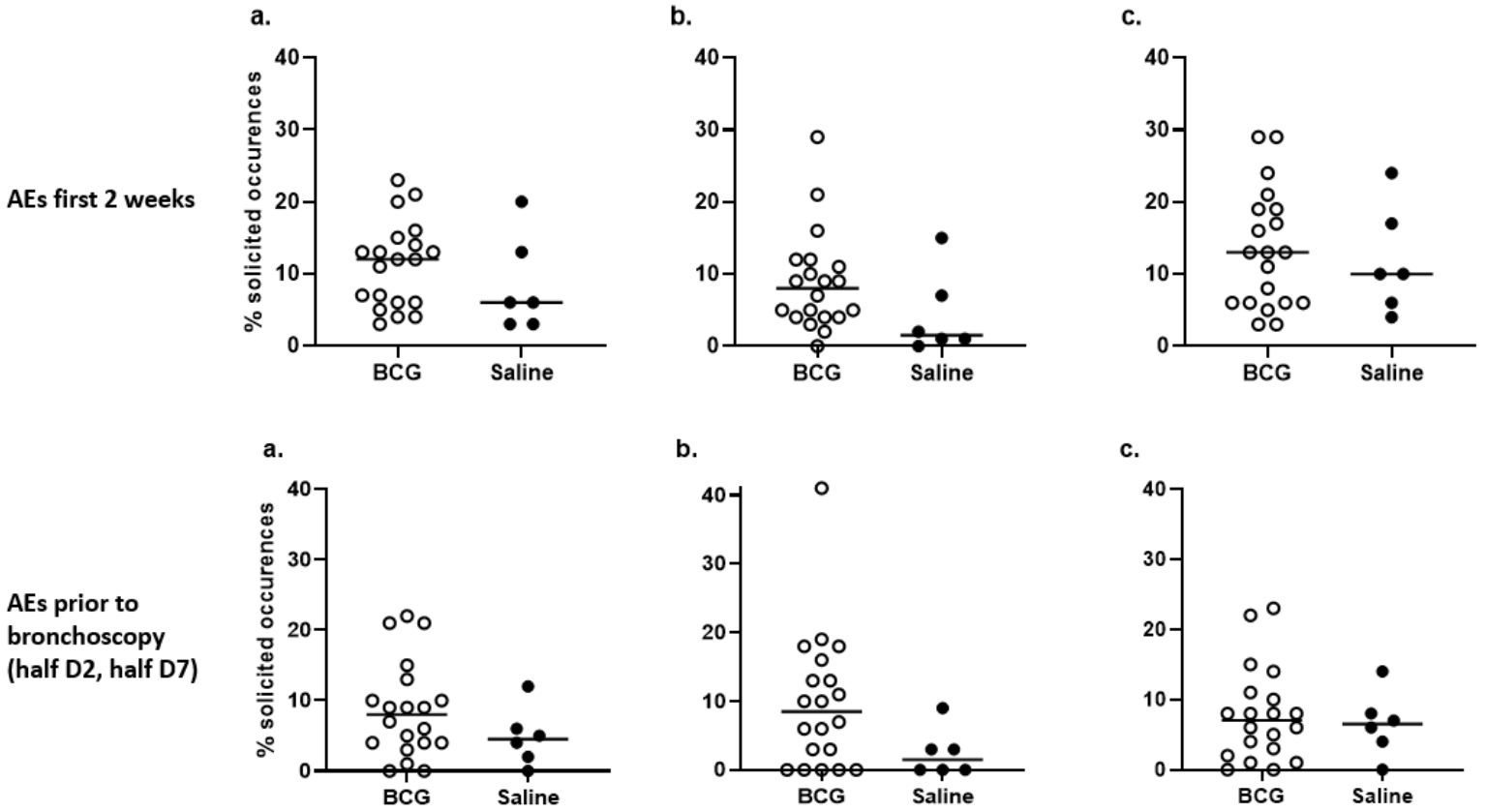


Figure 3.13. TB043: Percent of (a) total, (b) systemic, or (c) respiratory AEs by volunteer out of total solicited occurrences following BCG Danish 1x10^7 cfu or saline inhalation. Bronchoscopy occurred D2 (Group 1) or D7 (Group 2) following aerosol infection. AEs shown for full 2 weeks following infection or only prior to bronchoscopy (Group 1 volunteers 2 days of AEs, Group 2 volunteers 7 days of AEs) . AEs were collected every 12 hours for 2 days following challenge then daily. For "AEs first 2 weeks", total solicited occurrences calculated as, Total: 17 AEs x 16 collected time points; Systemic: 8 systemic events x 16 time points; Respiratory: 9 respiratory AEs x 16 collected time points. For "AEs prior to bronchoscopy" total solicited occurrences calculated as, Total: 17 AEs x 4 collected time points (Group 1) or 9 time points (Group 2); Systemic: 8 systemic events x 4 collected time points (Group 1) or 9 time points (Group 2); Respiratory: 9 respiratory AEs x 4 collected time points (Group 1) or 9 time points (Group 2). N=20 BCG and 6 saline. No difference between groups by Mann Whitney. Dots represent individual values, lines show median and IQR.

Table 3.7. TB043 Severity by group. Volunteers reporting AEs only. Significant results highlighted.

a. Number of Mild & moderate versus Severe adverse events

Time period	BCG versus Saline	Significant difference?*
Pre bronchoscopy, post challenge (2 days Group 1, 7 days Group 2)	Total (Respiratory and systemic)	No
	Respiratory only	No
	Systemic only	No
First 2 weeks post challenge	Total (Respiratory and systemic)	No
	Respiratory only	No
	Systemic only	No
Post bronchoscopy (12 days Group1, 7 days Group 2)	Total (Respiratory and systemic)	No
	Respiratory only	No
	Systemic only	No

b. Number of Mild versus Moderate & severe adverse events

Time period	BCG AEs > Saline AEs	Significant difference?*
Pre bronchoscopy, post challenge (2 days Group 1, 7 days Group 2)	Total (Respiratory and systemic)	No
	Respiratory only	No
	Systemic only	No
First 2 weeks post challenge	Total (Respiratory and systemic)	Yes, p=0.0092 BCG>saline
	Respiratory only	No
	Systemic only	Yes, p=0.0084 BCG>saline
Post bronchoscopy (12 days Group1, 7 days Group 2)	Total (Respiratory and systemic)	Yes, p=0.0204
	Respiratory only	No
	Systemic only	Yes, p=0.0082 BCG>saline

*Chi square (Fisher's exact)

Table 3.8. Adverse events in the first 48 hours following BCG Danish challenge by duration and severity

Adverse Event	Number of volunteers (frequency, max severity)			Median duration of AE Days (range)		
Route	Aerosol	Saline	p-value	Aerosol	Saline	p-value
Respiratory						
Cough	8 (0.40, 1)	4 (0.67, 1)	0.365	1.0 (0.5-4)	1.0 (0.5-4.5)	0.731
Sore throat	7 (0.35, 1)	4 (0.67, 1)	0.348	1.0 (0.5-2)	1.75 (0.5-8)	0.439
Tickly throat	8 (0.40, 1)	2 (0.33, 1)	>0.999	0.75 (0.5-1.5)	0.75 (0.5-1)	>0.999
Wheeze	2 (0.10, 1)	0	>0.999	1.25 (0.5-2)	-	*
Dyspnoea	3 (0.15, 1)	0	0.562	0.5 (0.5-1.5)	-	*
Phlegm	8 (0.40, 1)	0	0.132	0.5 (0.5-2)	-	*
Chest tightness	6 (0.30, 1)	1 (0.17, 1)	0.647	1.0 (0.5-2)	0.5	*
Chest pain	3 (0.15, 1))	0	0.562	1.0 (0.5-1.5)	-	*
Systemic						
Temperature	3 (0.15, 2)	0	0.562	0.5 (0.5-1)	-	*
Arthralgia	4 (0.20, 3)	0	0.543	1.0 (0.5-1)	-	*
Myalgia	8 (0.40, 2)	1 (0.17, 1)	0.436	1.0 (0.5 -1.5)	0.5	*
Feverish	8 (0.40, 3)	0	0.123	1 (0.5-1.5)	-	*
Headache	10 (0.50, 2)	2 (0.33, 1)	0.577	1 (0.5-1.5)	0.5 (0.5-0.5)	0.197
Fatigue	12 (0.60, 2)	1 (0.17, 1)	0.094	1 (0.5-1.5)	0.5	*
Nausea	1 (0.05, 1)	0	>0.999	0.5	-	*
Malaise	7 (0.35, 3)	0	0.167	1 (0.5-3)	-	*

Aerosol BCG Danish $1x10^7$ cfu challenge n =20; Aerosol saline n=6. Grading range 1-3. Adverse events presented here are any that occurred within 48 hours of BCG aerosol challenge. No haemoptysis recorded in any volunteers within 48 hours BCG challenge

3.5.5.3 Lung Function

Spirometry results are shown in Figure 3.6. Figure 3.6c compares BCG and saline readings. As with TB041, there was no difference in percent change in spirometry following BCG or saline inhalation on peak median AUC analysis. (FEV_1 median reduction in aerosol -32.7 (IQR -61.3

to 8.6); saline -25.0 (IQR-35.4 to 27.6), p=0.533. FVC median reduction in aerosol -11.9 (IQR -69.3 to 19.8); saline –19.8 (IQR -35.2 to 14.0), p=0.976 on Mann Whitney.). Figure 3.6d separates the groups to highlight the potential influence of bronchoscopy. Again, there was no difference between groups. There was also no difference in FEV1/FVC ratio (data not shown).

As TLCO was recorded 7 days after BCG infection, half the volunteers had not yet had their bronchoscopy (closed versus open circles; Figure 3.14). There was no difference between BCG and saline groups at D7 (p=0.533), and no difference between the pre-bronchoscopy BCG volunteers (Group 2A) and post bronchoscopy BCG volunteers (Group 1A) on Mann Whitney (p=0.739).

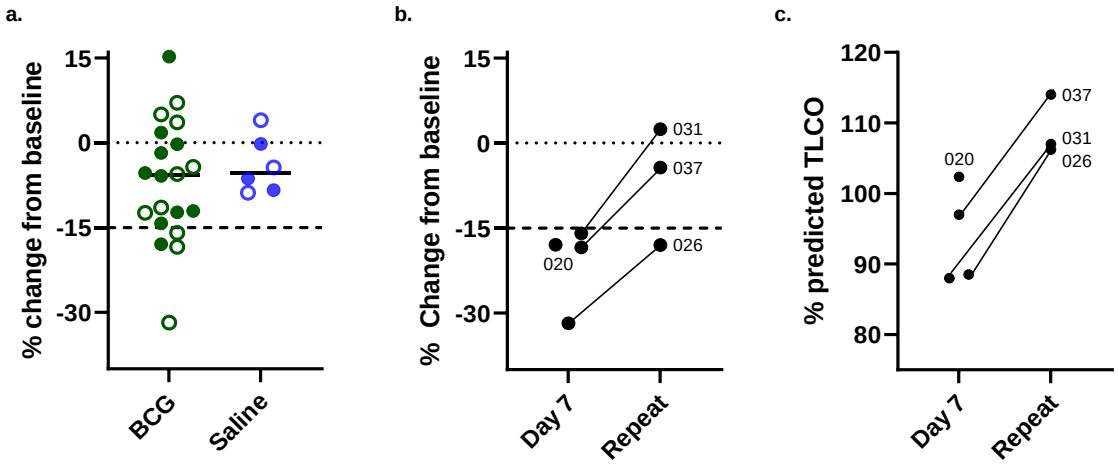


Figure 3.14. Gas transfer for TB043 volunteers; TLCO (transfer capacity of the lung for carbon monoxide uptake). (a) Change from baseline in TLCO at Day 7 by volunteer. Closed circles: Group 1, TLCO performed 5 days post bronchoscopy. Open circles: Group 2, TLCO performed prior to bronchoscopy. >15% change in TLCO deemed clinically significant drop (volunteers below dashed line). Group medians shown, no difference by Mann Whitney (p=0.533). (D2 and D7 BCG volunteers also compared, no difference by Mann Whitney (p=0.739)). (b) TLCO at Day 7 and then repeat at Day 84 or 168 for those BCG volunteers with clinically significant drop in TLCO. Numbers are volunteer identifiers. (c) % predicted TLCO on these volunteers

Of the four BCG volunteers who recorded a clinically significant drop in their TLCO at D7 (defined as >15% change in baseline reading), three volunteers had not yet had a bronchoscopy. Despite this drop, all four volunteers still recorded a TLCO of >80% predicted

(Figure 3.14c). There was one Group 1 volunteer (TBT-04301017) who recorded a TLCO 80% below predicted, but less than 15% drop from their baseline reading. This volunteer had had their bronchoscopy five days earlier. However, when corrected for haemoglobin, their value normalised.

Volunteer TBT-04301020 was five days post bronchoscopy and recorded a drop of 17.8% from baseline, however their result remained >100% predicted (Figure 3.14c). The volunteer failed to attend repeat pulmonary function test appointment and then moved abroad. They were contacted and were confirmed to have no clinical symptoms or concerns. The decision was made by the Chief Investigator not to repeat their pulmonary function test.

Volunteer TBT-04301037 recorded a drop of 18.4% TLCO. However, they also recorded a drop in their alveolar volume reading (VA) and were asymptomatic at the time. A low VA likely explains the drop in their TLCO (TLCO = KCO x VA per unit pressure, where KCO is the carbon monoxide transfer coefficient). This drop is likely a technical error, as an acute drop in lung volume would result in clinically significant respiratory symptoms.[522] Reassuringly, the volunteer's repeat was normal (Figure 3.14b).

Volunteer TBT-04301026. This volunteer's repeat pulmonary function test (PFT) at D84 still showed a greater than 15% drop from the volunteer's baseline (-18%). Their PFT was repeated again at D168 and their TLCO had fallen to -24% baseline screening. However, the screening value, which was performed at the NHS facility, and not with our own machine, was significantly higher than all other values the volunteer recorded. The predicted values from this NHS PFT were also significantly higher on NHS calculations than when we calculated them ourselves using the European Society technical standards for TLCO Global Lung Function Initiative calculator.[523] Hence it was likely that the baseline reading for this volunteer was incorrect. We were unable to repeat the PFT at the NHS facility due to the COVID-19 pandemic. Given this volunteer's predicted repeated value was above 100% and

they were asymptomatic, the Chief Investigator determined that there was no need to repeat this PFT.

Hence, after correcting for technical errors, of the 20 volunteers who inhaled BCG Danish, the majority recorded no clinically significant drop in their TLCO at D7 and all maintained their TLCO above a normal value of 80% predicted.

3.5.5.4 Blood parameters

CRP was not routinely performed in TB043 volunteers unless volunteers presented with systemic symptoms. There were no laboratory AEs deemed related to BCG infection.

3.5.5.5 Unsolicited AEs

These are presented at the end of the chapter in Table 3.16. Related unsolicited AEs were unremarkable and there were no possibly or probably related grade 3 unsolicited AEs. There were no recurring patterns of unsolicited AEs that were probably or definitely related to BCG aerosol infection.

3.5.5.6 Symptoms of note in aerosol BCG Danish volunteers

There were no symptoms of note, or significantly unwell volunteers amongst the TB043 volunteers.

3.5.5.7 BCG Bulgaria versus BCG Danish

I investigated whether there was a difference in AEs following aerosol inhalation of BCG Danish compared with BCG Bulgaria, prior to bronchoscopy. There was no difference in systemic, respiratory or all solicited AE data combined (Figure 3.15).

However, there was a significant difference when analysing the incidence of AE severity on Chi square, where volunteers inhaling BCG Bulgaria were more likely to report severe AEs than volunteers inhaling the same dose of BCG Danish (Table 3.9).

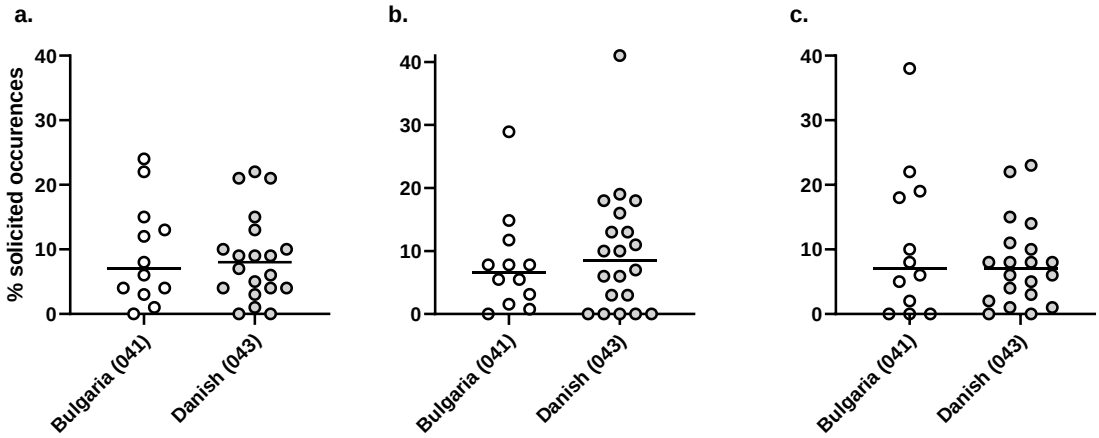


Figure 3.15. Percent of (a) total, (b) systemic, or (c) respiratory AEs by volunteer out of total solicited occurrences following BCG inhalation in TB041 and TB043 volunteers and prior to bronchoscopy. **"**Bulgaria (041)": BCG Bulgaria 1x10^{7}cfu aerosol infection (Group 2D/2E volunteers from TB041). "Danish (043)": BCG Danish AJV 1x10^{7}cfu aerosol infection (Group 1A and 2A volunteers from TB043).
For "Bulgaria 041" total solicited occurrences calculated as; Total AEs: 15 possible AEs x 16 collected time points; Systemic: 8 systemic events x 16 collected time points; Respiratory: 7 respiratory AEs x 16 collected time points. N=12. For "Danish 043" total solicited occurrences calculated as; Total: 17 AEs x 4 collected time points (Group 1) or 9 time points (Group 2); Systemic: 8 systemic AEs x 4 collected time points (Group 1) or 9 time points (Group 2); Respiratory: 9 respiratory AEs x 4 collected time points (Group 1) or 9 time points (Group 2). N=20. No difference between groups by Mann Whitney. Dots represent individual values, lines show median and IQR. AEs were collected every 12 hours for 2 days following challenge then daily. 2E volunteers (n=9) received aerosol BCG and intradermal saline. All other volunteers received BCG aerosol only.

Table 3.9. Number of Mild & moderate versus Severe adverse events following 1x10^7 cfu aerosol inhalation of either BCG Bulgaria (TB041) or BCG Danish (TB043). Significant results highlighted.

Time period	TB041 >TB043	Significant difference?*
Pre bronchoscopy, post challenge	Total (Respiratory and systemic)	Yes p=0.0108
	Respiratory only	No
	Systemic only	Yes p=0.0124
Post bronchoscopy	Total (Respiratory and systemic)	No
	Respiratory only	No
	Systemic only	No

*Chi square (Fisher's exact)

3.5.6 Post bronchoscopy AEs

AEs related to bronchoscopy have been well described.[524] The risks of bronchoscopy were discussed during volunteer consent and include reaction to sedation/local anaesthetic, sore throat, cough, transient hoarse voice, blood-stained phlegm, infection, hypoxia, airway damage, laryngospasm/bronchospasm, flu-like symptoms 1-2 days post procedure, and risk of death (<1 in 100,000).

The primary objective of collecting post-bronchoscopy AEs is to determine whether inhaling BCG increases risks of these AEs compared to saline inhalation (+/- intradermal BCG vaccination, TB041). TB041 volunteers had a 100ml saline bronchoalveolar lavage. The lavage in TB043 was 150ml to increase the immunologic analyses. TB043 volunteers also had up to six endobronchial biopsies. Due to these differences, TB043 volunteers might be expected to report more AEs, in particular transient haemoptysis, due to the expected minimal bleeding following airway biopsy.[524]

3.5.6.1 TB041

In the two weeks following bronchoscopy (2 weeks after BCG infection), there was no significant difference in frequency between Aerosol and ID groups by individual AE (Figure 3.16), nor in frequency and duration when comparing total, respiratory or systemic percent occurrences by volunteer (Figure 3.17). There was also no significant difference when comparing the frequency and duration of AEs between sub groups 2D and 2E (Figure 3.18). There was no significant difference in either frequency and severity (first column) nor AE duration (second column) of respiratory or systemic AEs arising within the first 48 hours following bronchoscopy, the period when the majority of bronchoscopy-related AEs would be expected to arise (Table 3.10).[524] This table (and Table 3.11, for TB043 below) was calculated

by including any new AE arising in the 48 hours following bronchoscopy. The AE was considered resolved if there were two consecutive days without symptoms. If the volunteer was one day symptom free and then the AEs returned, all days of symptoms reported were added together to give length of symptom. If the AE was present prior to bronchoscopy then it was only included if the grade increased.

When assessing differences in severity between groups over the two weeks following bronchoscopy by Chi square, there was no increased frequency of severe AEs between groups (Table 3.5a). However, there was a difference when comparing mild AEs with moderate & severe respiratory AEs (Table 3.5b), meaning that if volunteers did experience a respiratory AE in the two weeks after bronchoscopy that AE was less likely to be mild in volunteers who had received aerosol BCG vaccination than ID BCG.

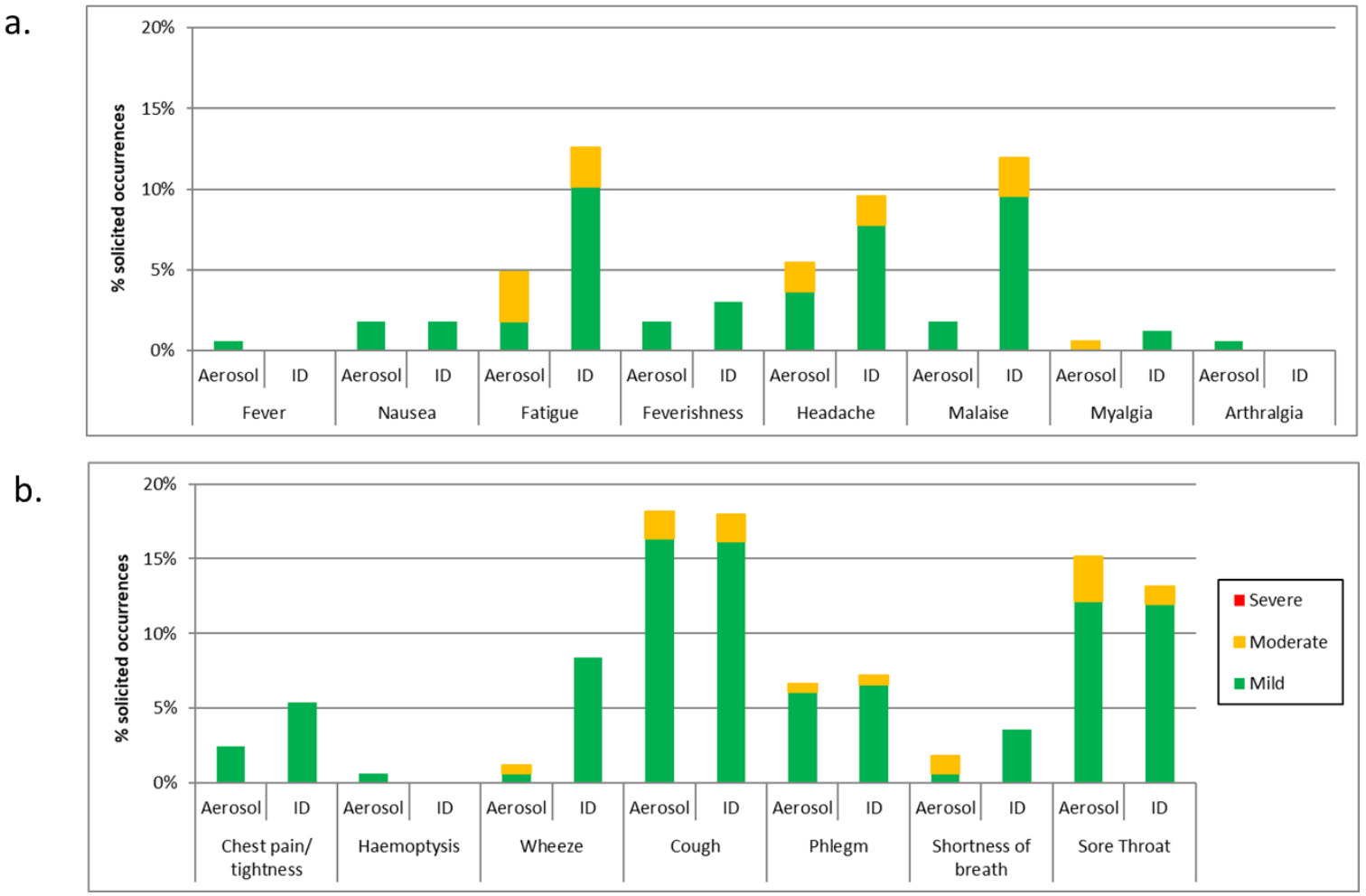


Figure 3.16. TB041: Systemic (a) or respiratory (b) AE occurrences by symptom, reported on volunteer electronic diary following bronchoscopy. Shown as percent of total solicited occurrences. Bronchoscopy occurred on D14 following BCG Bulgaria infection. Arm 2 high dose comparator groups. N=12 per group. ID: Group 2F (ID route). Aerosol: Group 2D (3 un-blinded volunteers aerosol BCG Bulgaria $1x10^7$ cfu), and 2E (randomised blinded volunteers aerosol BCG Bulgaria $1x10^7$ cfu and ID saline). Total solicited occurrences calculated as 12 volunteers x 15 time points.

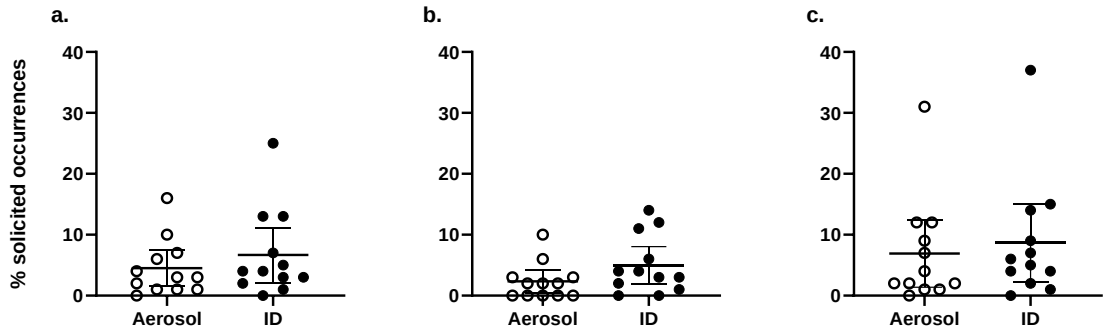


Figure 3.17. TB041: Percent of (a) total (b) systemic or (c) respiratory AEs by volunteer out of total solicited occurrences following bronchoscopy. Bronchoscopy occurred on D14 post BCG Bulgaria infection. AEs symptoms were collected daily. Total solicited occurrences calculated as; Total: 15 AEs x 14 collected time points; Respiratory: 7 respiratory adverse events x 14 collected time points; Systemic: 8 systemic events x 14 collected time points* N=12 per group. Aerosol: Group 2D/2E BCG Bulgaria $1x10^7$ cfu aerosol; ID: Group 2F BCG Bulgaria $1x10^6$ cfu ID. 8 possible systemic AEs, 7 respiratory AEs. No difference between groups by Mann Whitney. Dots represent individual values, lines show median and IQR.
*except 033, 057,056 and 058 who had a bronchoscopy at D15 so have x 13 timepoints.

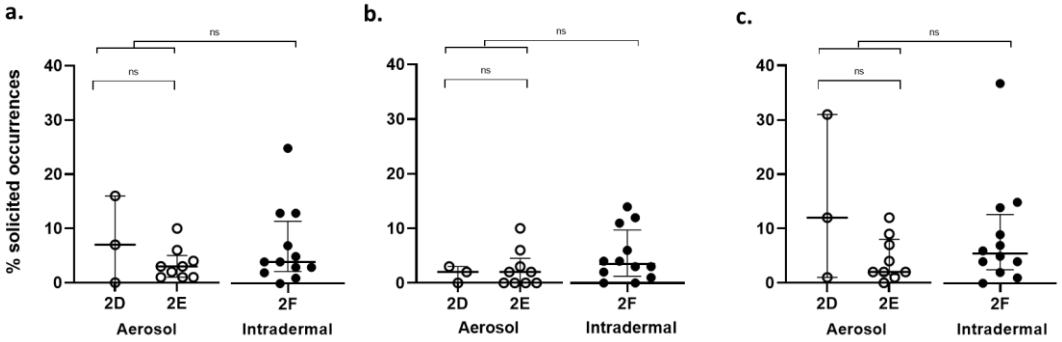


Figure 3.18. TB041: Percent of (a) total (b) systemic or (c) respiratory AEs by volunteer out of total solicited occurrences following bronchoscopy, Group 2D and 2E split. Bronchoscopy occurred on D14 post BCG Bulgaria infection. AEs were collected daily. Total solicited occurrences calculated as; Total AEs: 15 possible AEs x 14 collected time points; Systemic: 8 systemic events x 14 collected time points; Respiratory: 7 respiratory adverse events x 14 collected time points. Aerosol BCG Bulgaria $1x10^7$cfu: Group 2D n=3 unblinded, Group 2E n=9 blinded; ID BCG Bulgaria $1x10^6$ cfu: Group 2F n=12. p values calculated as difference in median by Mann Whitney. Dots represent individual values, lines show median and IQR.

Table 3.10. TB041: Adverse events arising in the first 48 hours following bronchoscopy by duration and severity. (14 days following BCG Bulgaria inhalation or injection)

Adverse Event	Number of volunteers (frequency, max severity)			Median duration of AE Days (range)		
Route	Aerosol	Intradermal	p-value	Aerosol	Intradermal	p-value
Respiratory						
Cough	5 (0.42, 2)	6 (0.5, 2)	>0.999	2 (1-10)	1.5 (1-5)	0.913
Sore throat	10 (0.83, 2)	8 (0.67, 2)	0.226	1 (1-6)	2 (1-5)	0.397
Wheeze	1 (0.08, 2)	4 (0.33, 1)	0.317	1	1.5 (1-4)	*
Dyspnoea	1 (0.08, 2)	1 (0.08, 1)	>0.999	1	4	*
Phlegm	1 (0.08, 2)	2 (0.17, 2)	>0.999	1	1 (1)	*
Haemoptysis	1 (0.08, 1)	0	>0.999	1	-	*
Chest pain	2 (0.17, 1)	3 (0.25, 1)	>0.999	1.5 (1-2)	2 (1-2)	>0.999
Systemic						
Temperature	1 (0.08, 1)	0	>0.999	1	-	*
Arthralgia	1 (0.08, 1)	0	>0.999	1	-	*
Myalgia	1 (0.08, 2)	1 (0.08, 1)	>0.999	1	1	*
Feverish	2 (0.17, 1)	2 (0.17, 1)	>0.999	1 (1)	1 (1)	>0.999
Headache	5 (0.42, 2)	6 (0.50, 2)	>0.999	1 (1-2)	1.5 (1-3)	0.394
Fatigue	4 (0.33, 2)	6 (0.50, 2)	0.665	1.5 (1-2)	1 (1-3)	0.500
Nausea	2 (0.17, 1)	2 (0.17, 1)	>0.999	1 (1)	1 (1)	>0.999
Malaise	2 (0.17, 1)	4 (0.33, 2)	0.479	1.5 (1-2)	2 (1-5)	0.667

Aerosol BCG Bulgaria challenge n = 12 volunteers 1 x 10^7 cfu. Intradermal BCG Bulgaria n = 12; volunteers received 1 x 10^6 cfu. Grading range 1-3.

3.5.6.2 Lung function

There was also no difference detected on spirometry in the two weeks following bronchoscopy between aerosol and ID BCG volunteers (Figure 3.6, Peak median AUC analysis: FEV_1 median reduction in aerosol -32.4 (IQR -62.1 to -7.8); intradermal -58.2 (IQR -101.9 to -17.5), p=0.242. FVC median reduction in aerosol -43.5 (IQR -59.9 to-9.4); intradermal –47.2 (IQR -89.4 to -26.3), p=0.514 on Mann Whitney).

3.5.6.3 TB043

As with TB041, there was no significant difference in frequency between BCG and saline groups by individual AE (Figure 3.19), nor frequency and duration when comparing total, respiratory or systemic percent occurrences by volunteer (Figure 3.20). (Group 1 and 2 data were combined, resulting in an average of 9.5 days of solicited AEs recorded post bronchoscopy (half volunteers 12 days, half 7 days)).

However, there was a trend for increased frequency of systemic AEs in the BCG group. This trend reached significance when interrogating the AEs which arose within the first 48 hours following bronchoscopy (Table 3.11), with a significance detectable in frequency of feverishness (50% BCG volunteers, 0 saline volunteers). In addition, while the other systemic AEs did not reach significance between conditions, no saline volunteer reported a fever, arthralgia or nausea.

When assessing differences in severity between groups by Chi square, as with TB041, while there was no difference in the incidence of severe AEs reported; in volunteers who experienced an AE following bronchoscopy, it was more likely to be mild if the volunteer had inhaled saline only (Table 3.7).

3.5.6.4 Lung function

There was no difference in spirometry readings taken in clinic following bronchoscopy (D7 and D14 readings Group 1, or D14 readings only Group 2, Figure 3.6, Peak AUC: FEV1 median reduction in aerosol -32.7 (IQR -61.3 to 8.6); saline -25.0 (IQR-35.4 to 27.6), p=0.533. FVC median reduction in aerosol -11.9 (IQR -69.3 to 19.8); saline –19.8 (IQR -35.2 to 14.0), p=0.976 on Mann Whitney.) TLCO findings have been discussed above.

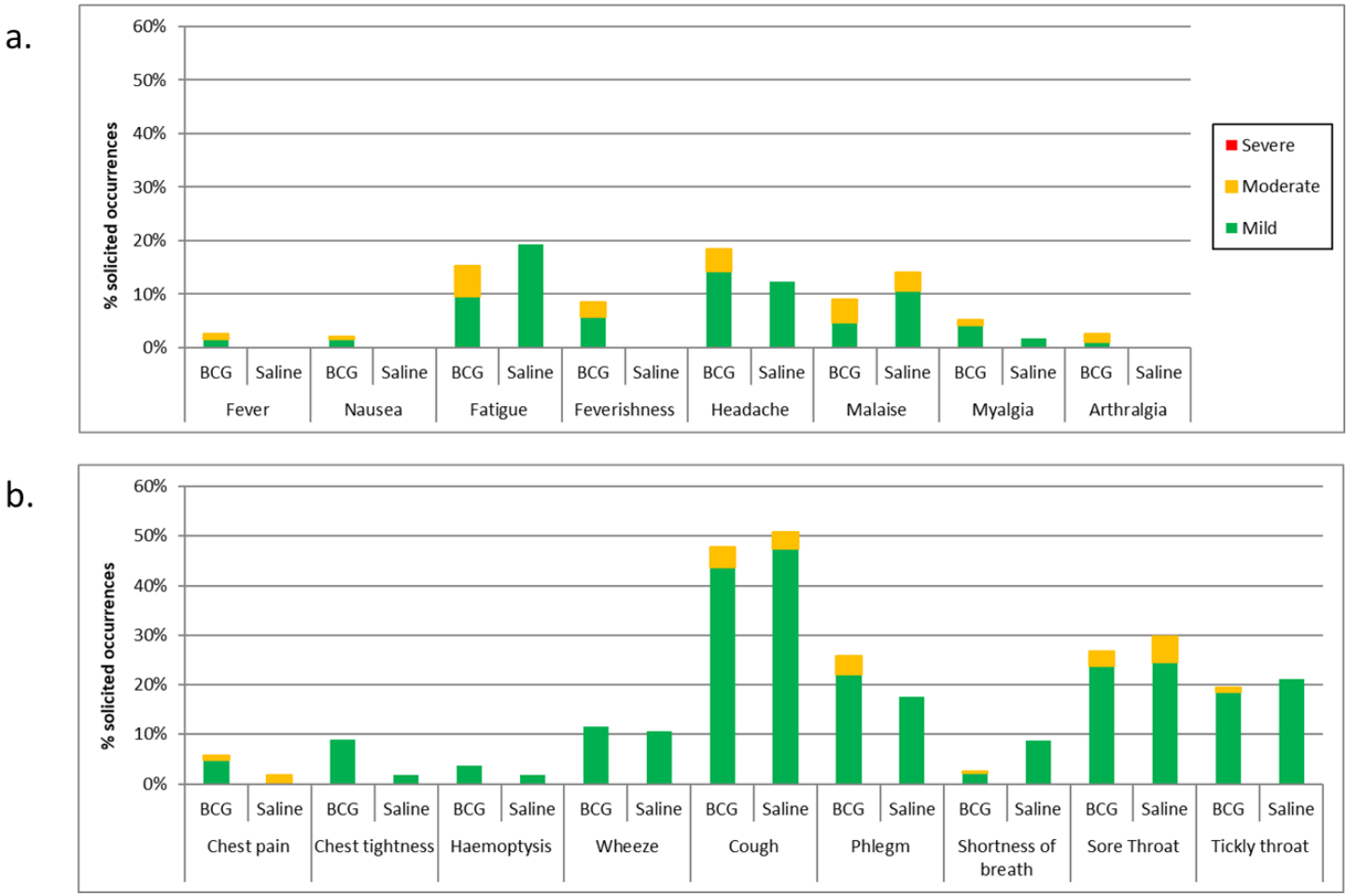


Figure 3.19. TB043: Systemic (a) or respiratory (b) AE occurrences reported on volunteer electronic diary following bronchoscopy. Shown as proportion of total solicited occurrences. Bronchoscopy occurred D2 (Grp 1) or D7 (Grp 2) following BCG SSI AJV infection.N=20 BCG N=6 Saline. Solicited occurrences calculated as no. of volunteers x 9.5 time points (average time points between groups: 12 time points for D2 volunteers and 7 time points for D7 volunteers, even numbers per group).

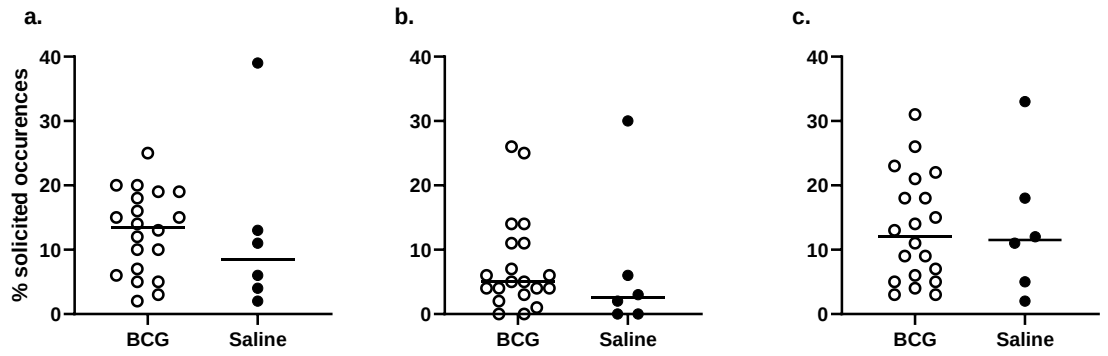


Figure 3.20. TB043: Percent of (a) total (b) systemic or (c) respiratory AEs by volunteer out of total solicited occurrences following bronchoscopy D2 (Grp1) or D7 (Grp 2) post BCG Danish AJV infection. AEs symptoms were collected daily. Total solicited occurrences per volunteer calculated as AEs x timepoints. Total: 17 AEs; Systemic: 8 AEs; Respiratory: 9 AEs. Collected timepoints: 12 (Grp 1) or 7 (Grp 2). N=20 BCG and 6 saline volunteers. No difference between groups by Mann Whitney. Dots represent individual values, lines show median.

Table 3.11. TB043: Adverse events arising in the first 48 hours following bronchoscopy by duration and severity. (Either D2 or D7 post BCG Danish or saline inhalation)

Adverse Event	Number of volunteers (frequency, max severity)			Median duration of AE Days (range)		
Route	Aerosol	Saline	p-value	Aerosol	Saline	p-value
Respiratory						
Cough	17 (0.85, 2)	5 (0.83, 2)	0.964	4 (1-8)	6 (1-8)	0.435
Sore throat	16 (0.80, 2)	5 (0.83, 2)	0.660	2 (1-6)	3 (1-7)	0.651
Tickly throat	8 (0.40, 2)	4 (0.67, 1)	0.365	1.5 (1-5)	2 (1-7)	0.535
Wheeze	7 (0.35, 1)	3 (0.50, 1)	0.644	1 (1-6)	1 (1-2)	0.750
Dyspnoea	3 (0.15, 2)	1 (0.17, 1)	>0.999	1 (1)	2	*
Phlegm	10 (0.50, 2)	3 (0.50, 1)	0.823	2.5 (1-9)	3 (1-4)	0.955
Haemoptysis	4 (0.20, 1)	1 (0.17, 1)	>0.999	1 (1-3)	1	*
Chest tightness	10 (0.50, 1)	1 (0.17, 1)	0.197	1 (1-3)	1	*
Chest pain	6 (0.30, 2)	1 (0.17, 2)	0.697	1 (1-3)	1	*
Systemic						
Temperature	3 (0.15, 2)	0	0.562	1 (1)	-	*
Arthralgia	5 (0.25, 2)	0	0.298	1 (1)	-	*
Myalgia	6 (0.30, 2)	1 (0.17, 1)	0.562	1 (1-2)	1	*
Feverish	10 (0.5, 2)	0	0.017	1 (1-3)	-	*
Headache	14 (0.70, 2)	2 (0.33, 1)	0.063	1 (1-6)	3.5 (1-6)	0.250
Fatigue	12 (0.60, 2)	4 (0.67, 1)	0.761	1 (1-8)	2 (1-5)	0.514
Nausea	3 (0.15, 2)	0	0.562	1 (1)	-	*
Malaise	11 (0.55, 2)	3 (0.5, 2)	0.736	1 (1-3)	1 (1-6)	0.698

Aerosol BCG Danish $1x10^7$ cfu challenge n =20; Aerosol saline n=6. Grading range 1-3.

3.5.6.5 BCG Bulgaria (TB041) versus BCG Danish (TB043)

BCG Danish (TB043) volunteers reported more respiratory and systemic AEs than BCG Bulgaria (TB041) volunteers after bronchoscopy (Figure 3.21), but there was no difference in severity (Table 3.9).

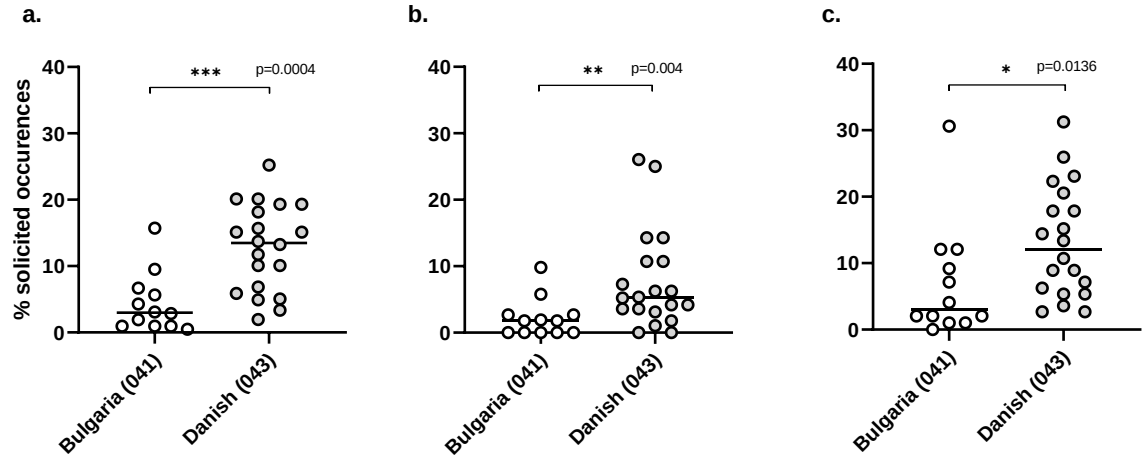


Figure 3.21. Percent of (a) total, (b) systemic, or (c) respiratory AEs by volunteer out of total solicited occurrences following bronchoscopy in TB041 and TB043 volunteers. "Bulgaria (041)": BCG Bulgaria $1x10^7$cfu aerosol infection (Group 2D/2E volunteers from TB041). "Danish (043)": BCG Danish AJV $1x10^7$cfu aerosol infection (Group 1A and 2A volunteers from TB043). AEs were collected daily following bronchoscopy. 2E volunteers (n=9) received aerosol BCG and intradermal saline. All other volunteers received BCG aerosol only. For "Bulgaria 041" total solicited occurrences calculated as; Total AEs: 15 possible AEs x 14 collected time points; Systemic: 8 systemic events x 14 collected time points; Respiratory: 7 respiratory adverse events x 14 collected time points. N=12. For "Danish 043" total solicited occurrences per volunteer calculated as AEs x timepoints. Total: 17 AEs; Systemic: 8 AEs; Respiratory: 9 AEs. Collected timepoints: 12 (Group 1) or 7 (Group 2). N=20. Statistical significance by Mann Whitney. Dots represent individual values, lines show median.

3.6 Administered BCG dose

Lypophilised BCG was reconstituted as outlined in Chapter 2. Materials and Methods. There was no difference in BCG AJV viability or clumping between the BCG reconstituted as per SmPC and my concentration method for volunteer inhalation, using diluent mixed with saline (Figure 3.22).

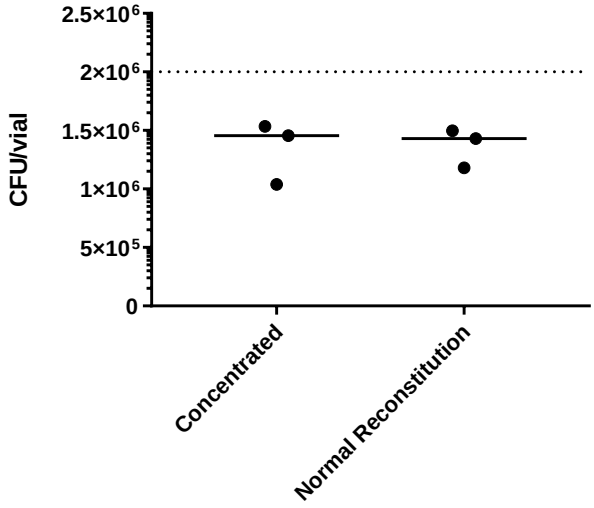


Figure 3.22. Median BCG Danish (AJV) cfu from plated triplicates to estimate total cfu in the vial. No difference between "concentrated" method compared with normal reconstitution on Mann Whitney. Black bar: medians. Dotted line: Lower limit of expected cfu/vial as per SmPC.

BCG was administered to volunteers by loading the Omron nebuliser or direct intradermal injection, and the remaining BCG was plated for quantification. The amount of viable BCG Bulgaria recoverable from reconstituted vials was consistently 0.5-1 log lower than that stated in the SmPC (Figure 3.23a). There was a significant difference between the viable BCG grown from vials administered to Group 2D volunteers (unblinded) compared to Group 2E (blinded). However, there was no significant difference between Group2D/E (aerosol) combined versus Group 2F (ID).

BCG Danish vials also grew fewer BCG colonies than expected, though the discrepancy wasn't as great (Figure 3.23b). The median for all vials was $1.44x10^6$ cfu, which was 0.5 log below the presumptive median cfu of $5x10^6$ on which we based our concentration calculations (dotted line). There was no significant difference between the recoverable BCG between Group 1 and Group 2.

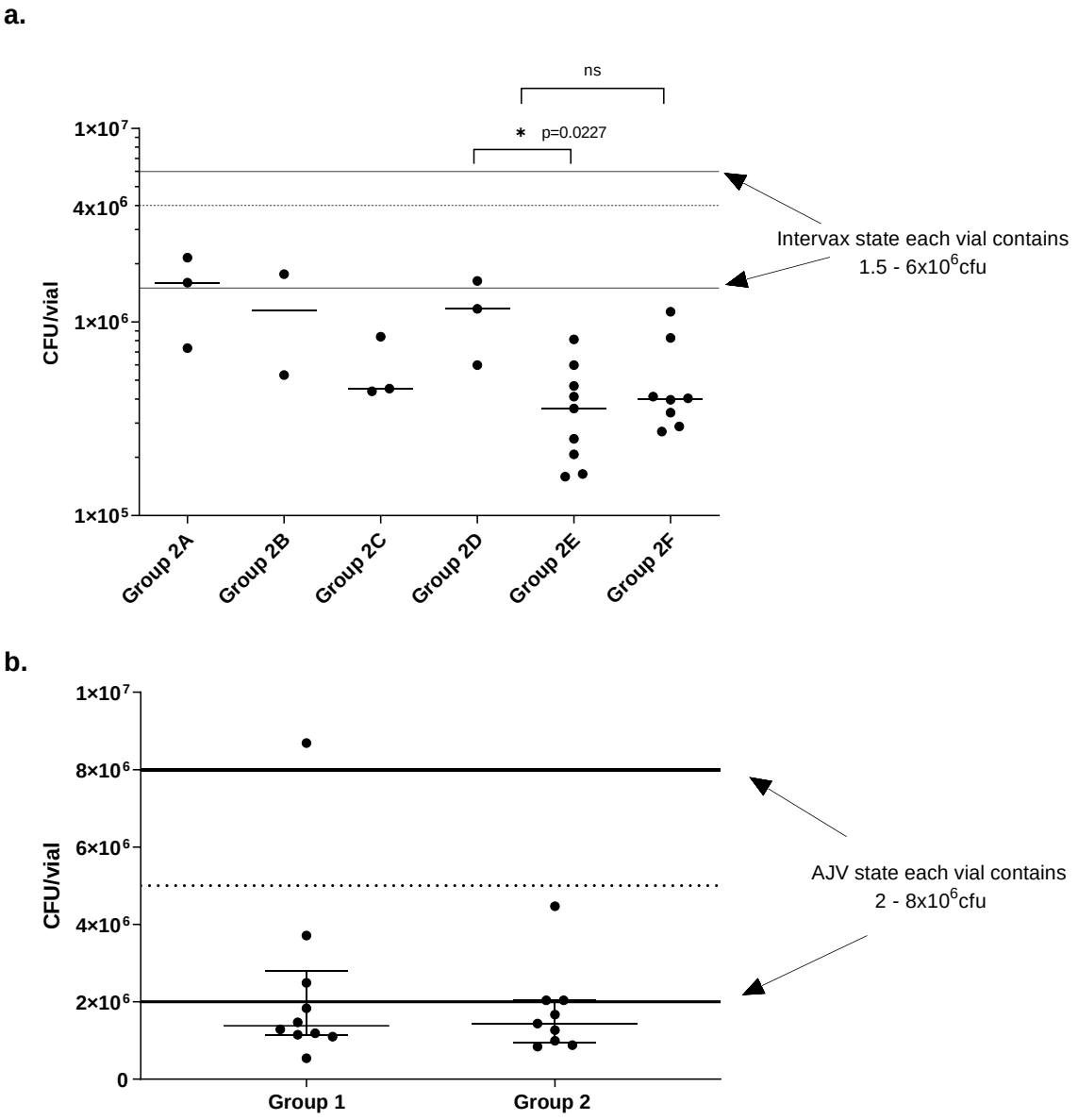


Figure 3.23. BCG Bulgaria (a) and BCG Danish (b) cfu count per vaccine vial. Calculated from plated reconstituted vials prior to dilution during BCG challenge. Vials matched to volunteer, bars median and IQR. (a) Combined Group 2D/E/F median 4.08 x10^5 cfu; range 1.59x10^5 – 1.63x10^6 cfu. (b) Combined Group 1 and 2 median 1.44 x10^6 cfu; range 5.39x10^5 – 8.69x10^6cfu. The dotted line shows the presumptive median cfu per vial that was used to calculate dilutions for doses (a. 4x10^6 BCG Bulgaria; b. 5.5x10^6 BCG Danish).

To examine the cause of this discrepancy, I investigated whether the shelf life might be impacting recoverable viable bacteria. All volunteers received BCG prior to the expiry date of the vaccine. However, Figure 3.24a shows that there was a significant positive correlation between the amount of viable BCG recoverable and time until expiry. CFU counts were lower than expected even when there was more than one year left before expiry. Interestingly,

despite less time to expiry for BCG Danish (compared with the Bulgaria vials; p<0.0001, Mann Whitney), there was no correlation between viable cfu recoverable and time to expiry (Figure 3.24b).

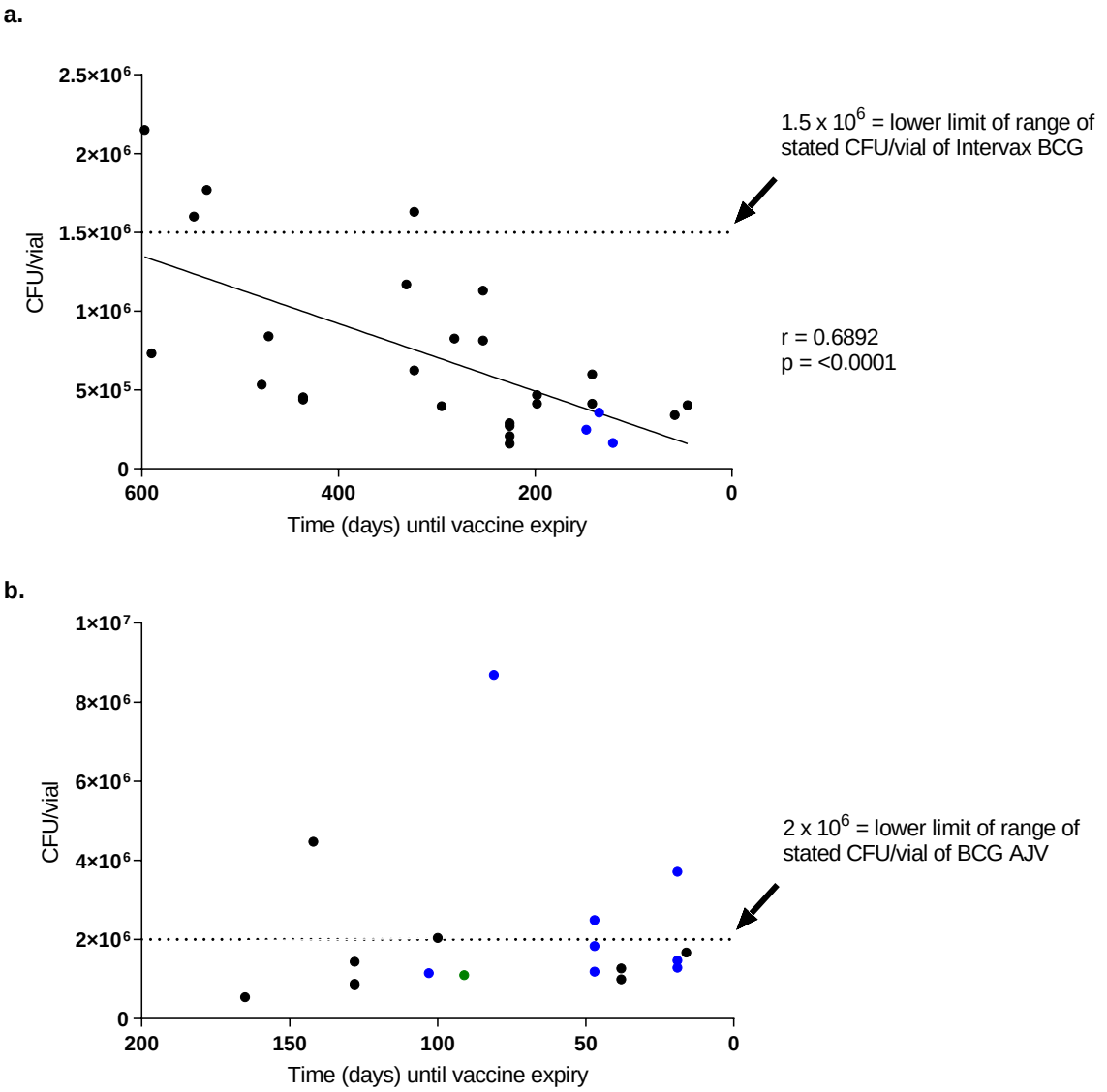


Figure 3.24. BCG Bulgaria (a) and BCG Danish (b) CFU/vial by time to expiry. (a) Groups 2A-2F. Black dots indicate batch 175-2. Blue dots indicate the three volunteers with the new batch 204-1. Two-tailed Spearman correlation r=0.6892 CI 0.4161 to 0.8483. (b) Groups 1A and 2A. No correlation. Note different axes. Green dot: Batch 117006A; Blue dots: Batch 117017A; Black dots: Batch 118009E. One Group 2 value missing due to issue with agar plate.

In TB041 all volunteers in the dose escalation groups and the ID group received the same batch, 175-2, expiry 30 November 2018. The trial took longer to complete than we originally

foresaw due to availability of bronchoscopy slots at the hospital. Because of this, the final three volunteers in Group 2E received a different batch; 204-1, expiry 28 February 2019. For TB043, as we were sourcing the BCG through NHS suppliers we were unable to purchase a single batch of vials to cover the study. One Group 1 volunteer received Batch 117006A expiring 30 April 2019; eight volunteers received batch 117017A expiring 31 August 2019; one Group 1 volunteer and all Group 2 volunteers received batch 118009E expiring 28 February 2020.

I then investigated the actual dose loaded into the nebuliser or syringe after mixing (diluting or concentrating). Figure 3.25 confirms that the dilution/missing SOP achieved stepped dosing, as planned, for TB041. However, likely reflective of the lower than expected cfu in each vial, the loaded dose was 0.5-1 log lower than the target dose for each group.

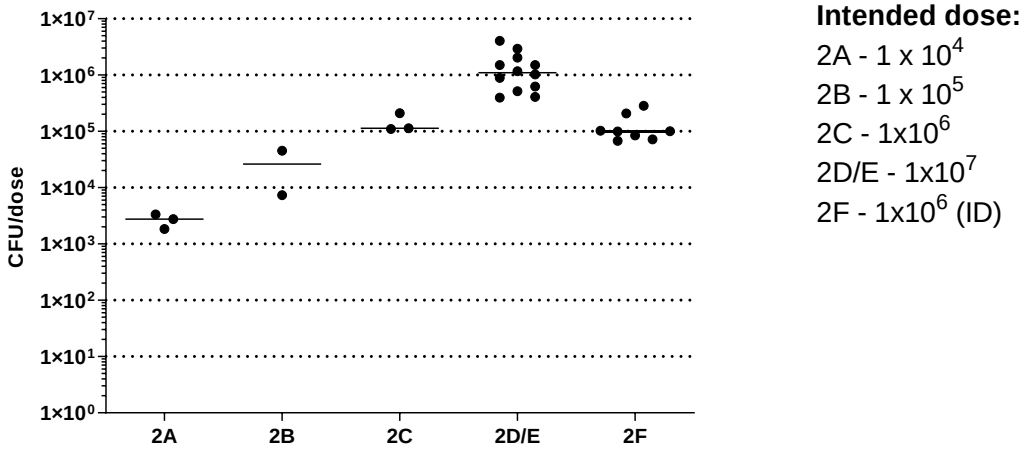


Figure 3.25. TB041: BCG Bulgaria dose loaded into nebuliser (2A-2E) or syringe (2F).
Dots represent vials. Where data points are less than volunteer number, one vial has been used for two or more volunteers that day (e.g. 2B, 2F)

There was a significant difference between the loaded dose of the high dose BCG aerosol groups in TB041 and TB043 ($1x10^7$cfu target dose for both; Figure 3.26). This is again likely due to the difference in viable bacteria recoverable from Intervax compared to AJV vials. Interestingly, despite delivering more live BCG Danish bacilli to volunteers, it was the BCG Bulgaria volunteers who reported more AEs.

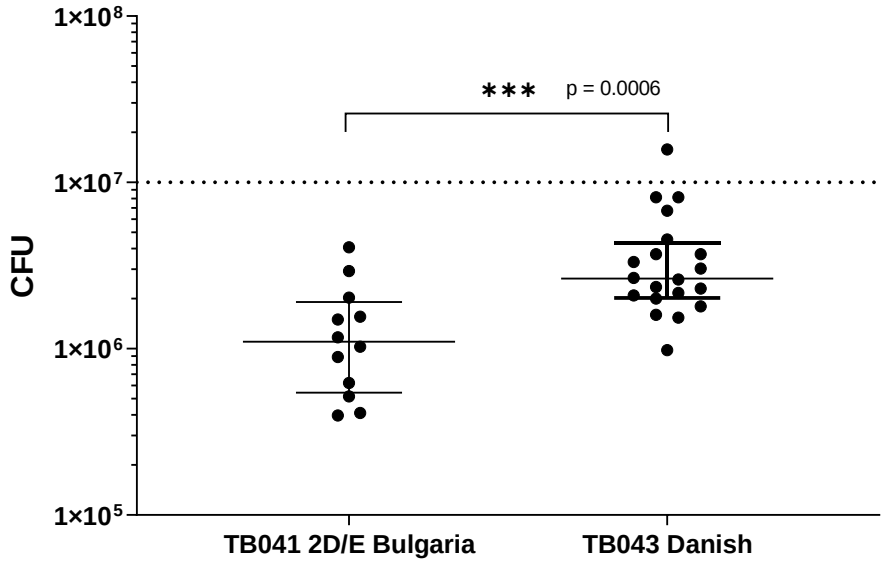


Figure 3.26. Dose loaded into nebuliser of BCG Bulgaria or BCG Danish
Dots represent each volunteer, lines median and IQR. Significant difference on Mann Witney. Plated from leftover dose kept in the dark at 2-8 degrees then plated onto 7H11 agar within 2 hours.

3.6.1 Rationale for dose

For adults, the intradermal licensed dose of BCG Bulgaria is 1.5-6 x10^5cfu in 0.1ml. For BCG Danish 1331 it is 2-8 x 10^5cfu in 0.1ml.[525,526] Our aim was to deliver this dose into the lungs of healthy adult volunteers. However, I hypothesised that, unlike ID injection where the full dose loaded into the syringe is injected under the skin (except for the miniscule amount of fluid retained in the syringe), this is likely not the case for nebulisation.

We developed an *in-vitro* model to quantify the actual delivered dose of nebulised BCG using a vacuum pump attached to an impinger to collect the aerosol expelled from the Omron nebuliser. The amount of BCG in this collected sample was quantified by plating. There was an average of 52.9% loss of BCG compared to the amount of BCG loaded into the nebuliser (Figure 3.27).

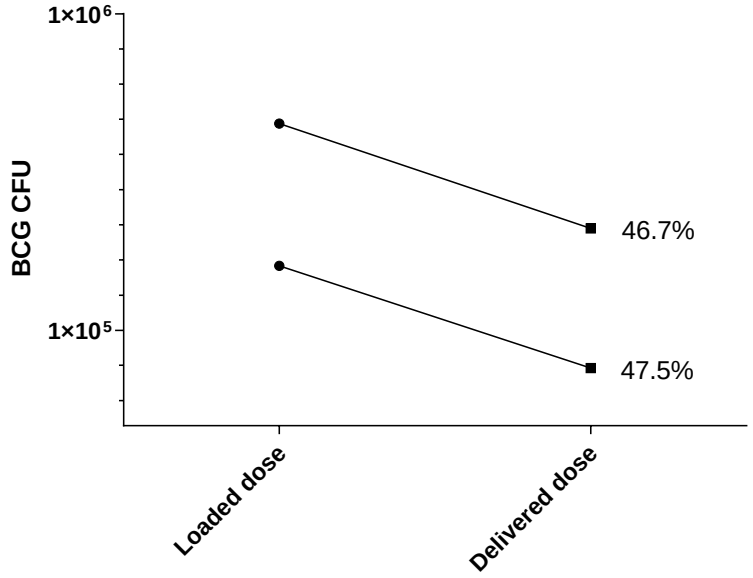


Figure 3.27. Percent of "delivered dose" compared with loaded dose after passing dose through the Omron nebuliser, using two separate BCG Intervax reconstituted vials (average 47.1%).

This model does not take into account the further loss that occurs in a clinical setting. Once started, the Omron nebuliser aerosolises the fluid continuously until dry, however as the volunteer does not breathe in continuously but inhales then exhales over the course of the 2-5 minutes of nebulisation, there is a significant amount of BCG not inhaled during this time. The normal inhalation to exhalation ratio (i/e ratio) at rest is 1:2. Volunteers were instructed to breathe deeply so we might conservatively estimate that the i/e ratio would be closer to 1:1 (i/e ratio on exertion).[527] Including this further 50% loss on nebulisation not captured by our impinger experiment, I estimated a total delivered dose of approximately 23.55% loaded dose.

Using this calculation, I plotted the estimated delivered dose of BCG into the airways of our volunteers (Figure 3.28). This estimated delivered dose indicates that we delivered our target dose of approximately $5x10^5$ cfu (licenced dose as per product SmPCs) by using a prepared ("loaded") dose of $1x10^7$cfu.

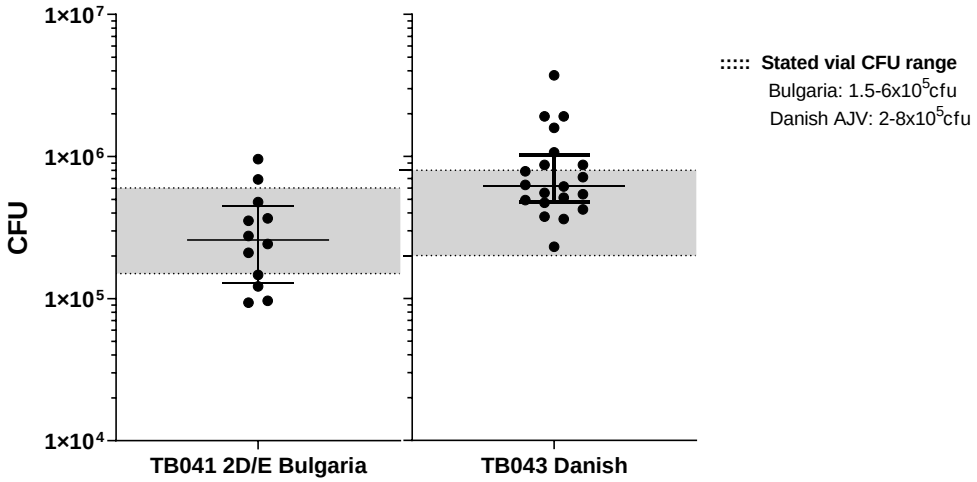


Figure 3.28. Approximate delivered dose of BCG Bulgaria or BCG Danish into lungs of volunteers.
Delivered dose calculated as plated cfu x 0.2355. Dotted line: Target delivered dose as per product SmPC. Bulgaria: Median $5.59x10^5$ cfu, range $9.35x10^4$ – $9.58x10^5$cfu; Danish: Median $6.2x10^5$ cfu, range $2.3x10^5$ – $3.72x10^6$cfu.

3.6.2 Comparing AEs with BCG dose

There was no correlation between solicited AEs following inhaled BCG Bulgaria or Danish and delivered dose (Figure 3.29).

There was also no significance when examining correlations separately by Grade. However, there was a positive trend (r_s>0.5) when comparing moderate and severe respiratory AEs only with dose, in both TB041 Bulgaria or TB043 Danish challenges (Figure 3.30).

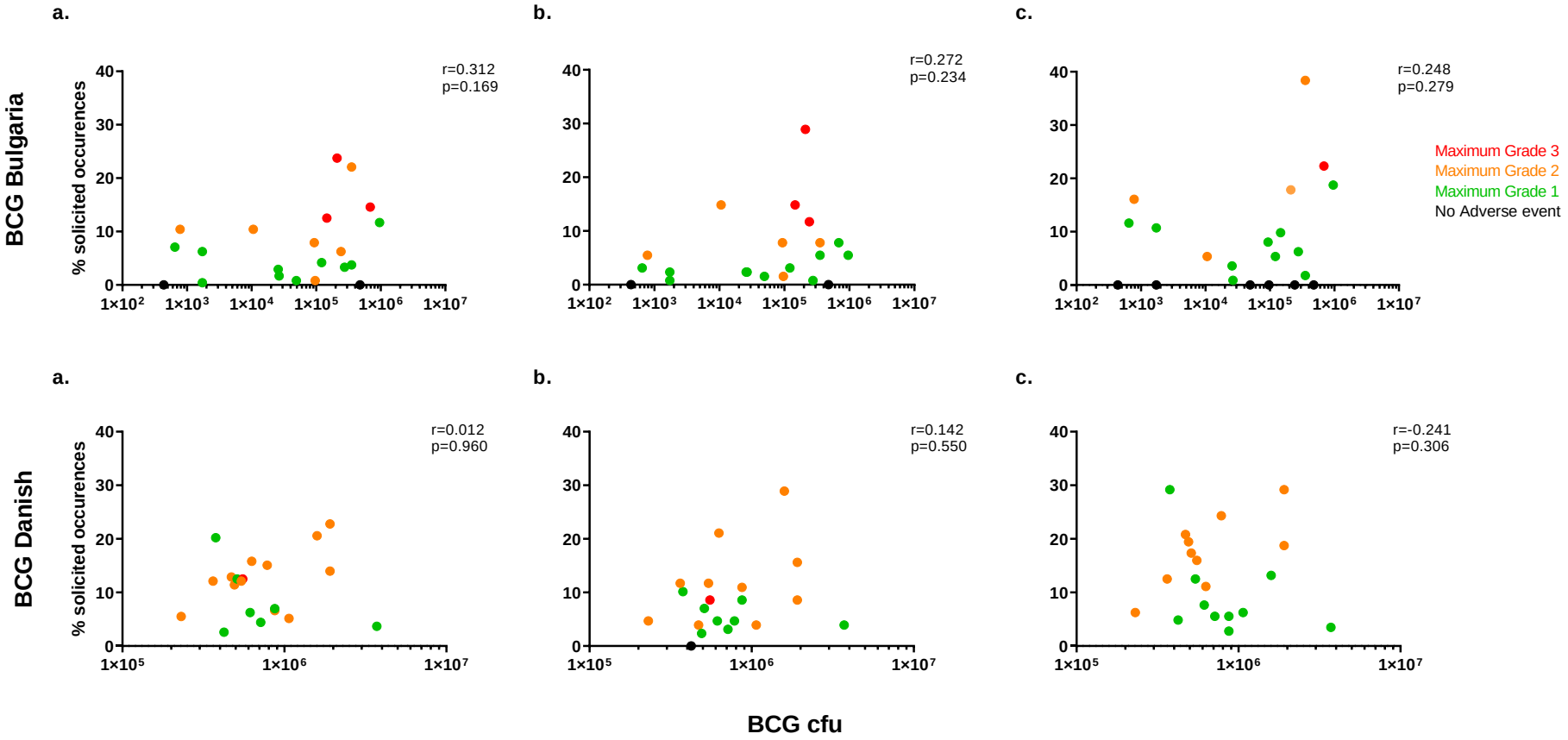


Figure 3.29. Frequency and severity of solicited AEs (a) total (b) systemic and (c) respiratory, following BCG aerosol inhalation, and prior to bronchoscopy by delivered dose and maximum grade. Each dot represents an individual. Note different scales on x-axis. BCG cfu delivered dose calculated based on 23.55% of plated cfu of mixed dose after loading into the nebuliser. BCG Bulgaria includes dose escalation groups 2A-2C (loaded dose target $1x10^4$-$1x10^6$cfu). No correlation on Spearman two tailed test.

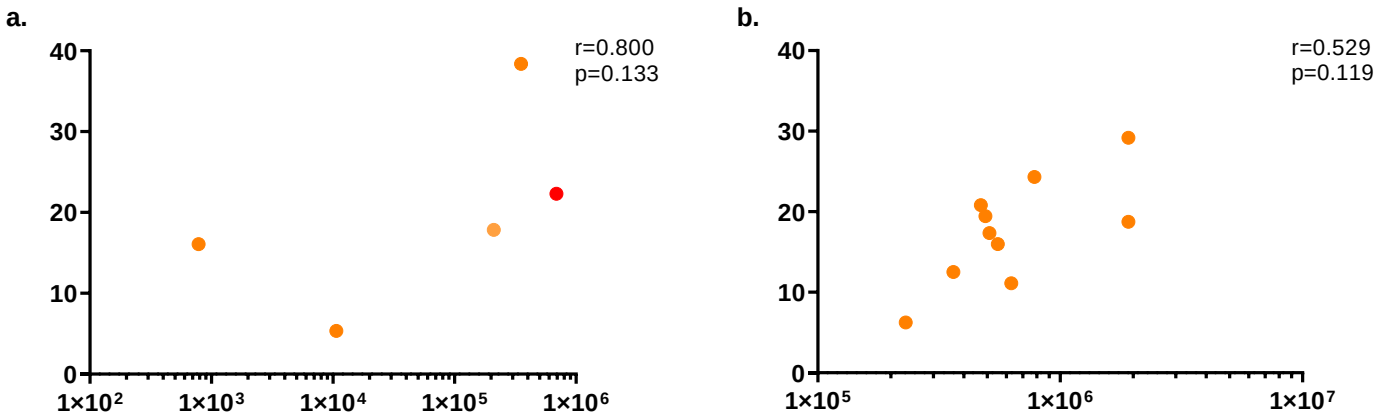


Figure 3.30. Frequency and severity of respiratory solicited AEs in (a) TB041 or (b) TB043, following BCG aerosol inhalation, and prior to bronchoscopy by delivered dose and maximum grade (Grade 2 moderate or Grade 3 severe only). Each dot represents an individual, orange Grade 2, red Grade 3.BCG cfu delivered dose calculated based on 23.55% of plated cfu of mixed dose after loading into the nebuliser. BCG Bulgaria includes dose escalation groups 2A-2C (loaded dose target $1x10^4$-$1x10^6$cfu). No significant correlation on Spearman two tailed test.

3.7 Detection of BCG in BAL and sputum samples

All TB043 volunteers had BCG detected in their BAL fluid by PCR HAIN genotyping at D2 or D7 but only 58% of TB041 volunteers (Table 3.12). A lower proportion of samples at each timepoint had mycobacteria detectable by live culture (MGIT). There were no NTMs definitively identified using the HAIN genotyping kits of 46 NTM spp.

Table 3.12. Mycobacterium detection in BAL fluid. n: Number of volunteers positive per group (% of volunteers positive in each group). Live mycobacteria culture detection by MGIT, species detection using HAIN genotyping. TB043 Group 1 or Group 2 n=10; TB041 Group 2D/E n=12. MGIT: BACTEC Mycobacterial Growth Indicator Tube

Group	BCG	Bronchoscopy timepoint	MGIT positive n (%)	BCG on HAIN n (%)	NTM on HAIN
TB043 Group 1	Danish	D2	6 (60)	10 (100)	0
TB043 Group 2	Danish	D7	2 (20)	10 (100)	0
TB041 Group 2D/E	Bulgaria	D14	5 (42)	7 (58)	0

TB043 aerosol BCG volunteers had induced sputum collected at D84 and D168 (Table 3.13). There was no BCG detected in any samples. Detected NTMs were *M.smegmatis, M.avium, M.celatum, M.chelonae* and *M.gordonae*. Only two volunteers had an NTM detected at both timepoints. TBT-04301018 cultured live *M.chelonae* at both D84 and D168. TBT-04301021 cultured live *M.gordonae* at D84 and live *M.celatum* at D168.

Table 3.13. TB043: Mycobacterium detection in sputum of TB043 volunteers. Live mycobacteria culture detection by MGIT, species detection using HAIN genotyping. n: Number of positive volunteers (% of volunteers positive out of total volunteers in Group 1 and 2). TB043 Group 1 and Group 2 combined D84 n=17, D168 n=11

Timepoint	MGIT positive n (%)	BCG detected n (%)	NTM detected n (%)
D84	6 (35)	0	8 (47)
D168	4 (36)	0	5 (45)

3.8 Discussion

In this chapter I have shown that delivering BCG Bulgaria or BCG Danish 1331 at a loaded dose of $1x10^7$ cfu by Omron nebuliser to healthy BCG-naïve and *M.tb* naïve adults is safe and well-tolerated.

3.8.1 Demographics

There were no differences in any characteristics between groups in either TB041 or TB043. The majority of volunteers were UK born and in TB043, caucasian (92%). This might reflect a slight bias in recruitment, as only 78% of Oxford residents were white British or white "other" in the most recent UK census (2011).[528] There are known variations in immune response related to genetics and country of birth. Caucasian *M.tb*–infected patients have a different inflammatory profile to matched African counterparts.[529] Therefore, results described in this thesis may not be reflective of responses in other population groups.

We enrolled a high proportion of volunteers in their twenties as the majority of older English adults have had the BCG vaccine as part of historic vaccination programmes.[530] Older volunteers tended to be from countries with different BCG vaccination policies. Excluding chronic illness also biased enrolment towards the younger decades.

The majority of volunteers were females, with the exception of TB043 D7 saline (2B). There is a greater incidence of TB amongst men than women.[1] The reasons for these differences are not fully understood and are likely to be multifactorial. Social and cultural factors, such as reporting rates and health seeking behaviours, likely impact this male bias. However differences between genders in innate and adaptive immune response to infection have been reported.[531] Male mice had lower protective immunity against *M.tb*-challenge than females, with concurrent higher levels of pro-inflammatory mediators such as IL-6, IFN-γ, TNFα and IL-

1.[532] By contrast, human females report more AEs following viral vaccination than males.[531,533] The more robust female immune response may result in symptomatic vaccine reactogenicity. In our study there was no significant difference in the frequency or severity of AEs in Group 2B compared with other groups, however numbers are likely too small to detect difference, due to the inherent baseline intra-volunteer variability.

3.8.2 Safety

The AE profile following BCG inhalation was reassuring. Dose escalation proceeded without meeting any safety stopping rules, and there were no SUSARs in either study. Most volunteers reported at least one AE following challenge, but most AEs were mild, and frequency did not differ between infection route (TB041) nor following saline alone (TB043). As local AE data following ID BCG vaccination is well documented, this was not formally collected in TB041, though the injection site was monitored to ensure normal healing. There was no abscess formation following ID BCG injection, despite delivering a higher than licensed dose.

3.8.2.1 Systemic AEs following BCG infection

By either route (ID or aerosol), post-BCG, some volunteers experienced the systemic syndrome of fever, headache, joint or muscle aches, fatigue, malaise and/or nausea, collectively described as a “flu-like illness” (FLI). This “vaccine” reactogenicity is a result of a systemic immune cascade, driven by the innate immune response.[534] PAMPs on the BCG cell wall interact with TLRs and other PRRs with spill over of inflammatory mediators, such as CRP and IL-6, into the systemic circulation.[9] This leads to an increase of prostaglandin E2 in the brain with resultant fever and other symptoms consistent with a FLI.[534] Symptoms tend to peak at 24 hours following vaccination and usually resolve at 48-72 hours, which was the

time course reported here. CRP was elevated in all volunteers who reported a systemic syndrome of vaccine reactogenicity, and there were also a handful of asymptomatic volunteers with a low level rise (<30mg/L).

As expected, there was a trend for TB043 volunteers infected with BCG to report more systemic AEs than volunteers who inhaled saline only. This difference did not reach significance, and this may be due to the small numbers in the saline groups. The most frequently reported systemic AE was headache and fatigue. The documented incidence of headache following ID BCG vaccination is 1 in 100.[526,535] However our ID volunteers reported headache at a rate of 1 in 5 and headache in TB043 saline volunteers was 1 in 10. Hence an alternative explanation for the lack of statistical significance of systemic AEs between TB043 BCG and saline groups might be AEs from concurrent upper respiratory tract infections, or other unrelated illnesses, confounding results. This highlights the importance of including control groups to help elucidate challenge-related AEs.

While there was no significant difference in frequency in TB041 by route, systemic AEs were more severe after aerosol than ID BCG. More aerosol BCG volunteers had an elevated CRP. In addition, two BCG aerosol volunteers experienced blood dyscrasias consistent with a systemic inflammatory cascade.[536,537] BCG introduced under the skin may be more easily contained and thus result in less of a systemic immune response. Why only some volunteers responded so vigorously to aerosol infection is likely due to inherent variation between immune activation and regulation, a consequence of both genetics, environment and prior pathogen exposure.[351,538-540] The severity of vaccine adverse reactions is variable between individuals and not directly correlated with immunoprotection.[541]

3.8.2.2 Respiratory AEs following BCG infection

There was no difference in respiratory AEs between those inhaling saline alone (with or without ID BCG infection), compared with those inhaling BCG suspended in saline. Saline volunteers frequently reported cough and sore throat. Unlike hypertonic saline, which is a known mild airway irritant, the 0.9% saline delivered here is physiologically isotonic. However, isotonic saline alleviates symptoms of bronchiectasis and improves cystic fibrosis outcome and, hence the clinical sequelae of 0.9% saline inhalation is not be neutral.[542,543] Therefore, saline may cause mild airway irritation, resulting in the AEs reported. Alternatively, as the majority of volunteers were enrolled over winter (TB041 and Group 1 TB043), a concurrent respiratory viral infection could explain these respiratory AEs. The respiratory AEs after BCG inhalation could therefore be related to BCG infection, however, as saline volunteers also reported a similar AE profile, other causes such as saline irritant, or concomitant viral infection cannot be excluded.

3.8.2.2.1 Chest pain and tightness

The one respiratory AE that aerosol BCG volunteers reported more than ID BCG volunteers was chest tightness or chest pain. Chest pain within 3 days of BCG inhalation could be due to immune hyperresponsiveness due to a concurrent viral infection or an underlying immune propensity as discussed above. A transient alveolitis would be expected following any inhaled infection as part of a normal immune response.[544,545] For the majority of volunteers, this process seemed not to cause any symptoms, but for the few, this could explain the chest pain symptoms. The cause of symptoms reported 10 days following infection is less clear. In mice, primed BCG-specific lymphocytes migrate into the local infection site 1-2 weeks post exposure.[48,54,545,546] Hence these chest symptoms might still be consistent with an alveolitis occurring around this time.

Our group has previously shown that BCG reaches peak growth under the skin 1-2 weeks after ID injection and is still detectable at 4 weeks.[480] In mice, BCG replicate for 4 weeks in the skin and up to 12 weeks in the draining lymph node.[502] In my volunteers there was likely a similar self-limiting BCG replication in the airways and lung parenchyma following BCG aerosol infection. If bacterial growth peaked at around 1-2 weeks, this would correspond with the second peak of chest symptoms reported here.

Systemic symptoms, in particular fever, would point to a disseminated BCG-osis. Volunteers reporting prolonged fever or other Grade 2 AEs had blood culture for mycobacteria. In all cases, blood cultures were negative, and volunteers' symptoms spontaneously resolved, excluding the possibility of a disseminated BCG infection at any timepoint.

3.8.2.3 Lung function tests

We recorded spirometry readings in TB041 to detect an inflammatory or hyperresponsive airway following BCG infection. When exposed to an irritative aerosol, airways can spasm, resulting in an obstructive lung picture on spirometry. Inhalation of the BCG or the diluent products might also cause transient irritation to the upper or lower respiratory system, with sore throat, cough or symptoms related to bronchospasm such as wheeze or shortness of breath. While some volunteers did report these symptoms, there was no difference in spirometry readings post-aerosol BCG compared with inhaling saline alone.

In TB043 we added TLCO as part of our baseline screening. TLCO decreases if there is a barrier to gas exchange across the interstitium. One cause for this is migration of immune cells and fluid into this space in response to inflammation (alveoli infiltrates) as discussed above.[544] Here the VA is low, KCO is low to "normal", and TLCO is markedly reduced.[547] This process may cause clinical symptoms, as was likely the case in the TB041 volunteer with chest pain. However, as this was not a routine screening test in TB041, but only performed when

clinically indicated, results for this volunteer were difficult to interpret acutely without a baseline reading. While TLCO readings did drop following BCG inhalation in TB043 in some volunteers, the majority were asymptomatic and all maintained their TLCO above a normal value of 80% predicted. A transient asymptomatic fall in TLCO might occur frequently as a marker of the expected physiological transient influx of cells and fluid into the interstitial space as a normal response following any aerosol infection. While asymptomatic transient changes in TLCO are not of particular importance for safety endpoints, they do help to understand the underlying physiologically processes, which in turn informs our understanding of the infection and immune processes, ultimately improving safety of aerosolised BCG delivery.

3.8.2.4 Bronchoscopy

Over the two studies only two D2 BCG aerosol volunteers had macroscopic changes detected on bronchoscopy. The respiratory consultant advised no further action as these minor changes were expected to self-resolve. Both volunteers reported mild respiratory AEs post-BCG infection, but these had resolved by the bronchoscopy and this airway erythema was asymptomatic. Local airway inflammation would be expected following an infection and it is interesting that most volunteers had no macroscopic inflammation. It is possible that some volunteers who had their bronchoscopy at D7 or D14 may also have had transient airway erythema around D2 which went undetected.

There was no difference in frequency of severe AEs after bronchoscopy in BCG versus saline volunteers, however, the AE was more likely to be mild if the volunteer had inhaled saline. While there was very little macroscopic evidence of airway inflammation, the difference in severity of AEs between groups may be due to a microscopic subclinical inflammation. A

further insult to the airways, in the form of a bronchoscopy, might exacerbate this, resulting in the symptomatology described.

3.8.2.5 TB041 Groups 2D and 2E

The increased frequency of respiratory AEs in Group 2D compared with 2E may be due to over reporting of AEs in Group 2D volunteers. These volunteers knew that they had inhaled BCG and that they were the first volunteers to inhale a higher dose. This highlights the importance of including blinding in clinical trial design.

An alternative explanation is the higher median dose of viable BCG that Group 2D volunteer received compared to Group 2E. This was due to the intra-vial variability in viable BCG cfu and association with time to expiry. However, as there was no significant correlation between BCG dose and AE reporting overall, this explanation is less likely.

3.8.2.6 Comparison with other studies

AEs were not directly comparable to Dheda's study due to the masking effect of their bronchoscopic BCG instillation procedure, and their different population cohort.[206] 70% of their volunteers reported AEs which were deemed related to bronchoscopy. However, these AEs were similar to those seen in TB041 and TB043 post aerosol BCG, and included SOB, chest pain, cough and fever. They concluded there were no AEs in their study definitively related to BCG instillation. AEs following PPD instillation in Silver's study were similar to that reported post-BCG bronchoscopic installation.[409]

In the historic BCG challenge studies, no AEs were reported in the children exposed to BCG in a room (exposure estimated to be up to 2.2×10^4 "clones").[492] BCG-related AEs in the lung cancer patients (exposed to repeated high doses of BCG) are difficult to define given their health condition at the time of challenge.[493,494]

3.8.3 BCG dose

The amount of viable cfu plated from the vial was 0.5-1 log lower than that stated in the SmPC, particularly for BCG Bulgaria Intervax. There may have been loss of viable bacteria between the time of reconstitution and plating. However, vials were kept in the dark between 2-8 degrees and plated within 2 hours, well within the 4-6 hours post-reconstitution viability for vaccination advised by SmPC. Viable bacteria cfu was proportional to the remaining shelf life for BCG Bulgaria but not BCG Danish, despite BCG Danish being closer to expiry than BCG Bulgaria. Bulgaria has a longer shelf life and hence loss may be related to time since manufacture. In addition, BCG Bulgaria was supplied in a long-necked glass vial and there was visible loss of bacteria on opening and discarding the vial top.

Passing the BCG through the nebuliser led to a 50% loss of viable BCG. The length of a mycobacterial cell rod is 1.5-4 µm and the Omron nebuliser mesh MMAD is 4.2 µm.[516,548] This loss may reflect clumps of bacteria not passing through the mesh, with perhaps some larger bacterial rods also not passing through, or being shredded and losing viability.

I hypothesised a further conservative 50% loss of BCG through respiration and hence I demonstrated that the amount of BCG we delivered into the lungs of our volunteers was close to the target, licenced, dose of $5x10^5$cfu. An NHP study using the same nebuliser, estimated a similar delivered dose (1.08 log loss).[426] By contrast, modelling bioaerosol viability and particle dynamics, the Roy group estimated losses of 2-3 log.[515]

Not all inhaled BCG will enter the lower airways as bacteria will be captured in the upper airway and a proportion will be swallowed. The Omron nebuliser has been designed specifically to deposit particles into the lower airways, with estimation of >70% inhaled dose.[549] Additionally, volunteers were encouraged to inhale slowly and deeply, increasing particle deposition into the lower airways. What effect upper airway versus lower lung infection has on AEs and immune responses is uncertain.[550]

While there was no significant correlation between delivered dose and solicited AEs in my study, there was a trend for increased severity of respiratory AEs and dose. A repeat challenge using a higher dose might clarify if this trend reflects a true correlation, and whether systemic AE severity is also affected by dose. However, given the severe AEs reported in some volunteers at the current dose, repeating the study using a higher dose would not be advisable.

In addition, regardless of AEs, dose may affect immune response. Dheda's group saw significantly lower immune responses after the $1x10^3$ compared with the $1x10^4$ dose in their bronchoscopic instillation of BCG in *M.tb*-exposed patients.[206] Immune responses will be described in the next two chapters.

3.8.4 BCG Bulgaria compared to BCG Danish

Volunteers inhaling BCG Bulgaria (TB041) were more likely to report severe AEs than volunteers inhaling the same dose of BCG Danish. Due to differences in time periods, this data is not directly comparable and the difference might be underestimated. AEs from TB043 was presented for an average of 6.5 timepoints (half volunteers 4 timepoints, half 9) compared with TB041 which was over 16 timepoints. Hence, as the majority of AEs occurred in the 2-3 days following BCG, the percent solicited AEs would be underestimated in TB041 compared with TB043. Further, post-bronchoscopy data from TB043 may also include some AEs related to BCG infection given the closer proximity in time from inhalation (D2 or D7 compared with D14 for TB041), again inflating TB043 AE post-bronchoscopy data compared with TB041.

The fact that volunteers reported more AEs following BCG Bulgaria (TB041) than BCG Danish (TB043), despite receiving less viable BCG, could be explained by difference in strain.

BCG Danish is from phylogenetic group DU2 Group III and BCG Bulgaria from DU2 Group 1 (RD Russia).[551] The two strains have similar safety profiles when administered intradermally however Bulgaria is slower growing than Danish on culture media.[552] Whether strains from different groups vary in efficacy is a matter for ongoing debate.[6,553] *In-vitro* BCG culture with human PBMCs results in a quantifiable difference in response by BCG strain, for example the proportion of Mycobacteria-specific single cytokine or Th1 polyfunctional CD4+ T cells was higher in the PBMCs of infants or adults vaccinated with Danish or Japan strains compared with Russia.[554-556] It remains unclear whether this difference translates to protection, as there has been no significant difference found between strains on systematic review.[6]

A further explanation is that our vaccine vials of BCG Bulgaria may have been particularly slow growing, resulting in lower counts at 3 weeks, without reflecting original cfu vial count. Slow growth rate of BCG was associated with larger BCG scar formation and PPD responses in Guinea-Bissau infants.[557] While there was no increase in AEs in these infants, there was an increase in reported cases of suppurative lymphadenitis in Singapore infants given the same batch.[558]

An alternate explanation for the difference in AE profile seen between Bulgaria and Danish could be a difference in viable bacteria. Loss of viability over time, means an increased proportion of dead to live bacteria within the vial, resulting in more dead BCG delivered to BCG Bulgaria volunteers. Dead bacteria, with associated extravasation of cell contents and DNA fragments might be more reactogenic than competent BCG, despite the replicating capacity of live BCG. However, in *in-vitro* studies, non-viable bacteria are associated with reduced Th1 cytokine production, inflammasome activation and innate trained immunity.[552,559]

3.8.5 Detection of Mycobacteria in BAL and sputum

Live BCG was detected in BAL at all timepoints (D2, D7 and D14) after BCG Danish or Bulgaria inhalation. A lower proportion of volunteers had BCG DNA detected in their BAL at D14 compared with D7 or D2, however D14 TB041 samples are likely not directly comparable to D2 and D7 TB043 samples as we used a greater lavage volume in TB043 (150ml vs 100ml). While all TB043 volunteers had BCG detected in their BAL, fewer volunteers had live BCG at D7 compared with D2. Whether this is reflective of better BCG growth control at D7 is unclear. All human cells were spun off from the BALF for immunology prior to BCG culture, and so the BCG detected here is likely not representative of BCG load in the airway. A follow on study is planned for comprehensive quantification which will also include intracellular BCG from the BAL. Dheda's group reported BCG culture from 9% volunteers 3 days post-BCG bronchoscopic instillation in *M.tb*-exposed volunteers, which is less than seen here at any timepoint.[206] However, only 10ml fluid was cultured and it is unclear whether this also included lavage cells.

I was unable to quantify the amount of BCG recovered per individual in this study. Despite using a standard saline lavage volume, BAL return varies between patients, due to anatomical differences and variable saline absorption. [560] There is no validated method to calculate dilution factor to standardise BAL fluid.[560] Furthermore, BCG cfu was not quantifiable from MGIA as BAL TTP results corresponded to less than 1 cfu using our standard curve. This may be due to a delay in commencement of BCG replication after lung retrieval. Work to resolve these issues is ongoing.

Reassuringly, there was no BCG detected on induced sputum by 3 months after aerosol infection. Induced sputum may be less sensitive to bronchoscopic lavage for detection of sub-clinical infection, though a recent review concluded induced sputum was as sensitive as bronchoscopy for pulmonary TB disease diagnosis in smear negative patients.[561] While this is

not definitive proof of clearance, as BCG may have tracked to LNs or other tissues,[562] as volunteers had no ongoing symptoms and all had robust immune responses, clearance is a likely hypothesis.

Almost 50% of volunteers had NTM detected in their sputum. Environmental contamination is one hypothesis, as collection occurred as per standard clinical protocol, and not under sterile conditions. For this reason, serial tests are recommended for clinical diagnosis of NTM disease.[563] One volunteer had the same NTM detected at both timepoints, making environmental contamination less likely. While incidence of NTM infection is difficult to determine in the UK as colonisation surveys on asymptomatic people are not routine practice, UK prevalence of NTM disease is rising.[564,565] However, such high colonisation levels are surprising and warrant further investigation to confirm these findings.

3.9 Conclusions

- Delivering BCG Bulgaria or BCG Danish 1331 at a loaded of dose of $1x10^7$ cfu by Omron nebuliser to healthy BCG-naïve and *M.tb* naïve adults is safe and well-tolerated.
- In the high dose groups, approximately $5x10^5$ aerosol BCG was likely delivered into the lungs of our volunteers.
- The majority of all reported AEs were mild, all were transient and all resolved spontaneously.
- There was no significant difference in solicited respiratory AEs or lung function between BCG aerosol groups and either ID BCG groups (TB041) or inhaled saline alone (TB043).

- Volunteers inhaling BCG were more likely to report systemic AEs or these AEs were more likely to be severe than following intradermal BCG infection, or inhaled saline alone.
- Following BCG inhalation, some volunteer reported a self-resolving pleuritic chest pain or tightness, with or without associated transient reduction in gas transfer on TLCO. This is likely reflective of a transient alveolitis, though it may also be symptomatic peak BCG growth.
- Volunteers unblinded to aerosol route are more likely to report respiratory symptoms.
- For both BCG strains, viable bacteria were consistently less than that stated on the SmPC. For BCG Bulgaria this was correlated with time to expiry.
- BCG Bulgaria inhalation resulted in increased AE reporting compared with BCG Danish, despite lower numbers of viable bacteria being delivered.
- BCG was recovered from all volunteers in the BAL at D2 or D7, and in more than 50% volunteers at D14. There was no BCG cultured from induced sputum from 3 months post-infection.

Table 3.14. TB041: Unsolicited AEs, observation and laboratory AEs occurring in the first 28 days following aerosol BCG Bulgaria challenge. Coded using MedDRA LLT 2020

Volunteer	Timepoint	Adverse event	Duration	Grading	Relatedness
Group 2D					
TBT-0411033	Day 1	Diarrhoea (one episode loose stool)	Once	1	Possible
	Day 8	Cold symptoms/rhinorrhoea Associated with general malaise, chest pain and shortness of breath	4 days	2	Possible
	Day 9	Dizziness	2 days	3	Possible
	Day 9	Tachycardia	2 days	2	Possible
	Day 10	Loss of consciousness-assessed by paramedics at the scene. NAD. No treatment given	Few minutes	3	Possible
	Day 13	Laryngitis ("lost voice")	1 days	2	Possible
TBT-0415503	Day 0	Rhinorrhoea	2 days	1	Possible
TBT-0415504	Day 1	Sinusitis	3 days	2	Possible
	Day 2	Tachycardia to 103, detected incidentally at visits	Resolved by Day 7	1	Possible
	Day 10	Epistaxis	Once	1	Possible
	Day 21	Insomnia	Ongoing	1	Not related
	Day 26	"Sore tonsils"	55 days	1	Possible
Group 2E					
TBT-0411052	Day 2	CRP 9.8 mg/L	Resolved by Day 7	Not gradable	Probable
TBT-0411057	Day 0	Bad taste	< 12 hours	2	Probable
	Day 0	Hypersensitivity pneumonitis Likely secondary to BCG inhalation. Symptoms: Flu like illness, Chest pain, SOBOE recorded in e-diary D1-D7 (solicited symptoms). Respiratory viral PCR negative on nasal swab. TLCO 66.8% predicted 24 Oct (D8). No baseline. Referral to respiratory consultant. Repeat TLCO 1 month later (6 December) was low normal. Pneumonitis deemed resolved discharged from respiratory clinic	2 months (51 days)	3	Probable
	Day 2	CRP 90 mg/L with concurrent "flu-like symptoms" as recorded under solicited AEs	Resolved by Day 7	Not gradable	Probable
	Day 8	Cold sore lip (herpetic)	7 days	1	Possible
BT-0411057	Day 14	Increased white cell count	Resolved Day 21	1	Probable
TBT-0415513	Day 2	Diastolic hypertension	Resolved by Day 14	1	Possible
	Day 10	Chest heaviness	2 days	1	Possible
TBT-0415516	Day 1	Sensation of throat swelling (volunteer states common symptoms for her when unwell)	2 days	1	Probable
	Day 2	CRP 70 mg/L with concurrent "flu-like symptoms" as recorded under solicited AEs	Resolved by Day 7	Not gradable	Probable
	Day 7	Anaemia: Haemoglobin 113 g/L at Day 7 visit.	Resolved by Day 14	1	Unlikely
TBT-0411050	Day 2	CRP 29.1 mg/L with concurrent cough and chest pain as recorded in solicited AEs	Resolved by Day 7	Not gradable	Probable
TBT-0415510	Day 2	CRP 40 mg/L with concurrent "flu-like symptoms" as recorded under solicited AEs	Resolved by Day 7	Not gradable	Probable
	Day 28	Sore throat	3 days	1	Possible
TBT-0415515	Day 2	CRP 10 mg/L	Resolved by Day 14	Not gradable	Probable
	Day 2	Grade 2 Diastolic hypertension (Grade 1 present prior to challenge)	Resolved by Day 7	2	Possible

Table 3.15 TB041: Solicited and unsolicited AEs, observations and laboratory AEs 1-6 months following aerosol BCG Bulgaria challenge. Coded using MedDRA LLT 2020

Subject ID	Timepoint	Adverse events	Duration	Grading	Relatedness
Group 2D					
TBT-0411033	8 years prior to trial commencement	Hypermobility syndrome: Intermittent usual joint pain (knees and hips)	Intermittent throughout	2	Not related
	5 months (D160)	Right knee dislocation with pain	Ongoing	3	Not related
	5 months (D160)	Vomiting secondary to Tramadol (taken for pain due to knee dislocation)	1 day	3	Not related
	3-6 months	Fatigue- exact start date unknown	Ongoing	1	Not related
TBT-0415503	3-6 months	Sore throat –exact start date unknown	Unknown, not ongoing	1	Unlikely
TBT-0415504	5 months (Day 155)	"head cold" and sinusitis	ongoing	1	Unlikely
Group 2E					
TBT-0411050	2 months (Day 61) 2.5 months (Day 77)	Migraine - not unusual for the volunteer, not really affecting activities. Not needing medication.	1 day 1 day	1	Not related
TBT-0411052	5 months (Day 146)	Headache. Possibly related to work/screen use/ tension	1 day	1	Not related
TBT-0411056	5 months (Day 153)	Fatigue and malaise. Dose of sertraline was changed from 25mg to 100mg.	3 days	2	Not related
TBT-0411057	 Day 67 Day 69 Day 69 Day 69 Day 69	Chest infection: Sore throat Malaise Feverishness Shortness of breath on exertion Coughing – non-productive	 2 days 10 days 2 days 5 months 5 months	 1 1 1 2 2	Possible
	4 months (Day 121)	Chest infection: Cough Shortness of breath on exertion	14 days	 2 2	Unlikely
	5 months (Day 141) Day 142	Tonsillitis diagnosed by GP, treated with Antibiotics. Associated with nausea, fatigue and feeling generally unwell. Fever	9 days 8 days	3	Unlikely
TBT-0415513	3 months (Day 85)	Headache	5 days	1	Not related
	5.5 months (Day 161) 6 month	Hypertension Diastolic hypertension at final 6 month visit	1 day	1 1	Not related
TBT-0415515	Day 52	Pain due to left 7cm ovarian cyst – admitted to hospital for pain relief. Discharged with analgesia. Reported as SAE	4 days	3	Not related

Table 3.16 TB043: Unsolicited, observations and laboratory AEs from D0 and post-ediary solicited adverse events following BCG Danish aerosol inhalation. Unsolicited AEs in bold**.** Coded using MedDRA LLT 2020.

BCG	Timepoint	Adverse event	Duration	Max grade	Relatedness
Group 1					
TBT-04301003	Day 11	Sneezing more than usual	2 days	1	Possible
	Day 12	Cough	3 days	1	Probable
	Day 14	Chest tightness (during spirometry)	1 day	1	Probable
	Day 81	Sore throat	3 days	1	Unlikely
	Day 81	Cough productive of phlegm	3 days	1	Unlikely
	Day 81	Bedtime wheeze	3 days	1	Unlikely
	Day 81	Conjunctivitis left eye	2 days	1	Unrelated
TBT-04301007	Day 51	Tickly throat from hay fever	3 days	1	Unlikely
TBT-04301008	Day 22	Fatigue	5 days	1	Unlikely
	Day 70	Cough	2 days	1	Unlikely
	Day 70	Headache	2 days	1	Unlikely
TBT-04301010	Day 2	Cough post bronchoscopy	13 days	2	Probable
	Day 30	Sore throat	2 days	1	Possible
	Day 65	Chronic sinusitis (pre-existing)	Ongoing	1	Unlikely
TBT-04301015	Day 0	Increased mucous	1 day	1	Probable
	Day 2	**Hoarse voice** (from bronchoscopy)	1 day	1	Possible
	Day 28	Feverishness	1 day	2	Possible
	Day 29	Headache	1 day	1	Possible
	Day 60	Headache	1 day	1	Unlikely
	Day 65	Malaise	4 days	1	Unlikely
	Day 65	Sore throat	4 days	1	Unlikely
	Day 112	Feverishness	6 days	2	Unlikely
	Day 112	Headache	6 days	1	Unlikely
	Day 112	Fatigue	6 days	2	Unlikely
	Day 112	Nausea	6 days	1	Unlikely
	Day 112	Malaise	6 days	2	Unlikely
	Day 112	Cough, with phlegm	13 days	2	Unlikely
	Day 112	Shortness of breath	6 days	1	Unlikely
	Day 112	Chest tightness	6 days	1	Unlikely
	Day 115	Wheeze	1 day	2	Unlikely
TBT-04301017	Day 0	Dry lips	12 hours	1	Probable
	Day 2	**Vomit** x 1 following bronchoscopy	-	1	Possible
	Day 3	**Back ache** (period pain)	2 days	1	Unlikely
	Day 14	Cough	1 day	1	Probable
	Day 14	Tickly throat	1 day	1	Probable
	Day 44	Headache	6 days	1	Unlikely
	Day 44	Sinus congestion	6 days	1	Unlikely
	Day 50	Nausea	3 days	1	Unlikely
	Day 78	Blocked nose	3 days	1	Unlikely

BCG	Timepoint	Adverse event	Duration	Max grade	Relatedness
Group 1					
	Day 80	Headache	14 days	2	Unlikely
	Day 98	Feverishness	1 day	2	Unlikely
	Day 98	Nausea	1 day	1	Unlikely
	Day 98	Malaise	1 days	2	Unlikely
TBT-04301018	Day 44	Headaches (intermittent)	10 days	1	Unlikely
	Day 75	Arthralgia	5 days	1	Unlikely
	Day 75	Fatigue	5 days	2	Unlikely
	Day 76	Headache	2 days	2	Unlikely
	Day 126	Earache	14 days	2	Unlikely
	Day 126	Sinus pain	14 days	1	Unlikely
	Day 126	Fatigue	14 days	1	Unlikely
TBT-04301019	Day 0	Chills, feeling freezing	2 days	2	Probable
	Day 4	Neck pain	Ongoing	2	Unrelated
	Day 14	Panic attacks, low mood	30 days	2	Unrelated
	Day 15	Fatigue, related to stress	40 days	2	Unlikely
	Day 137	Feverishness related to sinusitis	6 days	2	Unlikely
	Day 137	Sinus infection	6 days	3	Unlikely
	Day 137	Fatigue	Ongoing	2	Unlikely
	Day 166	Headache, related to scoliosis	1 day	3	Unrelated
TBT-04301020	Day 1	Sore eye	2 days	1	Unlikely
	Day 8	Diarrhoea	2 days	1	Unlikely
	Day 21	Coughing up phlegm	31 days	1	Possible
	Day 57	Coughing up phlegm	2 days	1	Unlikely
	Day 56	Sore throat	3 days	2	Unlikely
	Day 67	Feverishness (due to tonsillitis)	13 days	2	Unlikely
	Day 67	Headache	13 days	1	Unlikely
	Day 67	Fatigue	13 days	3	Unlikely
	Day 67	Malaise	13 days	2	Unlikely
	Day 67	Cough	13 days	1	Unlikely
	Day 67	Sore throat	13 days	3	Unlikely
	Day 113	Fatigue, possibly due to diet	5 days	2	Unlikely
TBT-04301021	Day 1	Rough sounding voice	2 days	1	Probable
	Day 2	Indigestion/ bloating	1 day	1	Unlikely
	Day 3	**"lung pain"** post bronchoscopy	1 day	1	Probable
	Day 58	Feverishness	4 days	1	Unlikely
	Day 58	Myalgia	4 days	1	Unlikely
	Day 58	Fatigue	4 days	1	Unlikely
	Day 58	Malaise	4 days	2	Unlikely
	Day 58	Cough	4 days	2	Unlikely
	Day 58	Sore throat	4 days	2	Unlikely
	Day 58	Tickly throat	4 days	2	Unlikely

BCG	Timepoint	Adverse event	Duration	Max grade	Relatedness
Group 2					
TBT-04301024	Day 1	Feeling faint/dizzy	1 day	2	Probable
	Day 1	Trouble sleeping	1 day	2	Possible
	Day 10	Sneezing	1 day	1	Possible
	Day 10	Itchy eyes	1 day	1	Possible
	Day 10	Runny nose	1 day	1	Possible
TBT-04301024	Day 26	Eye pain and redness	8 days	1	Unrelated
	Day 42	Malaise	4 days	1	Unlikely
	Day 42	Cough	4 days	1	Unlikely
	Day 42	Sore Throat	4 days	1	Unlikely
	Day 42	Runny nose	4 days	1	Unlikely
	Day 118	Headache (due to period pains)	1 day	2	Unrelated
TBT-04301026	Day 1	Chills at night	1 day	1	Probable
	Day 78	Fractured foot	26 days	3	Unrelated
	Day 154	Watery eye	7 days	2	Unrelated
	Day 156	Sore throat	2 days	1	Unlikely
	Day 156	Malaise	2 days	1	Unlikely
	Day 160	Dry cough	4 days	1	Unlikely
	Day 160	Wheeze (on exercise)	4 days	1	Unlikely
	Day 160	SOB (on exercise)	4 days	1	Unlikely
TBT-04301027	Day 0	Joint pain at base of the neck	1 day	1	Unlikely
	Day 2	Urinary frequency	1 day	1	Unrelated
	Day 2	Dysuria	1 day	1	Unrelated
	Day 21	Headache	1 day	1	Possible
	Day 35	Headache and cold symptoms	1 day	2	Unlikely
	Day 35	Malaise	3 days	1	Unlikely
	Day 35	Sore throat	1 day	1	Unlikely
	Day 35	Runny nose	3 days	1	Unlikely
	Day 167	Headache	3 days	2	Unlikely
	Day 167	Runny nose	3 days	1	Unlikely
TBT-04301028	Day 0	Sneezing	1 day	1	Possible
	Day 9	Lost voice	5 days	1	Possible
	Day 12	Blocked nose	3 days	1	Possible
	Day 41	Cough, with phlegm	10 days	1	Unlikely
	Day 41	Tickly throat	10 days	1	Unlikely
	Day 41	Blocked nose	10 days	1	Unlikely
	Day 163	Feverishness	2 days	1	Unlikely
	Day 163	Headache	2 days	1	Unlikely
	Day 163	Runny nose	2 days	1	Unlikely
	Day 163	Sore throat	2 days	1	Unlikely
TBT-04301029	Day 9	Rash on chest	2 days	1	Unlikely
	Day 9	Hormonal, low mood	2 days	2	Unlikely
	Day 9	Cough with phlegm	40 days	2	Probable
	Day 13	Intermittent headache	11 days	1	Probable
	Day 13	Malaise	1 day	1	Probable
	Day 13	Sore throat	1 day	1	Probable
	Day 13	Tickly throat	1 day	1	Probable

BCG	Timepoint	Adverse event	Duration	Max grade	Relatedness
Group 2					
	Day 15	Myalgia	10 days	1	Possible
	Day 15	Arthralgia	10 days	1	Possible
	Day 20	Fatigue	27 days	1	Possible
	Day 22	Malaise	5 days	3	Possible
	Day 22	Sore throat	3 days	3	Possible
	Day 50	SOB on exertion	3 days	3	Unlikely
	Day 50	Chest tightness	3 days	3	Unlikely
	Day 50	Nasal congestion	2 days	2	Unlikely
	Day 50	Runny nose	2 days	2	Unlikely
TBT-04301030	Day 24	Malaise, cold symptoms	3 days	1	Possible
	Day 24	Cough, phlegm	3 days	1	Possible
	Day 24	Sore throat	3 days	1	Possible
	Day 50	Malaise	4 days	1	Unlikely
	Day 50	Cough	4 days	1	Unlikely
	Day 50	Sore throat	4 days	1	Unlikely
	Day 50	Phlegm	8 days	1	Unlikely
	Day 51	Blocked nose	7 days	1	Unlikely
TBT-04301031	Day 4	Sores in mouth	4 days	1	Possible
	Day 8	Testicle discomfort	1 day	1	Unlikely
	Day 8	Felt lightheaded	1 day	2	Possible
	Day 8	Phlegm on throat (not cough)	Ongoing	1	Possible
	Day 15	Back pain	1 day	1	Unrelated
	Day 187	Left calf/ sole pain (after running)	3 days	1	Unrelated
TBT-04301042	Day 0	Rash on right hand	2 days	1	Possible
	Day 24	Headache	2 days	1	Possible

Chapter 4.

Local immune responses

4 Local Immune Responses

4.1 Introduction

The previous chapter demonstrated safety of an aerosol attenuated *M.tb* species human challenge model in healthy *M.tb*-naive adults. This chapter aims to characterise the early local mucosal immune responses following this infection. To my knowledge, these BCG aerosol studies are the first to investigate immune responses following an aerosol inhaled BCG infection in *M.tb* naïve people, and TB043 is the first to investigate mucosal responses in humans 1 week following mycobacterial infection challenge. Results from TB043 are presented here. For my thesis I analysed the first two groups. Each group had 10 volunteers who inhaled 10^7cfu BCG Danish (AJV) and 3 volunteers inhaled aerosolised saline. Group 1 had a bronchoscopy at D2 and Group 2 a bronchoscopy at D7.

4.2 Statement of Authorship

All assays were developed by J. Marshall unless otherwise stated. Flow panels titrations and media culture experiments were performed by J.Marshall with the help of R.Wittenberg. BAL and biopsy processing was performed by J.Marshall. Additional hands were provided on bronchoscopy processing days as required by S.Harris, I.Satti, R.Wittenberg, R.Tanner, E.Hoogkamer and H.Morrison. J.Marshall performed all flow cytometry staining and analysis. 10x library preparation and sequencing was performed by the Wellcome Trust Centre for Human Genomics. M.Surakhy cleaned and QC'd the data and performed all R analysis. J.Marshall manually assigned cluster names and interpreted all data including DGE analysis. M.Surakhy produced the figures in collaboration with J.Marshall.

4.3 Results

4.3.1 Endobronchial Biopsies:

Endobronchial biopsies were obtained for all participants. In Group 1, the first four biopsies were sent for histology and if the volunteer was tolerating the procedure, an additional two were obtained for flow cytometry. These were digested and stained for the innate panel. 7 volunteer samples were obtained and cell counts are shown in Figure 4.1.

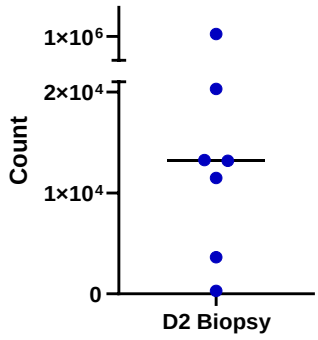
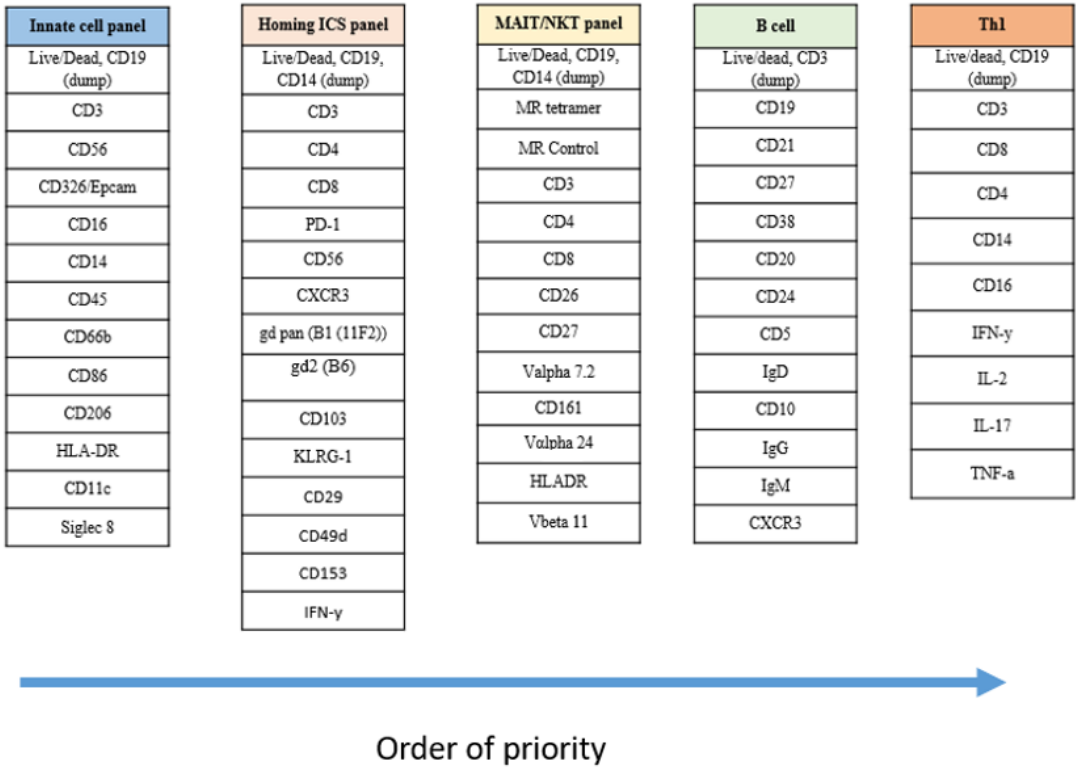


Figure 4.1. Cell count of endobronchial biopsies taken at the D2 bronchoscopy, following digestion and staining. From innate flow cytometry panel after gating out dead cells, doublets and debris. Median:$1.32x10^4$cells (line), range $2.95x10^2$ - $1.02x10^6$.

Only one sample had sufficient cells for analysis. Hence for Group 2, all biopsies were sent for immunohistochemistry staining. Due to delays from the COVID-19 pandemic this analysis is still ongoing and will not be presented in my thesis.

4.3.2 Bronchoalveolar lavage fluid

Figure 4.2 shows the order of priority of flow cytometry panels and the number of volunteer samples stained for each panel.



	Group 1 (Day 2)		Group 2 (Day 7)		Total
Volunteer	Total BCG	Total Ctr	Total BCG	Total Ctr	
Innate	10	3	10	3	26
Hom ICS	3	2	7	3	15
Hom surf	6	1	3	1	11
MAIT	3	0	4	1	8
B cell	1	0	3	0	4
Th1 ICS	0	0	2	0	2

Figure 4.2. Five flow panels with markers, order of priority, and number of volunteer samples stained per panel. Hom: Homing. ICS: Intracellular cytokine staining. Ctr: Saline Control. Th1: Polycytokine panel.

Despite randomisation, all three D7 saline control bronchoscopies were performed on the same day. On flow cytometry, these samples had high percentage staining for the majority of markers, despite gating out dead cells and doublets (Figure 4.3).

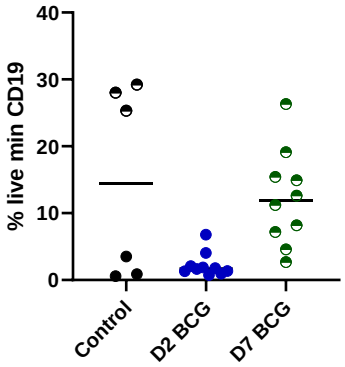


Figure 4.3. An example figure of flow cytometry results. Open circles D7.

I analysed whether there was any difference in the sample processing (Figure 4.4).

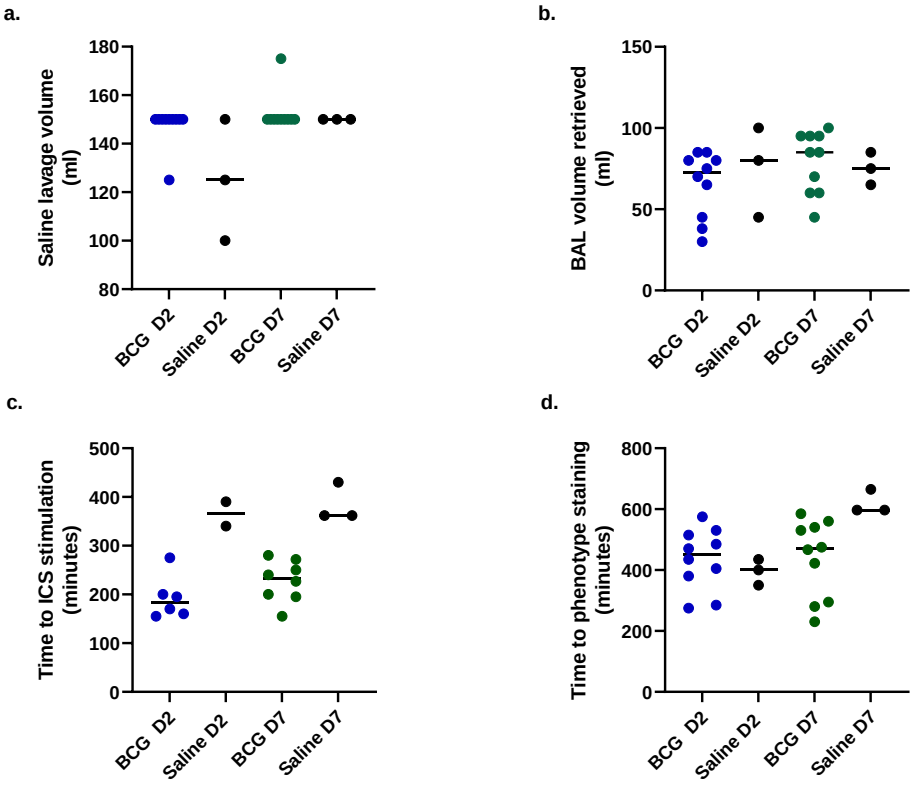


Figure 4.4. BAL cell processing by group. (a) Saline wash volume, (b) BAL volume retrieved, (c) time from BAL retrieval to commencing phenotype staining, (d) time from BAL retrieval to commencing ICS stimulation.

While there was no difference in volume instilled or retrieved, there was a longer time from retrieval to ICS stimulation for controls than BCG samples (median BCG 200 min, saline 362 min, p=0.0001, Figure 4.4c). There was also a longer time from retrieval to phenotype stain for D7 controls compared to BCG or D2 control, Figure 4.4d. (Median D2 saline 400min, D7 saline 597min, D7 BCG 471min. D7 BCG vs D7 saline p=0.007, on Mann-Whitney. Note however that D7 saline only had a sample size of 3 and so analysis may not be accurate).[566]

I had planned to pool saline controls for analysis, but due to the differences between D2 and D7 controls this wasn't possible. Without further control samples, I also couldn't be certain if the D2 controls or the D7 controls were a representative sample. Hence, I have restricted the flow data results to BCG volunteers only.

D2 BCG samples had more total cells and live cells than D7 BCG samples (Figure 4.5).

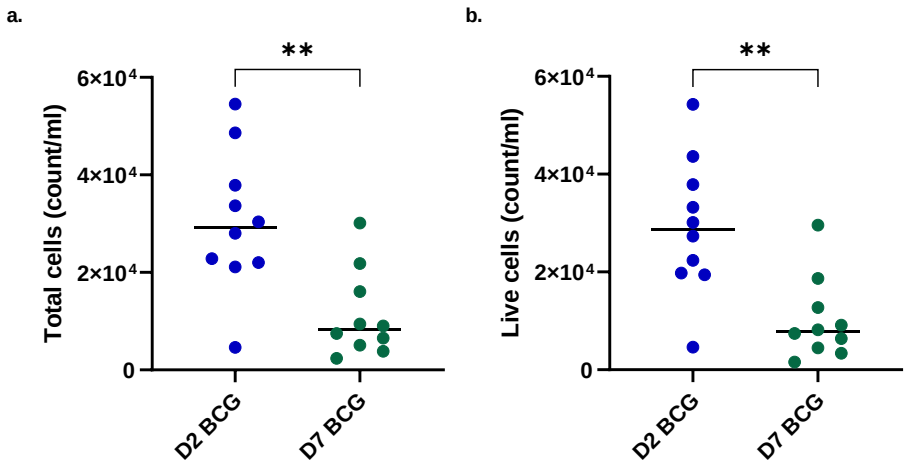


Figure 4.5. BAL cell counts. (a) Total cell count/ml BAL retrieved, (b) live cell count/ml BAL retrieved using the innate flow cytometry panel after gating out doublets. Bar: median. (a) p=0.0039, (b) p= 0.0021 on Mann-Whitney after normality testing.

Only three D2 BCG samples had sufficient cells for homing ICS staining (Figure 4.2). To improve power, I wanted to investigate whether I could combine homing panel results from unstimulated BAL samples stained on the same day with samples which were

incubated overnight and stained the next day. I used samples from three sources. 3 blood samples (PBMC) from BCG-naïve donors; 1 blood sample (PBMC) from a volunteer infected with aerosol BCG 7 days prior and 1 BAL sample from a BCG-naïve D7 control volunteer. All samples were unstimulated and processed in two ways; same day staining or overnight incubation then staining with the homing flow cytometry panel (see Methods for Homing flow cytometry protocol).

There was no significant difference in frequency of cells or subsets between the two processing methods (Figure 4.6). All phenotype staining results from the homing panel are therefore presented with samples pooled from the two methods.

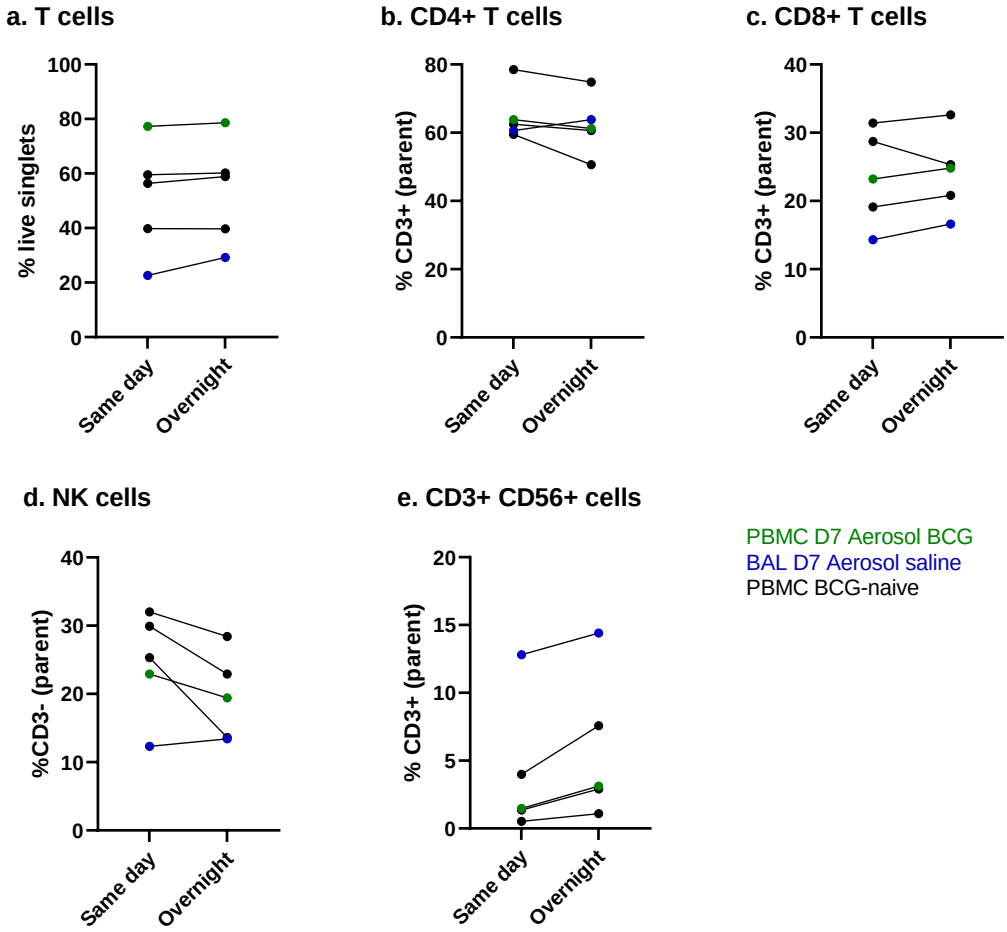


Figure 4.6. Results of homing flow cytometry comparator experiment using two different methods of staining: same day staining or staining overnight incubation of samples. (a) T cells, (b) CD4+ T cells, (c) CD8+ T cells, (d) NK cells, (e) CD3+ CD56+ cells. Subsets also compared, data not shown here. No significant difference on Wilcoxon in any cell type.

4.3.3 10x single cell sequencing

BAL cells from three BCG-infected and three control volunteers from each group were processed for single cell RNA sequencing. 78775 cells were sequenced and 43 cluster types identified at Seurat Resolution 2.0 (Figure 4.7).

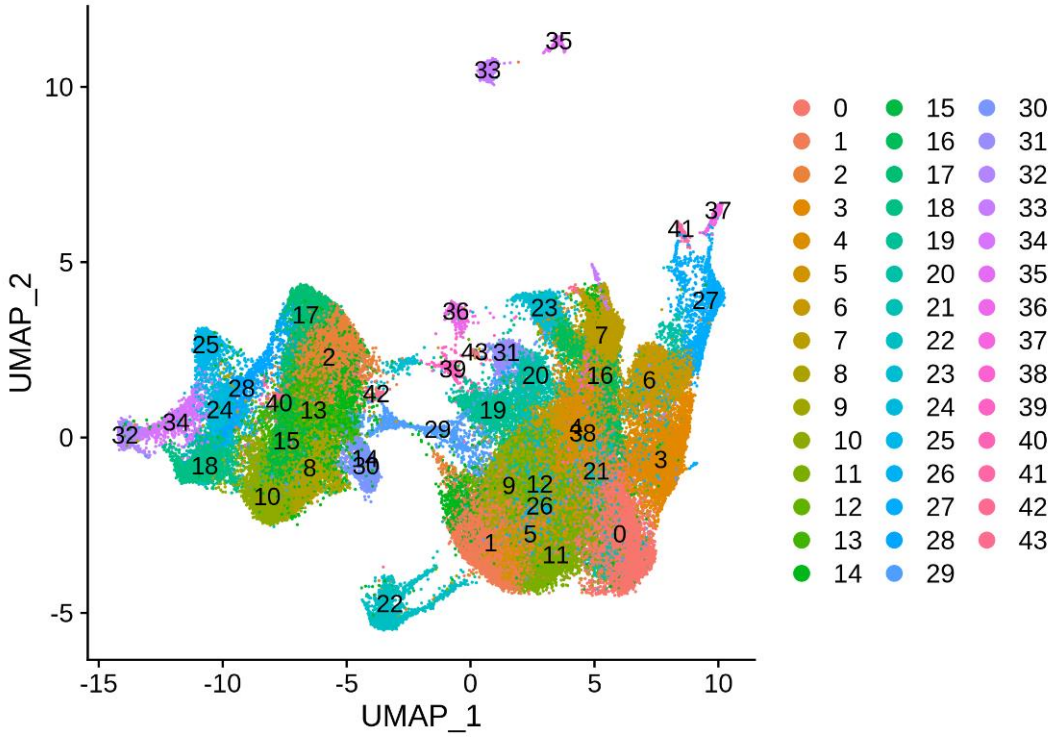


Figure 4.7. UMAP Feature plot showing distribution of cell clusters. Seurat Resolution 2.0 resulted in 43 cluster types.

Cluster identity is shown in Figure 4.8 on UMAP (Uniform manifold approximation and projection). The explanation for how I assigned identity is discussed under each cell type.

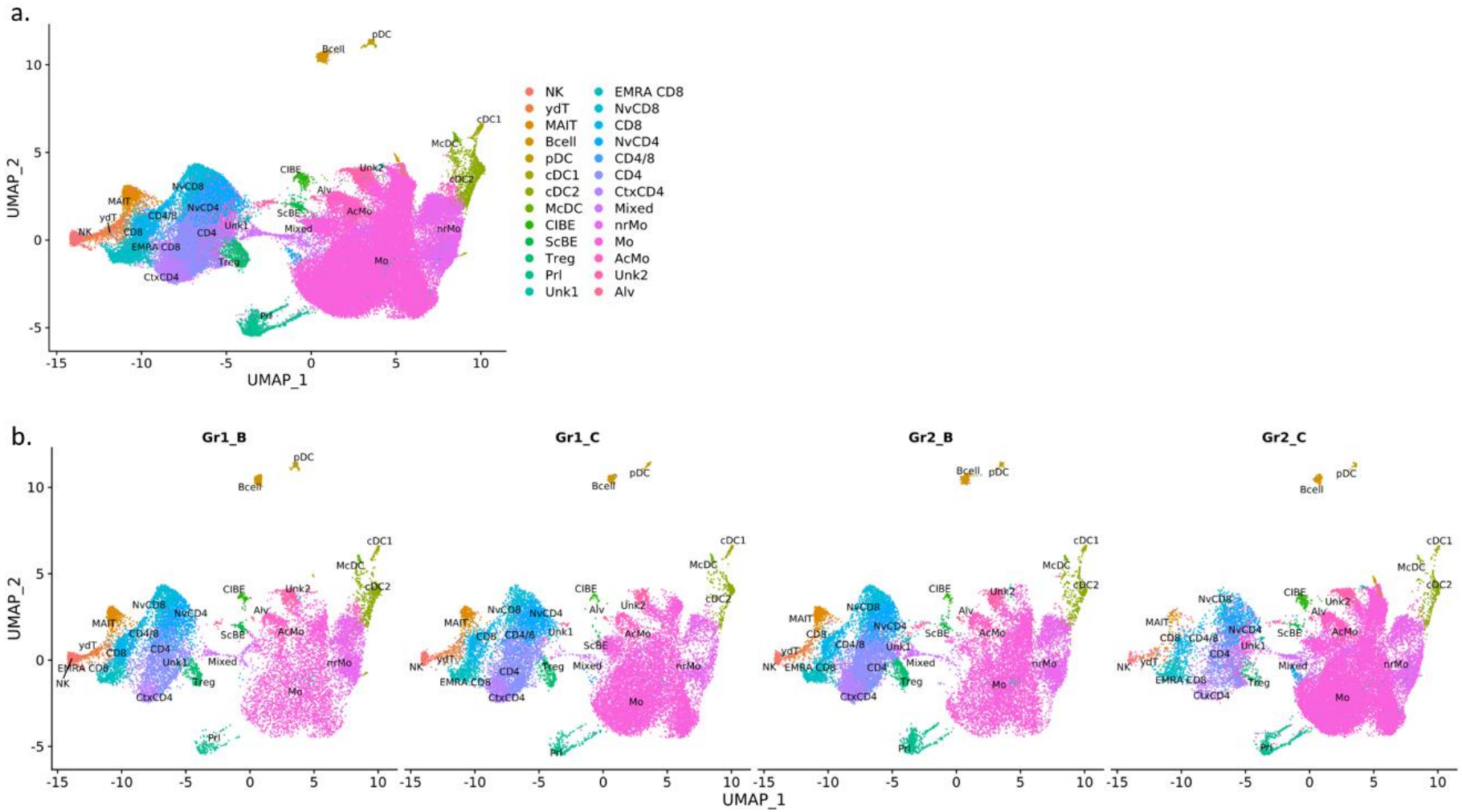


Figure 4.8. Cluster identity on single cell RNA-seq UMAP plot coloured by cell type. (a) Identity of each cluster, (b) distribution of cells by group. “NK”: NK cell, “ydT”: γδ T cell, “pDC”: plasmacytoid DC, “cDC”: classical DC, “McDc”: Migratory DC, “CIBE”: Cilliated bronchial epithelial cell, “ScBE”: Secretory bronchial epithelial cell, “Treg”: T regulatory cell, “Prl”: Proliferating cells, “Unk”: unidentified cell type, “EMRA CD8”: “terminally differentiated effector memory cells re-expressing CD45RA”-like CD8+ T cell, “Nv”: Naïve, “Ctx”: Cytotoxic-like, “nrMo”: non-resident Macrophage, “Mo”: Macrophage, “AcMo”: Activated macrophage “Alv: Alveolar Type 2 cell.

Figure 4.9 shows the percentage of each cell type by group and Figure 4.10 by volunteer as determined by 10x. Controls had more Alveolar Macrophages (AMs; all BAL macrophages combined) and less T cells than after D2 or D7 BCG infection (Figure 4.8b, Figure 4.9, Figure 4.10). One saline D2 volunteer had more CD4+ T cells and less AMs than the other controls (Volunteer "C2b" in Figure 4.10). However for all controls, the proportions of cell types are in keeping with healthy controls from the literature where percentage of macrophages in BAL on single cell sequencing ranges from 50%-99%, and T cells up to 18% total BAL cells.[567]

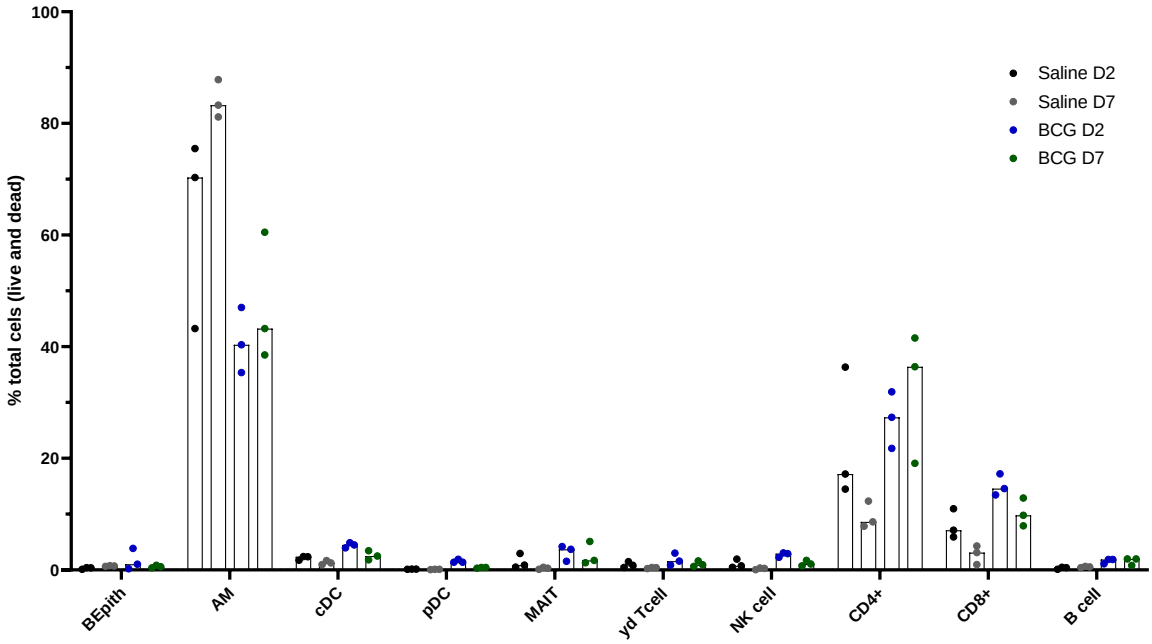


Figure 4.9. Proportion of cell types (combined clusters) on single cell RNA-seq by group. Sample size was too small for statistical analysis. Bars: median.

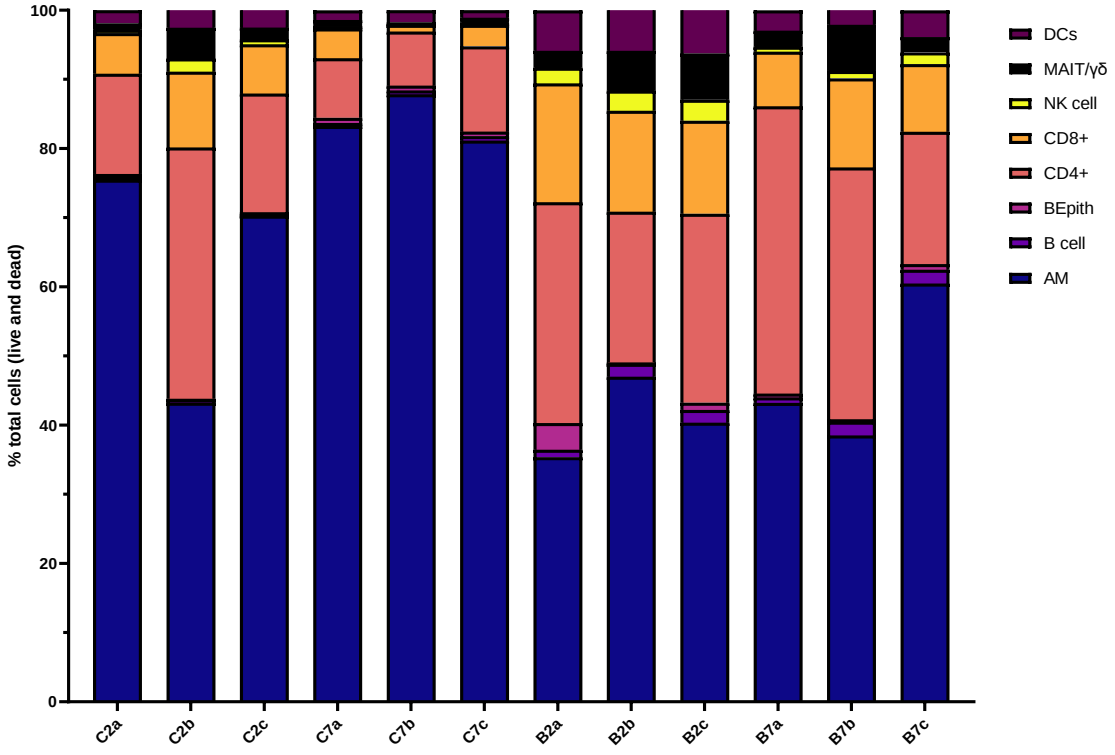


Figure 4.10. Cell types shown as % total cells by each volunteer. "C": Saline control; "B": BCG infection; "2/7": Day of bronchoscopy; "a-c": individual volunteer (3 volunteers for each condition)."DCs": Dendritic cells. "AM": Alveolar Macrophages. "BEpith": Bronchial epithelial cells. "CD4+": CD4+ T cells. "CD8+": CD8+ T cells. Total cell count sequenced per volunteer: C2a 5563, C2b 7905, C2c 5173; C7a 8247, C7b 8150, C7c 9807; B2a 3209, B2b 7977, B2c 3502; B7a 7455, B7b 6682, B7c 5105.

All D2 BAL samples, but no D7 samples, were incubated with CD3+ MACs beads and passed through a magnetic column before being recombined for library preparation. This should not affect the RNA expression of cells and hence gene expression analysis should still be comparable between groups.[504] However the MACs bead sorting step may have affected cell proportions.

To investigate this, I processed a saline control BAL sample taken at D14 by both methods. Seurat resolution depth 1.2 was chosen as only aggregate cell types were required for this analysis. 7343 cells were sequenced; 3375 cells with no MACs and 3968 cells with MACs beads. I identified cell types by assessing mean gene expression of canonical markers.

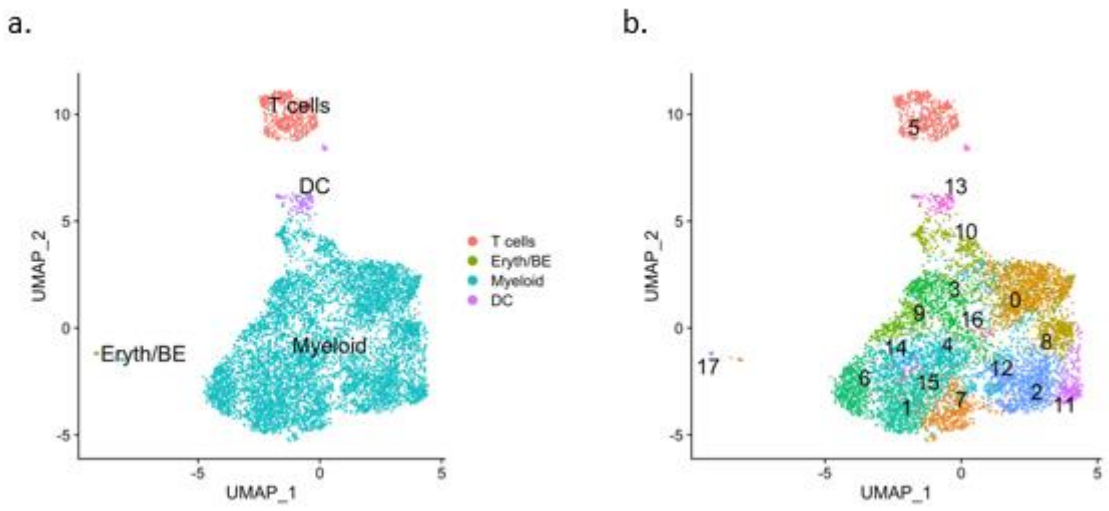


Figure 4.11 shows the clusters and cell types identified.

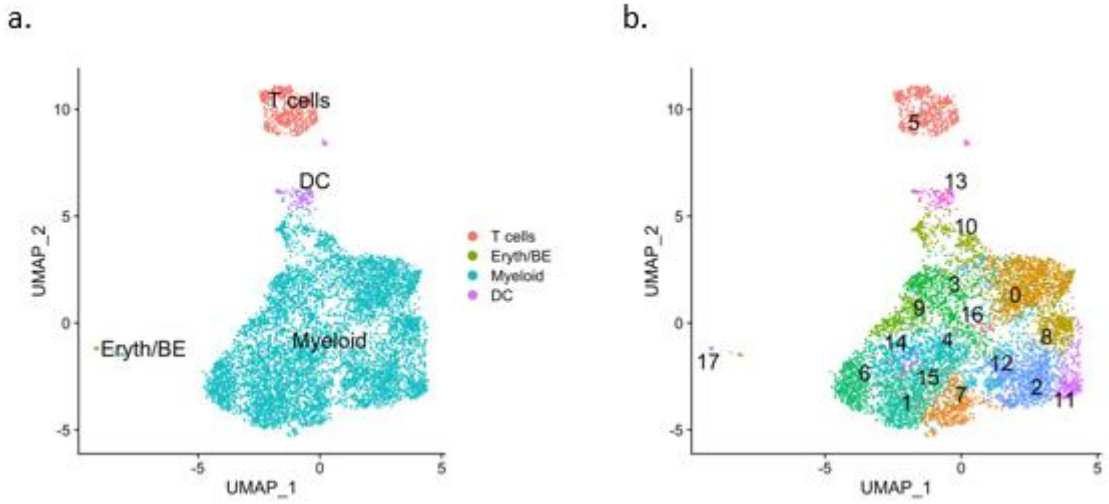


Figure 4.11. UMAP plot showing broad identity of each cluster (a) using Seurat resolution 1.2, which identified 17 initial cluster types (b). "Eryth/BE": Erythrocytes/bronchial epithelial cells. "DC": Dendritic cells. "Myeloid": Non-DC myeloid cells.

There was no difference in proportion of cell types between the CD3+ MACs bead and non-MACs bead sorted sample by Wilcoxon (Figure 4.12).

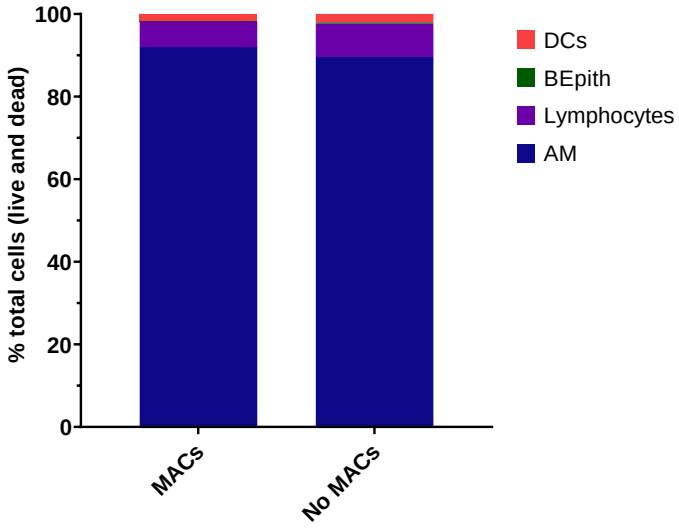


Figure 4.12. Comparing cell proportions on MACs and no-MACs bead sorting. "DCs": Dendritic cells. "AM": All BAL Macrophages. "BEpith": Bronchial epithelial cells.

4.3.3.1 Determining cell proportions by flow cytometry and single cell RNA sequencing

Table 4.1 shows the percentage of cells out of total BAL cells for the main cell types identified in my study, using either flow cytometry or 10x chromium single cell RNA-seq.

Table 4.1. Differing BAL cell percentages identified by either flow cytometry or single cell RNA sequencing (scRNA-seq) as % total singlets at day 2 or day 7 (D2 or D7) following aerosol BCG Danish 1331 inhalation. Median and IQR (interquartile range). Note as n=3 for RNA-seq, IQR shows min and max. Percentages given as % live singlets for flow cytometry, and % live and dead singlets for single cell RNA-seq. Flow cytometry values come from the innate, homing or MAIT/iNKT panels. All cell values are from unstimulated conditions. Granulocytes and iNKTs unidentifiable from scRNA-seq (see further details under relevant chapter headings below).

Cell	**D2 median % singlets (IQR)**		**D7 median % singlets (IQR)**	
	Flow cytometry	scRNA-seq	Flow cytometry	scRNA-seq
Neutrophils	1.96 (1.1-5.1)	-	5.81 (3.1-8.2)	-
Eosinophils	0.41 (0.2-0.5)	-	0.63 (0.4-0.9)	-
Total MPs	56.53 (54.3-61.1)	40.31 (35.4-47.0)	36.76 (30.0-40.8)	43.21 (38.5-60.5)
DCs	5.16 (2.5-7.2)	5.90 (3.9-6.8)	2.56 (1.6-3.4)	2.92 (2.1-3.7)
NK cells	0.92 (0.5-1.3)	2.91 (2.3-3.0)	1.80 (1.5-2.5)	1.05 (0.7-1.7)
Total CD3+ cells	8.78 (4.6-10.8)	45.44 (40.7-50.0)	46.55 (37.2-55.8)	50.17 (29.7-54.2)
MAITs	0.34 (0.34-0.47)	3.71 (1.6-4.2)	2.07 (1.5-3.1)	1.73 (1.3-5.1)
iNKT cells	0.024 (0.01-0.3)	-	0.37 (0.2-0.8)	-
γδ2 T cells	0.61 (0.4-2.0)	1.63 (0.9-3.0)	6.67 (2.1-12.7)	0.91 (0.6-1.6)
CD4+ T cells	7.44 (3.8-9.2)	27.31 (21.8-31.9)	21.44 (13.6-33.3)	36.42 (19.1-41.5)
CD8+ T cells	3.11 (2.1-5.4)	14.61 (13.4-17.2)	5.78 (4.7-9.4)	9.83 (7.9-12.9)
B cells	0.11 (0.1-0.1)	1.91 (1.1-2.0)	1.34 (0.6-3.2)	2.02 (0.8-2.3)

There are a number of differences between the processing of the cells used for flow cytometry and 10x scRNA-seq which might explain why cell proportions are not comparable between the two methods. Firstly, flow cytometry excludes dead cells, while scRNA-seq does not. Hence the differences in proportions may be due to a greater tendency for one cell type to die, thereby affecting proportions. Further, I was unable to detect any granulocytes in the scRNA-seq data hence cell percentages are given as % total cells excluding granulocytes, while flow cytometry includes these. In addition, the cell markers used to identify cell types were different between flow cytometry and scRNA-seq (see below under relevant chapter sub-headings for details) which might also explain the differences seen here.

Only 3 samples were used for scRNA-seq which would have affected the median cell proportion if that sample sat at the extreme range of cell proportions amongst volunteer samples. However, cell percentages for any given individual did not match between flow cytometry and scRNA-seq and hence this explanation is less likely (e.g. see Figure 4.13).

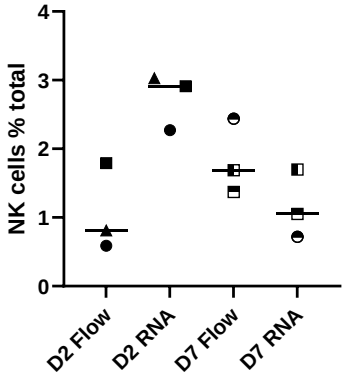


Figure 4.13. Mismatch in percentage of Natural Killer (NK) cells as determined by flow cytometry or single cell RNA sequencing, 2 or 7 days (D2 or D7) following BCG aerosol inhalation. % total singlets (live cells flow cytometry, dead and live cells scRNA-seq). "Flow": Flow cytometry. "RNA": 10x chromium single cell RNA sequencing. Line shows median. NK cells on flow cytometry identified as protein expression of: CD3- CD56+ cells with singlets excluding CD19+ cells. NK cells on scRNA-seq identified as: gene expression of NCAM1 (CD56), FCGR3A (CD16) and CD3G (CD3). Shapes represent separate individuals. D2: volunteer numbers TBT-04301007; TBT-04301020, TBT-04301021. D7: volunteer numbers TBT-04301026, TBT-04301029, TBT-04301030.

scRNA-seq cells were processed first, while cells for flow cytometry were often stained a few hours later. Markers used to identify cell types might be up or down regulated in this time (gene or protein expression), affecting the identification of cells. Cells reserved for scRNA-seq processing were kept on ice to comply with the 10x chromium protocol, but cells processed for flow cytometry were kept at room temperature to reduce death rate from chilling. Again this might explain the differences seen.

Finally, D2 and D7 scRNA-seq samples were processed differently to each other (with or without CD3+ MACs bead incubation). While there was no difference in proportion of cell types between the CD3+ MACs bead and non-MACs bead sorted sample (see above; Figure 4.12), this analysis was performed on only one saline sample. A larger sample size, which included BCG exposed samples and which also investigated cell subsets might detect differences. Because of this difference between processing for scRNA-seq samples, and also because identifying cell types via flow cytometry is a validated method with years of precedent, frequencies of cells are reported from flow cytometry only throughout this chapter, unless the cell type was only identifiable from 10x.

4.3.3.2 Differential gene expression of total cells via scRNA-seq

While cell proportion identified by scRNA-seq may not be a reliable method in this study due to the issues discussed above, MACs bead processing will not have affected gene expression of cells (see discussion for further treatment on this subject).

I performed Differential Gene Expression analysis (DGE) for all clusters combined. Table 4.2 shows the number of genes differentially expressed per condition. For every cell type there was a difference in number of genes upregulated between BCG and saline volunteers at both timepoints. The largest difference for every cell type was between D7

BCG and D7 saline samples, except for total macrophages ("AMs") where the largest difference was D2 BCG compared with D2 saline.

Table 4.2. Number of genes differentially expressed on DGE by cell cluster. Calculated as number of genes upregulated plus number downregulated between comparators.(): number of genes upregulated in D2BCG (first column); D7BCG (second column); D7BCG (third column).

	D2: BCG v Saline	D7: BCG v saline	D2 BCG v D7 BCG
All cells	310 (223)	946 (594)	242 (145)
AMs	586 (482)	514 (317)	231 (50)
pDCs	945 (689)	1579 (1013)	463 (190)
cDCs	415 (320)	538 (270)	281 (73)
NK cells	410 (317)	585 (357)	274 (126)
MAITs	297 (236)	458 (280)	220 (74)
γδ2 T cells	486 (371)	555 (362)	311 (122)
CD4+ T cells	150 (105)	755 (486)	437 (291)
CD8+ T cells	140 (82)	274 (151)	230 (103)

Criteria: log fold change ≥ 0.25 and expressed in ≥ 0.25 cells in at least one of the two groups.

Figure 4.14 shows the DGE pathway analysis using Metascape for total cells.[568] At D2 there was increased RNA expression in BCG volunteers compared with saline associated with cytokine signalling pathways, interferon signalling, NOD-like pathway, apoptosis, leucocyte activation and mycobacteria phagocytosis; and by D7 RNA expression for leucocyte migration and adaptive immune system pathways had increased for BCG volunteers compared with D7 saline and D2 BCG.

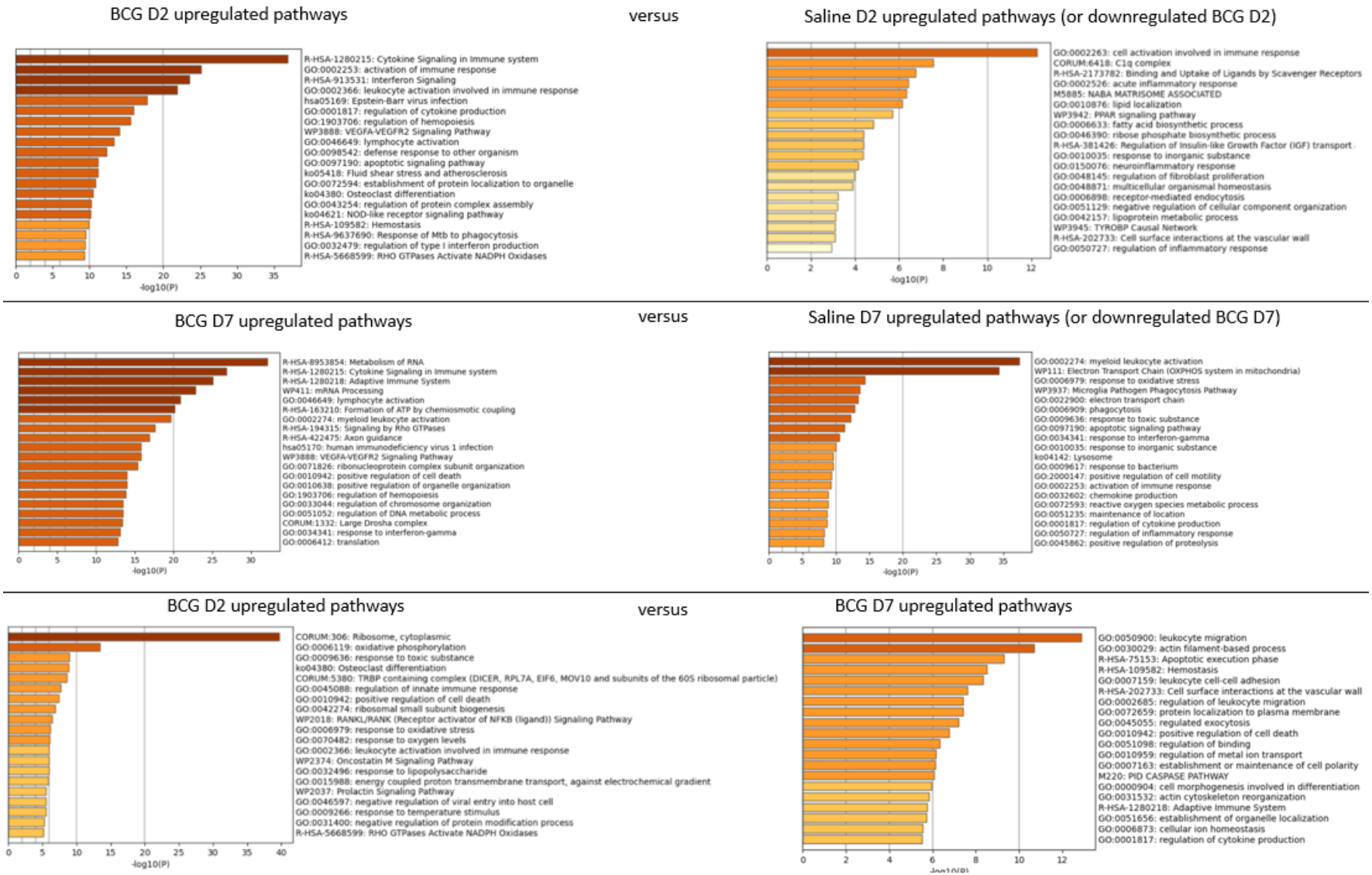


Figure 4.14. Total cell Metascape pathway enrichment analysis[568] of DGE by group.

4.3.4 Granulocytes

Neutrophils and eosinophils increased in the airway at D7 compared with D2 following BCG aerosol infection (Figure 4.15; % total CD3 negative cells).

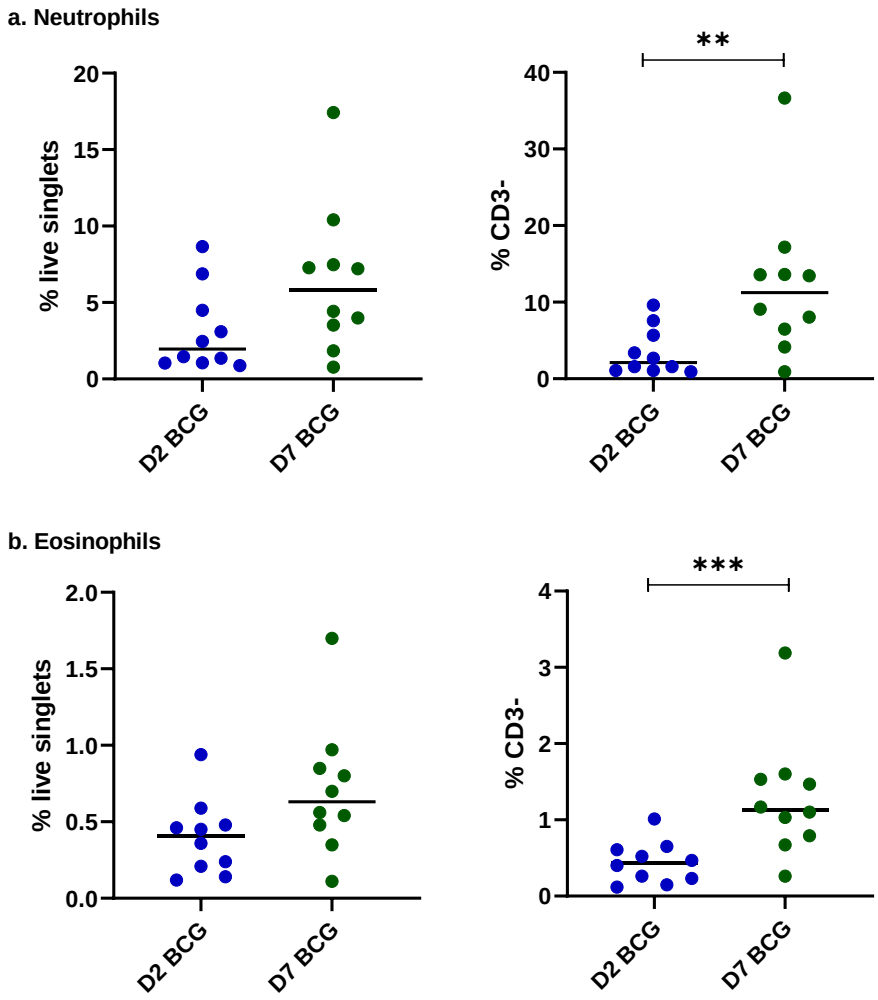


Figure 4.15. Frequency of (a) neutrophils and (b) eosinophils using the innate flow cytometry panel. Neutrophils defined as live singlets, Lin- (CD19, CD56, CD3), CD16+, CD66b+. Eosinophils defined as live singlets, Lin- (CD19, CD56, CD3), CD16- Siglec8+. Differences in cell frequency between groups calculated using Mann Whitney, lines show median, not controlled for multiple comparisons. Neutrophils % CD3-: p=0.0094; Eosinophils % CD3- p=0.0007.

In the 10x scRNA-seq data I was unable to identify any granulocyte populations despite using multiple combinations of markers to ensure cell populations weren't missed.[512,569,570] Cluster 31 cells expressed CD16b (FCGR3B), which is exclusively expressed by granulocytes, most abundantly by neutrophils (Figure 4.16).[571,572] However, there was no expression of

CD66b in this cell population and it seems unlikely that in both the saline and BCG samples, the only neutrophil population would be downregulated for CD66b RNA, which is expressed in activated neutrophils.[199,507,570,573] In addition, only a proportion of cells expressed FCGR3B (CD16b) in this cluster (Figure 4.16).

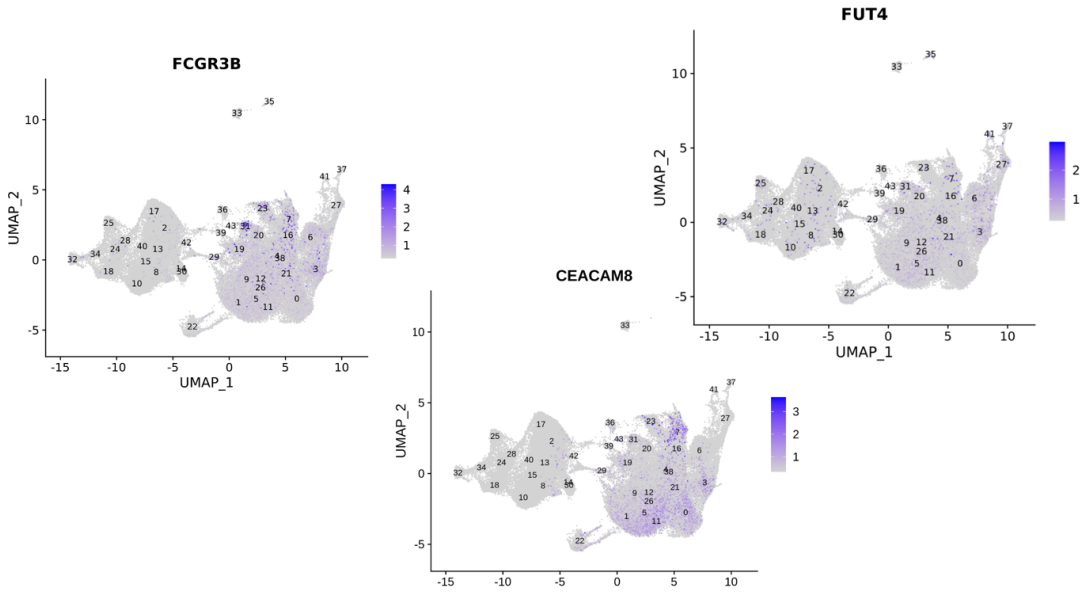


Figure 4.16. UMAP projection showing RNA expression of canonical neutrophil markers. FCGR3B (CD16b), CEACAM8 (CD66b), FUT4 (CD15).[512,569,570]

I also looked for other granulocytes using markers TPSAB1, TPSB2 and FCER1A (mast cells); Basophils (TPSAB1 CPA3 HDC GATA2 AKAP12 CLC SFXN1 PRNP ATP10D TNFSF11); Siglec 8 (eosinophil, basophil), but no cluster was definitive.[507,574]

Hence, while some clusters may contain a mix of cell types including some granulocytes, for the purposes of this analysis I concluded that there were no definitive granulocyte clusters in this data set.

4.3.5 MPs, Alveolar macrophages and Dendritic cells

4.3.5.1 Mononuclear phagocytes (MPs)

There was a higher frequency of MPs at D2 following BCG infection than at D7 both when looking at total singlets and also as percentage of total CD3- cells (Figure 4.17a,b). I

defined MPs as negative for CD19, CD3, CD56 and CD66b, and CD45 positive. This population likely contains a mix of dendritic cells, monocytes, macrophages and some granulocytes.[182,575-577]

There was no difference between timepoints in "activated" HLA-DR+ MPs (Figure 4.17c.).

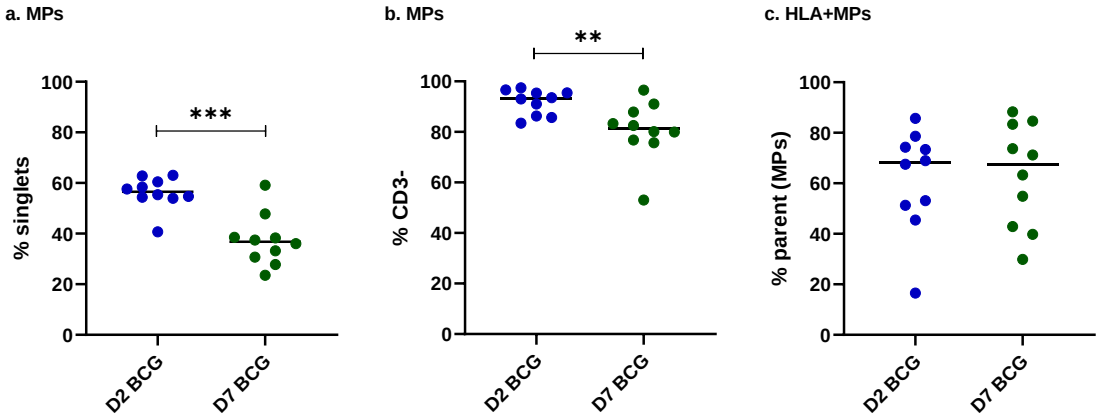


Figure 4.17. Frequency of Mononuclear phagocytes (MPs) using the innate flow cytometry panel. MPs defined as live singlets, CD19-CD3-CD56-CD66b- CD45+. (a) MPs as % live singlets (minus CD19, p=0.0007); (b) MPs as % CD3- cells, p=0.0052; (c) HLA-DR+ MPs as % MPs. Differences in cell frequency between groups calculated using Mann Whitney, lines show median, not controlled for multiple comparisons.

CD45 was not used to define other cell types due to inconsistencies with staining between groups. The CD45+ gate was used here however, to ensure only immune cells were analysed (given the gating strategy for MPs are all negative gates). I also analysed MPs without the CD45 marker and statistical significance between populations was the same.

4.3.5.2 Macrophages

I first confirmed that I was not losing macrophages through adherence to the tubes, by harvesting with EDTA and scraping, then counting cells on microscopy using cytospin, in the initial samples.[578]

I then employed a number of strategies to identify macrophages in the BAL. Assessing by size and granularity alone is problematic as these can vary greatly in BAL[579,114] and in my

flow cytometry plots, there was no consistent separation between granulocyte, mononuclear and lymphocyte populations. There was also no clear positive CD86 population and it was difficult to determine CD206lo and CD206hi populations (Figure 4.18).

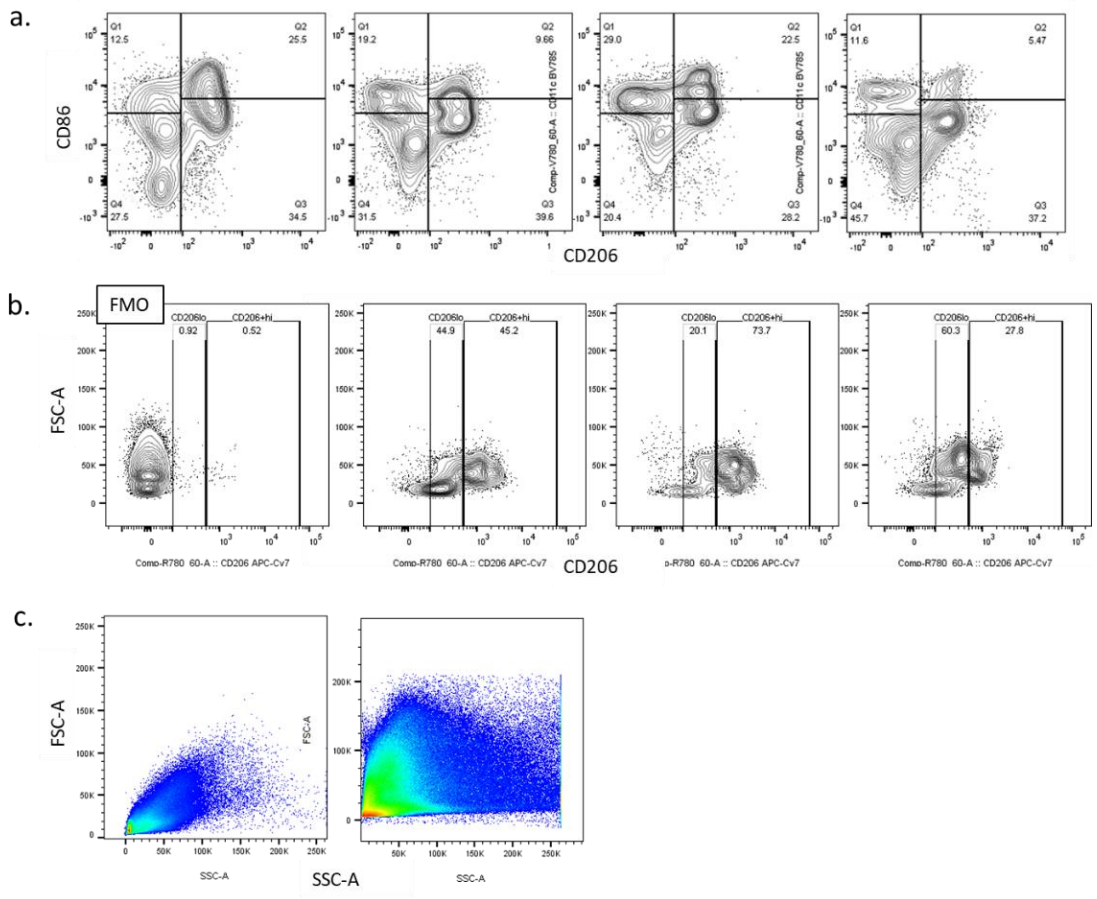


Figure 4.18. Typical innate flow cytometry plots from aerosol BCG volunteers highlighting the difficulties of defining AM subsets. (a) Gating against CD86 and CD206. (b) Gating CD206lo and CD206hi populations against the FMO (left plot). (c) Two example BAL samples showing poor separation between granulocyte and lymphocyte populations in the BAL.

Given mature AMs downregulate CD14, using CD14+ to define BAL macrophages is problematic. In addition, CD14+ cells were an ill-defined population due to autofluorescence (despite using Fc-receptor blocker to reduce this problem).[580] I was therefore unable to identify AMs using canonical markers CD86, HLA-DR, CD14, and CD206; nor identify monocytes using classic CD16/CD14 definitions.[114,117,120,161,165]

Given the clear requirement for ongoing assay workup to define the macrophage and monocyte populations on flow cytometry, I have limited results to the 10x data.

I identified the macrophages/monocyte population (together referred to as AMs) on 10x using UMAP distribution of CD3-, CD14, CD16 monocyte markers and canonical alveolar macrophage markers (Figure 4.19).

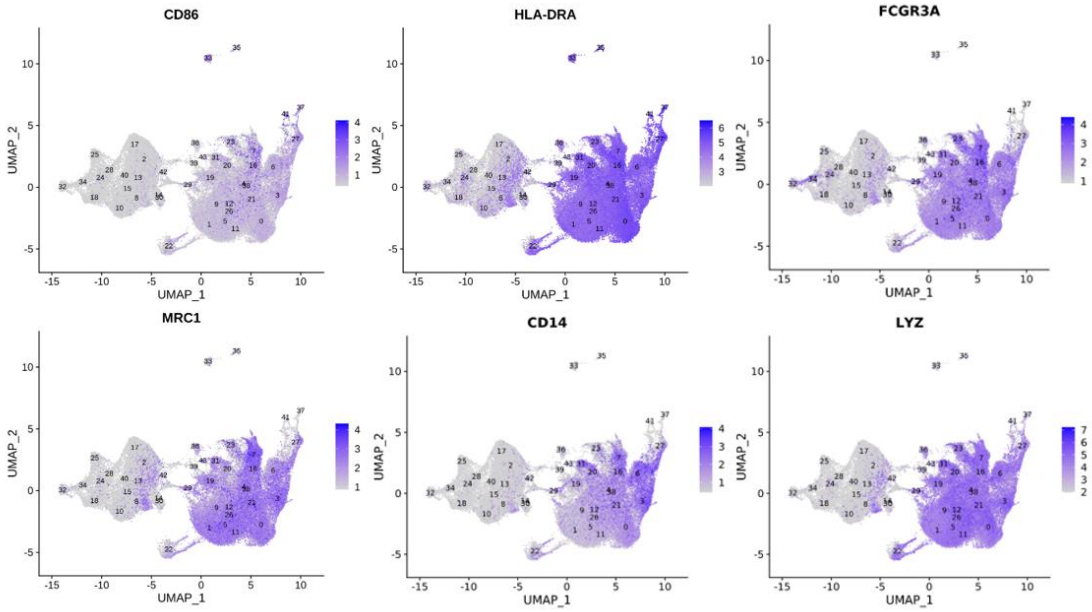


Figure 4.19. UMAP visualisation of canonical markers for alveolar macrophages and monocytes. **"**MRC1": mannose receptor/CD206, "LYZ": Lysozyme, "FCGR2A": CD16. CD68 plot not shown.[117,165]

After a literature search and gene expression analysis of 26 markers, I further subdivided the AMs into three effector subtypes based on seven markers as per Figure 4.20.[512,567,581]

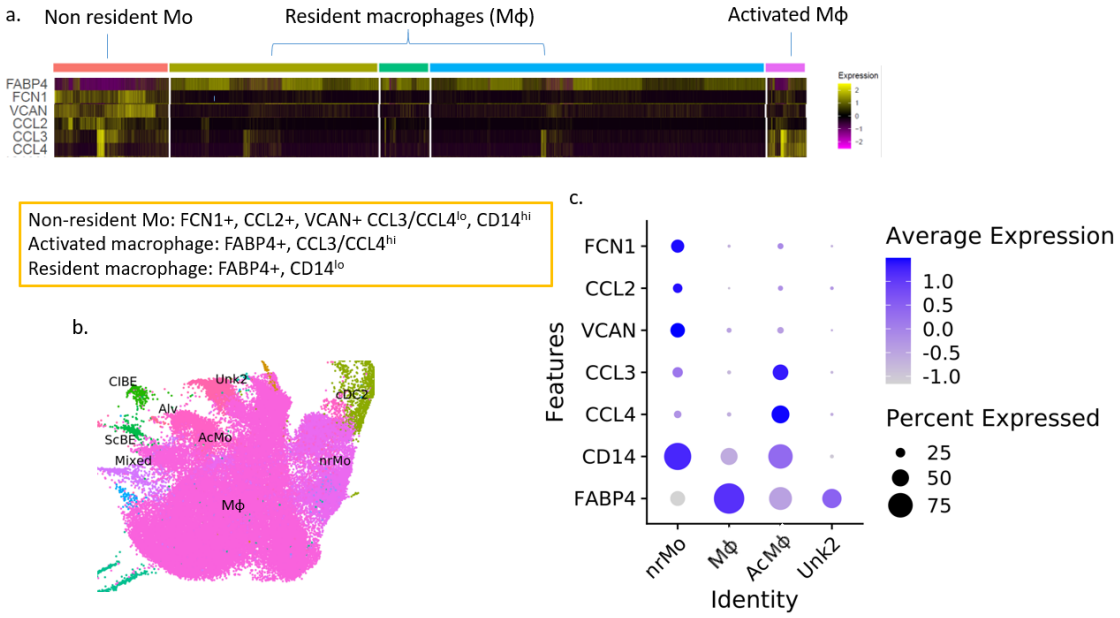


Figure 4.20. Gene expression markers used to identify 3 subsets of macrophages/monocytes in the BAL fluid. Showing (a) heat map, (b) UMAP plot and (c) gene expression dot plot.[512,567,581] "Mϕ": Resident Macrophage; "nrMo": non-resident monocyte/macrophage; "AcMϕ": Activated macrophage; "Unk2": unknown (likely also Mϕ).

CCL3 and CCL4 (Macrophage inflammatory proteins (MIP) 1α and 1β) are chemokines intrinsically involved in infection and inflammation.[160] FCN-1 (M-ficolin) is expressed in peripheral monocytes and immature alveolar macrophages, CCL2 and VCAN are associated with cell migration to infection site, and FABP4 is an adipokine produced by alveolar macrophages.[512,567,581]

While there was not a large difference in AM subsets, there was a trend for influx of non-resident macrophages following BCG infection, and a small increase in activated macrophages at D2 (Figure 4.21).

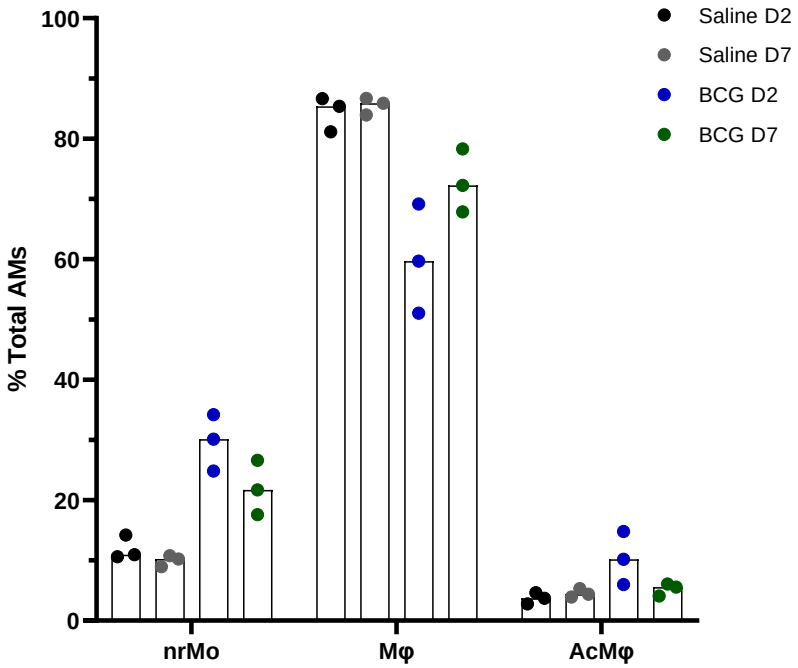


Figure 4.21. Percent of AM subsets out of total AMs by group. Sample size was too small for statistical analysis. Bars: median.

For further analysis I combined all AM subtypes. There was a higher proportion of AMs in saline controls than from BCG infected volunteers (Figure 4.9) and a difference in number of genes upregulated between saline controls and BCG volunteers on DGE at both D2 and D7 (Table 4.2).

Gene pathway enrichment analysis using Metascape showed increased cytokine expression, including IFN-y signalling; leucocyte activation, phagocytosis; MHC-II antigen processing and presentation pathways; in AMs D2 and D7 after BCG infection (Figure 4.22). At D2, saline controls were upregulating genes associated with the TNF-α /NF-κB pathway (or this was downregulated in D2 BCG volunteers). By D7 the NOD-receptor pathway was increased in BCG exposed volunteers.

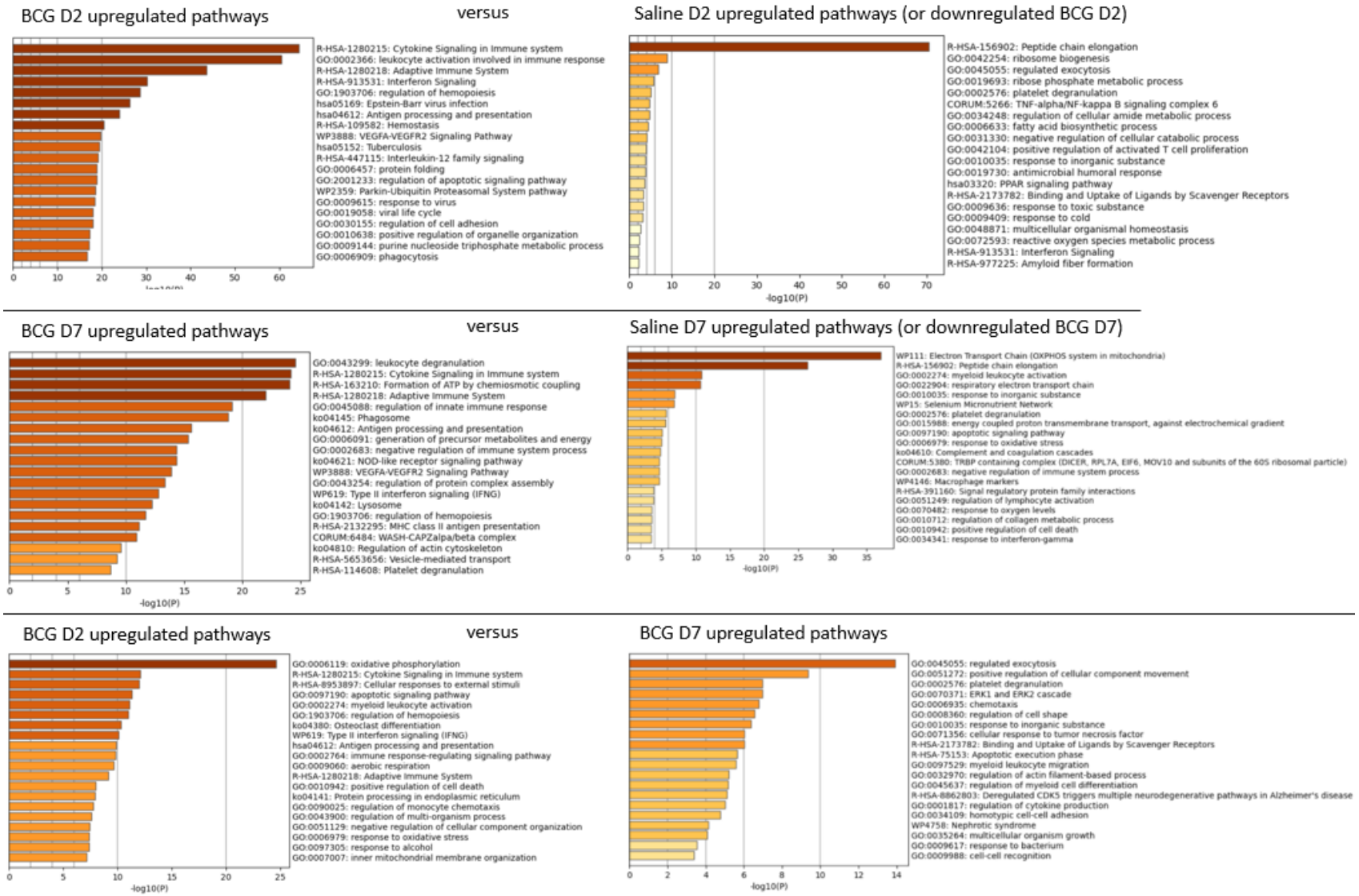
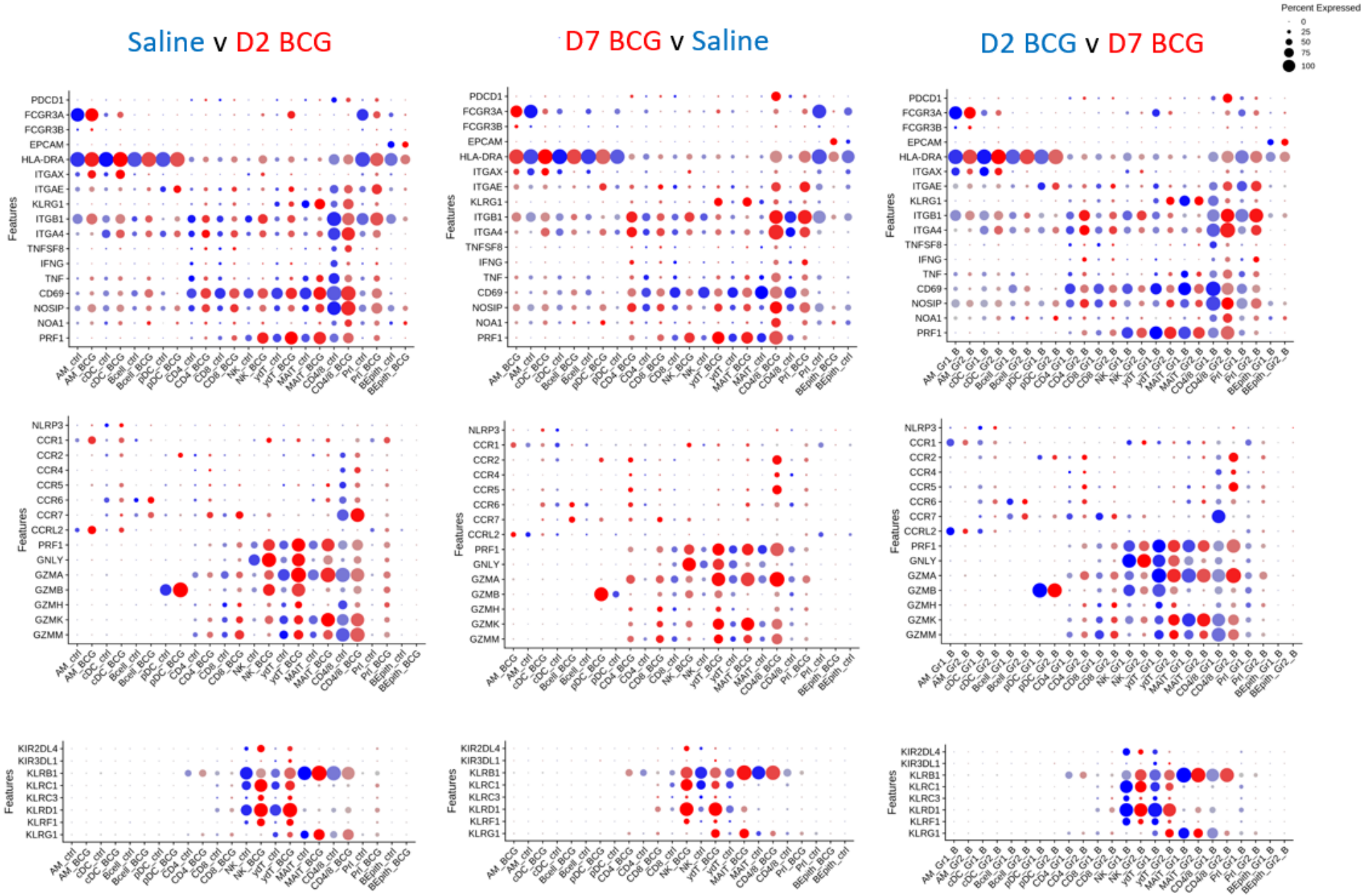


Figure 4.22. AM Metascape pathway enrichment analysis[568] of DGE by group. DGE criteria: Logfc ≥ 0.25 and expression ≥ 0.25 of the cells in at least one of the 2 groups.

I analysed expression of 100 genes that I selected for their association with TB immunity (Figure 4.23). If there was insufficient difference in gene expression between clusters, there were no results. For instance, I included all interleukins but only 8 yielded results. The same occurred with IFN signalling genes. Hence this method is inherently biased and provides an incomplete picture of immune mechanisms. However, it is a useful additional step for exploratory analysis, alongside the unbiased DGE pathway analysis above.

At D2, AMs showed increased expression of CXCL9, CD11c, CCRL2, IL17RA, IL-1β compared to D7 and controls, while at D7 there was increased CCL18 chemokine expression (Figure 4.23). There was no obvious change in expression of TNFα, IL-6 or its receptor, and no changes in GM-CSF expression in any cell type.


Saline v D2 BCG
D7 BCG v Saline
D2 BCG v D7 BCG
Percent Expressed
0
25
50
75
100
Features
PDCD1
FCGR3A
FCGR3B
EPCAM
HLA-DRA
ITGAX
ITGAE
KLRG1
ITGB1
ITGA4
TNFSF8
IFNG
TNF
CD69
NOSIP
NOA1
PRF1
NLRP3
CCR1
CCR2
CCR4
CCR5
CCR6
CCR7
CCRL2
PRF1
GNLY
GZMA
GZMB
GZMH
GZMK
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KLRC3
KLRD1
KLRF1
KLRG1

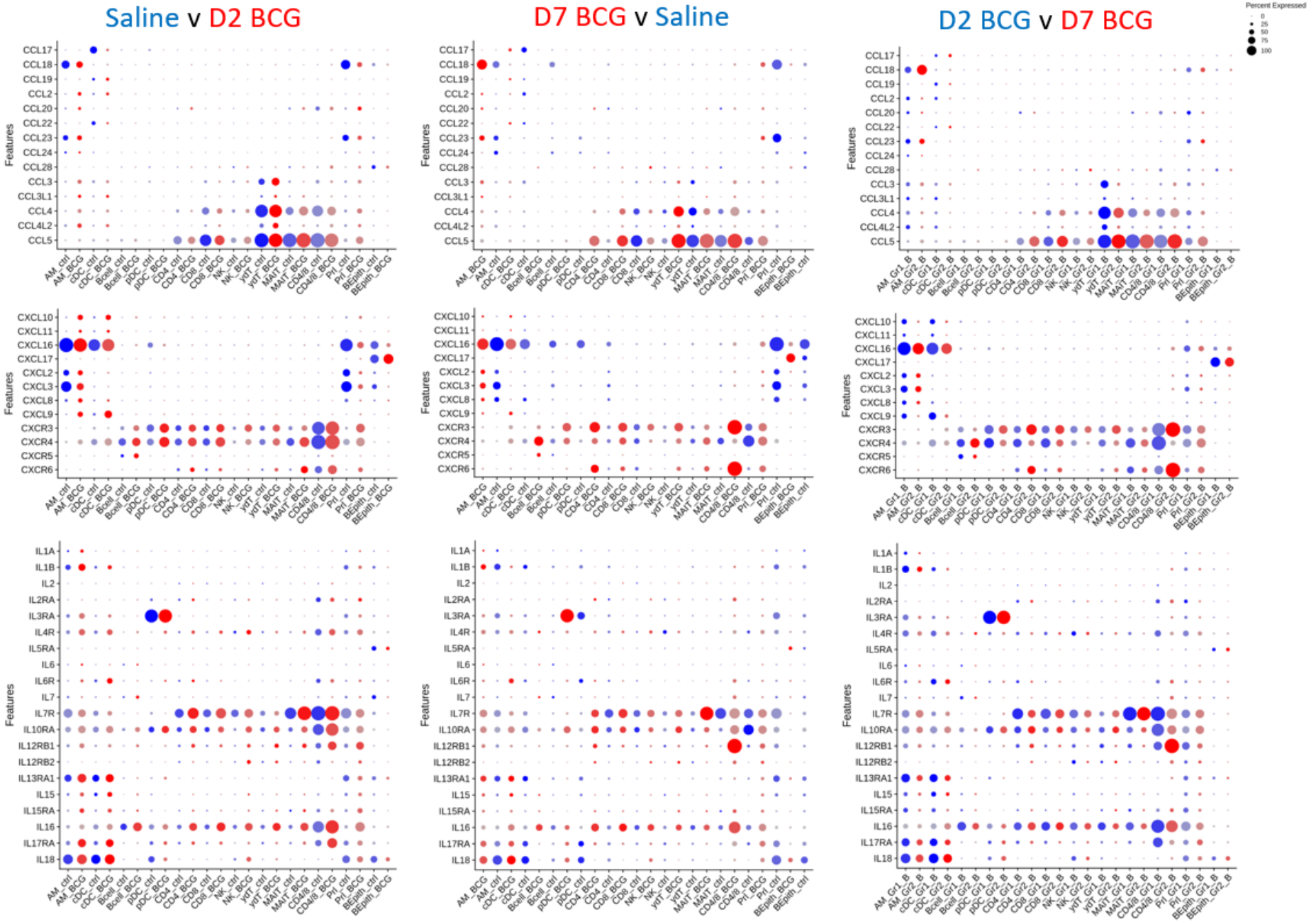

Saline v D2 BCG
D7 BCG v Saline
D2 BCG v D7 BCG
Percent Expressed
0
25
50
75
100
Features
CCL17
CCL18
CCL19
CCL2
CCL20
CCL22
CCL23
CCL24
CCL28
CCL3
CCL3L1
CCL4
CCL4L2
CCL5
CXCL10
CXCL11
CXCL16
CXCL17
CXCL2
CXCL3
CXCL8
CXCL9
CXCR3
CXCR4
CXCR5
CXCR6
IL1A
IL1B
IL2
IL2RA
IL3RA
IL4R
IL5RA
IL6
IL6R
IL7
IL7R
IL10RA
IL12RB1
IL12RB2
IL13RA1
IL15
IL15RA
IL16
IL17RA
IL18
AM_ctrl
AM_BCG
cDC_ctrl
cDC_BCG
Bcell_ctrl
Bcell_BCG
pDC_ctrl
pDC_BCG
CD4_ctrl
CD4_BCG
CD8_ctrl
CD8_BCG
Nk_ctrl
Nk_BCG
γdT_ctrl
γdT_BCG
MAIT_ctrl
MAIT_BCG
CD4/8_ctrl
CD4/8_BCG
Prl_ctrl
Prl_BCG
BEpith_ctrl
BEpith_BCG
AM_Gr1_B
AM_Gr2_B
cDC_Gr1_B
cDC_Gr2_B
Bcell_Gr1_B
Bcell_Gr2_B
pDC_Gr1_B
pDC_Gr2_B
CD4_Gr1_B
CD4_Gr2_B
CD8_Gr1_B
CD8_Gr2_B
Nk_Gr1_B
Nk_Gr2_B
γdT_Gr1_B
γdT_Gr2_B
MAIT_Gr1_B
MAIT_Gr2_B
CD4/8_Gr1_B
CD4/8_Gr2_B
Prl_Gr1_B
Prl_Gr2_B
BEpith_Gr1_B
BEpith_Gr2_B

Figure 4.23. RNA expression of 100 pre-selected genes, by group. Size of dots shows percent cells in cluster expressing RNA, brighter dots correspond to greater gene expression; data normalised and scaled. CEACAM8 (CD66b), FCGR3A (CD16A), FCGR3B (CD16B), GNLY (Granulysin), GZM (Granzyme), ITGA4 (CD49), ITGAE (CD103), ITGAX (CD11c), ITGB1 (CD29), KIR2DL4 (CD158), KLRB1 (CD161), KLRC1 (NKG2A), KLRC3 (NKG2E), KLRD1 (CD94), NOA1 (Nitric Oxide Associated 1), NOSIP (Nitric Oxide Synthase Interacting Protein), PDCD1 (PD1), PRF1 (Perforin), TNFSF8 (CD153).

4.3.5.3 Dendritic cells (DCs)

DCs were at lower frequency in the BAL at D7 post-BCG infection compared with D2, though this was only significant when looking at % singlets (Figure 4.24). The lack of difference when analysing DCs as % CD3- cells is likely due to total CD3- cells and MPs also falling at the same timepoint.

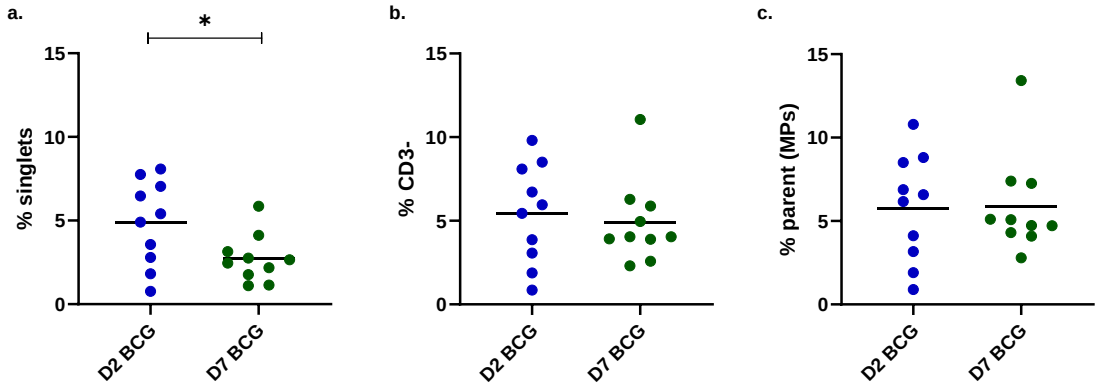


Figure 4.24. Frequency of Dendritic cells (DCs) using the innate flow cytometry panel. DCs defined as live singlets, negative for CD19, CD3, CD56, CD66b and CD16, positive for HLA-DR and CD11c. % singlets: p= 0.0323 using an unpaired t-test after normality testing. Lines show mean.

I was unable to further define these DCs due to the limitation of markers on the flow cytometry panel. This population is likely a mix of plasmacytoid DCs (pDCs), cDC1 and cDC2 (classical DCs), and other MPs such as monocyte or macrophage subsets.[182,575]

pDCs were identified as Cluster 36 using the canonical pDC marker IL3RA (CD123) as well as GZMB, SERPINF1 and ITM2C (Figure 4.25).[512,582,583]

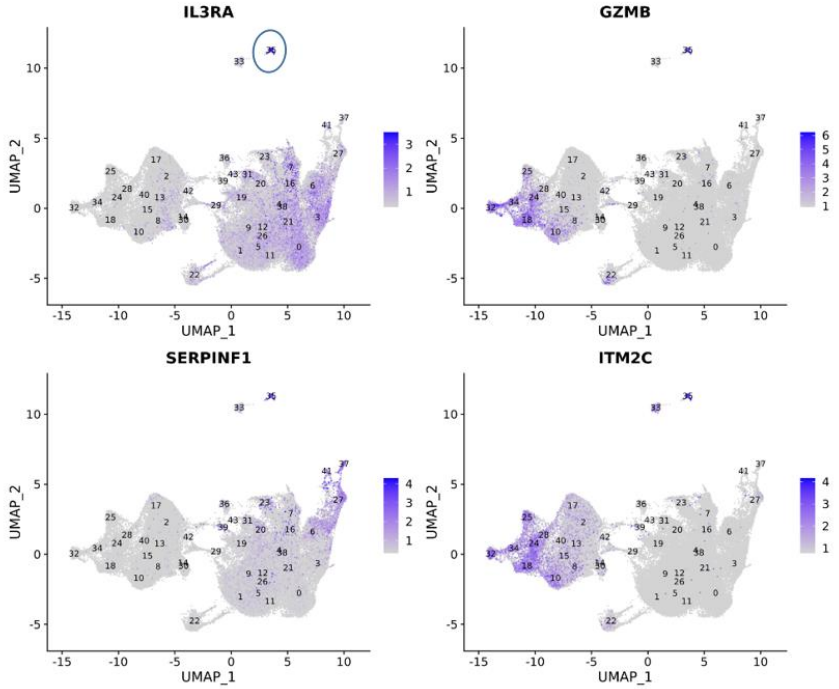


Figure 4.25. UMAP projection of canonical pDC markers, predicts pDC cell population as Cluster 36 (circled in IL3RA figure).[512,582,583] Gene abbreviations (from the Genecard database):[584] "IL3RA": Interleukin 3 Receptor Subunit Alpha, "GZMB": Granzyme B, "SERPINF1": Serpin Family F Member 1, "ITM2C": Integral Membrane Protein 2C.

I identified three cDCs populations (Figure 4.26). Cluster 27 was readily identifiable as cDC2 based on the expression of CD1c+, CCL17+ (and lack of CD3 expression; Figure 4.34), and cDC1 as cluster 37 due to the expression of CLEC9A.[582,583] I identified Cluster 41 as a cDC due to geographical clustering with cDC1 and cDC2 and high expression of CCL17 (especially compared to the other CD3 negative populations).[512,585] I identified this cluster as migratory cDCs due to the expression of CCR7, which allows migration to the lymph nodes.[586]

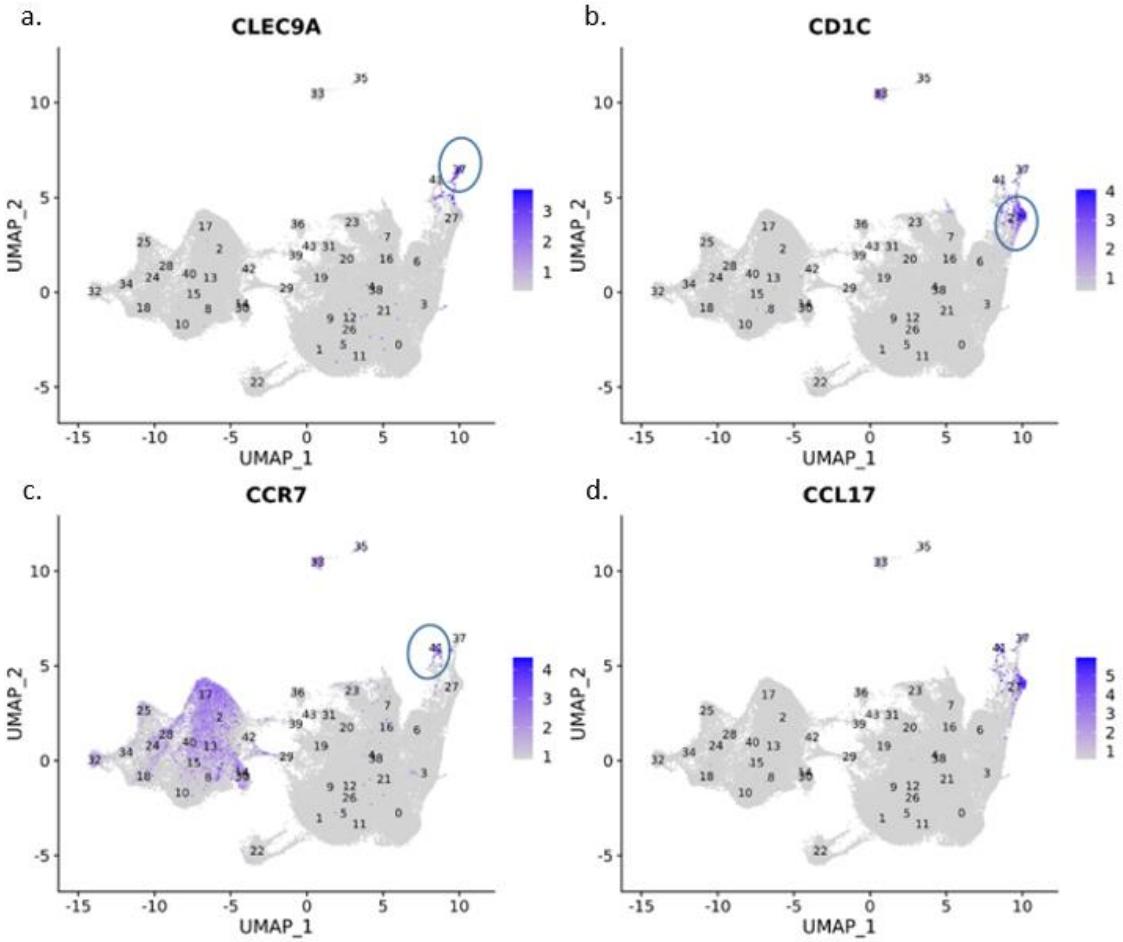


Figure 4.26. UMAP projection showing RNA expression of cDC markers, predicts cDC1 cell population as Cluster 37 (circled in CLEC9A figure, (a)), cDC2 cell population as Cluster 27 (circled in CD1C figure, (b)) and migratory cDC (circled in CCR7 figure, (c)).[582-586] Gene abbreviations (from the Genecard database):[584] "CLEC9A": C-Type Lectin Domain Containing 9A, "CD1C": CD1c, "CCR7": C-C Motif Chemokine Receptor 7, "CCL17": C-C Motif Chemokine Ligand 17.

Figure 4.27 shows percentage of cells defined by RNA expression. Consistent with the flow cytometry data, there was a trend for more DCs at D2 than D7 post-BCG infection.

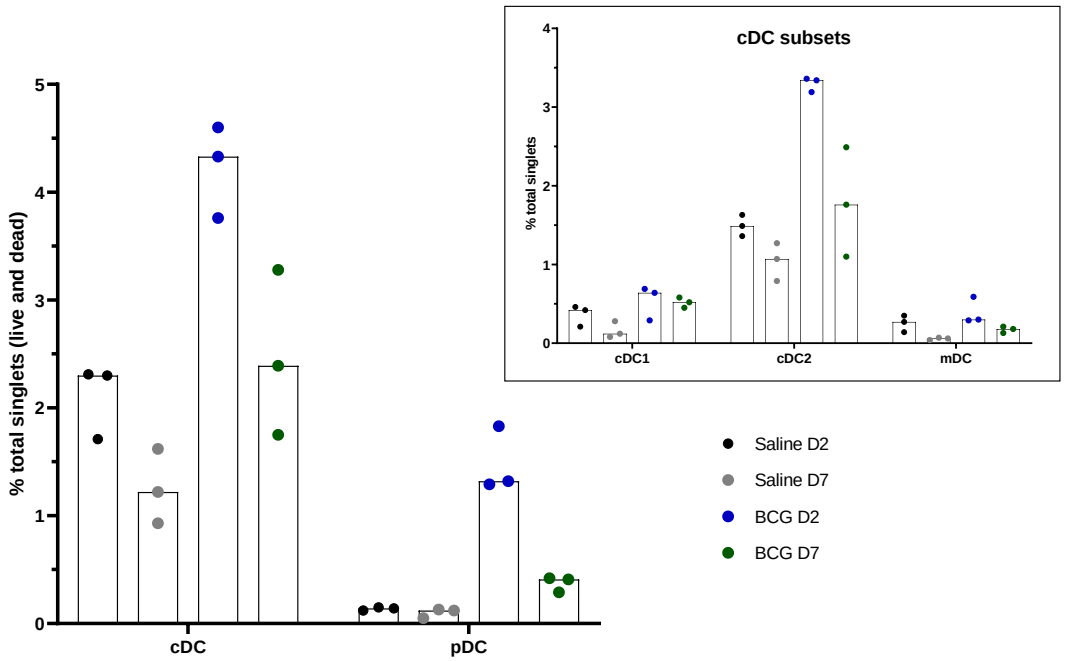


Figure 4.27. Percent of DCs by group. Main graph: Three cDC clusters combined (37, 27 and 41) and pDCs (Cluster 36). Inset: 3 clusters of cDCs. As there are only 3 subjects per group, numbers were too small to perform any statistical analysis. Bar: median.

I combined all three cDC clusters for the DGE analysis. There was a difference in number of genes upregulated between saline controls and BCG volunteers in DCs (Table 4.2) with the largest number of genes seen in pDCs following BCG infection compared to saline controls at either D2 or D7. The results of the metascape pathway analysis are shown in Figure 4.28 (cDCs) and Figure 4.29 (pDCs). At D2 post-BCG, cDCs cells had upregulated pathways involved in Th1 cytokine signalling and response, antigen presentation through MHC-II, activation of TLRs, response to lipopolysaccharide, phagosome production, leucocyte activation, and apoptosis. By D7 cDCs had upregulated pathways related to cell migration, DC differentiation and T cell activation including T cell mediated cytotoxicity pathways. There was some negative regulation of immune processes at D2 and D7. In pDCs post-BCG infection, there was increased RNA expression associated with cytokine signalling and cell activation.

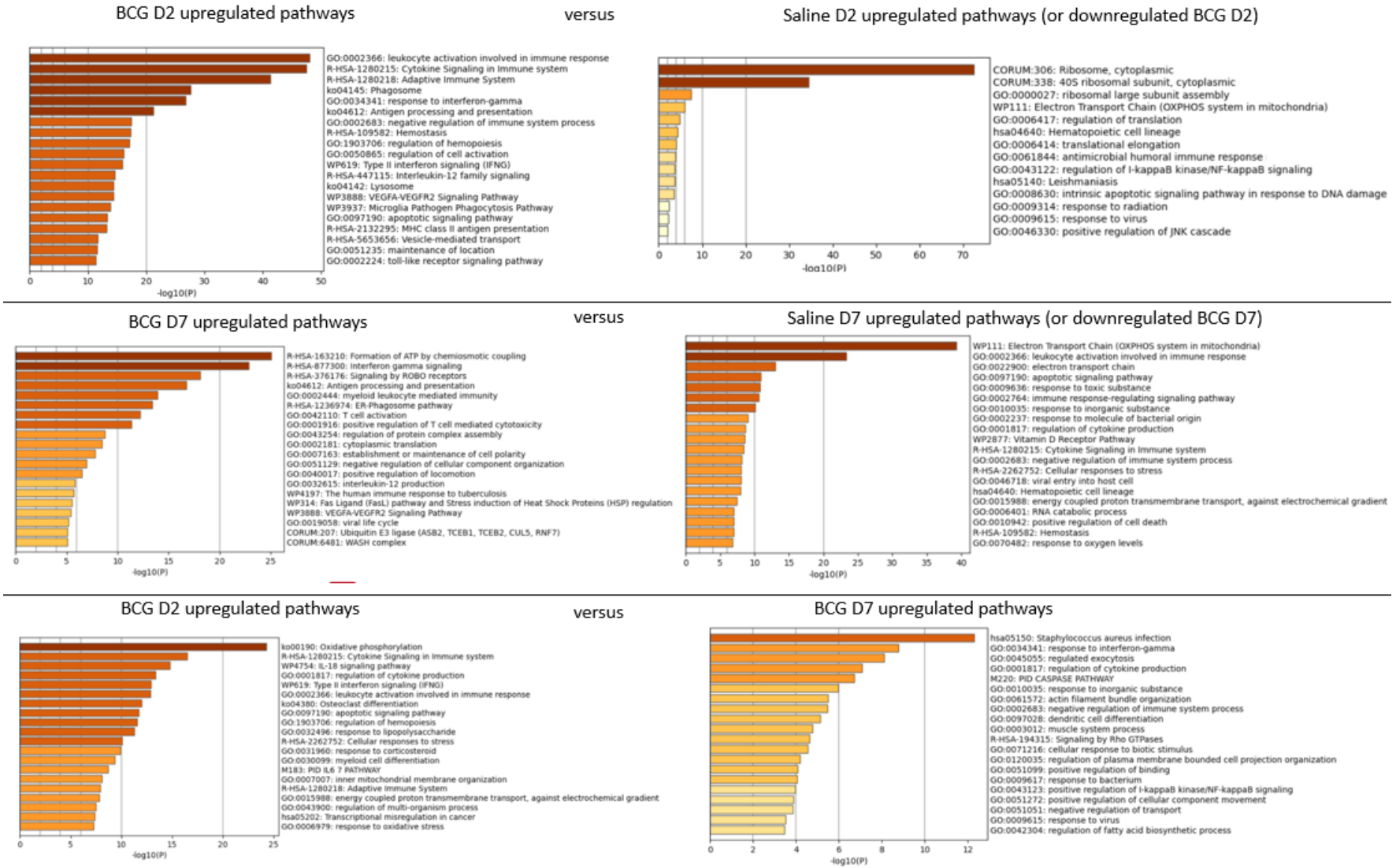


Figure 4.28. cDCs Metascape pathway enrichment analysis[568] of DGE by group.

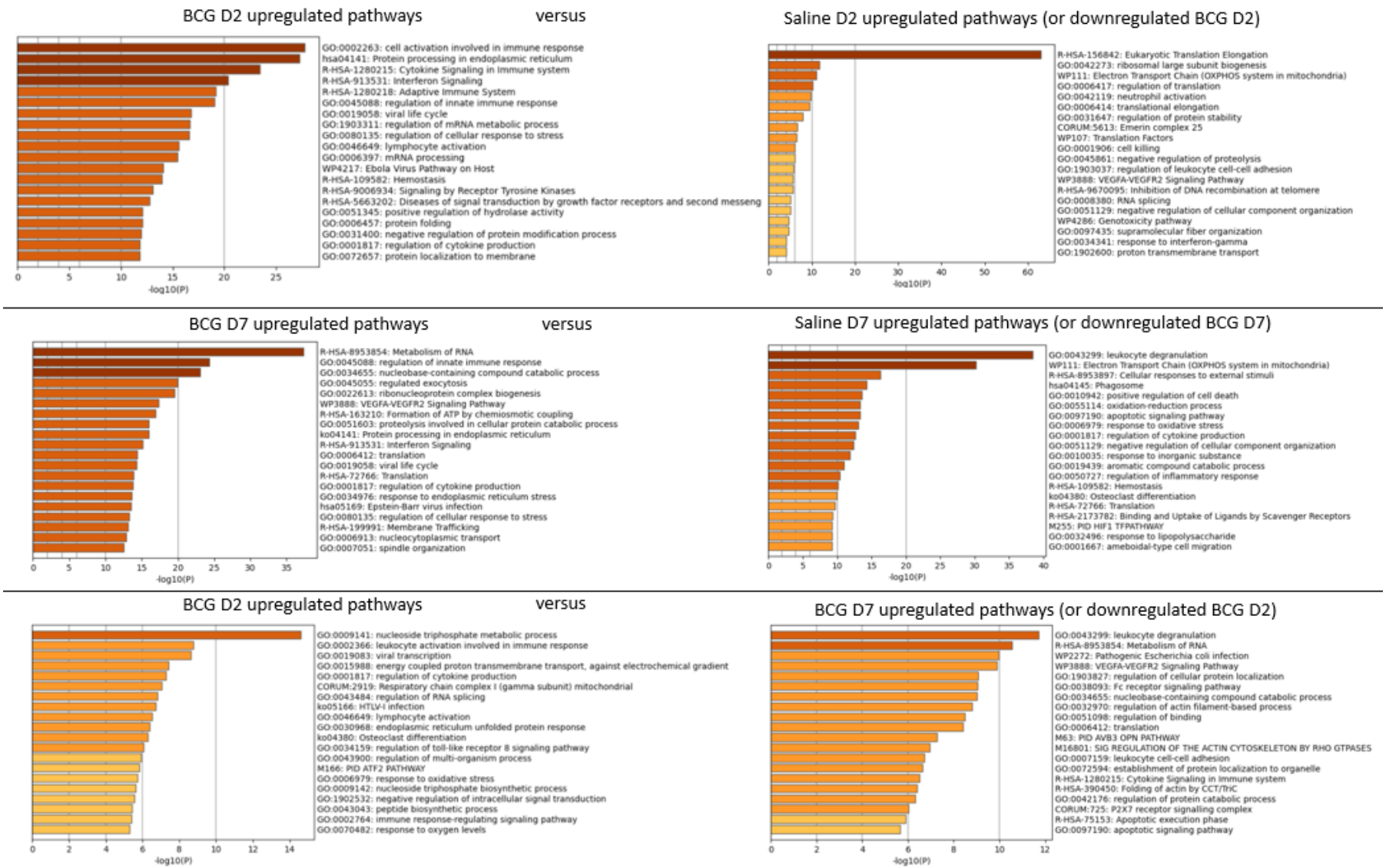


Figure 4.29. pDCs Metascape pathway enrichment analysis[568] of DGE by group.

For cDCs the expression changes in the 100 pre-defined genes is subtle (Figure 4.23). At D2 but not D7 there was increased IL17RA as well as increased CXCL9, CXCL10, IL-1B and IL-6R, all markers associated with trained immunity in TB.[94,299,395] pDCs had upregulated expression of lung specific chemokines CXCR3 and CXCR4 and strong expression of the cytotoxic Granzyme B at D2 and D7 post-BCG compared with saline volunteers.

4.3.6 NK cells

Compared to D2 post-BCG infection, there was an increase of NK cells in the BAL at D7 defined by flow cytometry (Figure 4.30). Within these NK cells, CD16+ NK cells increased and HLA-DR+ NK cells decreased at this time point.

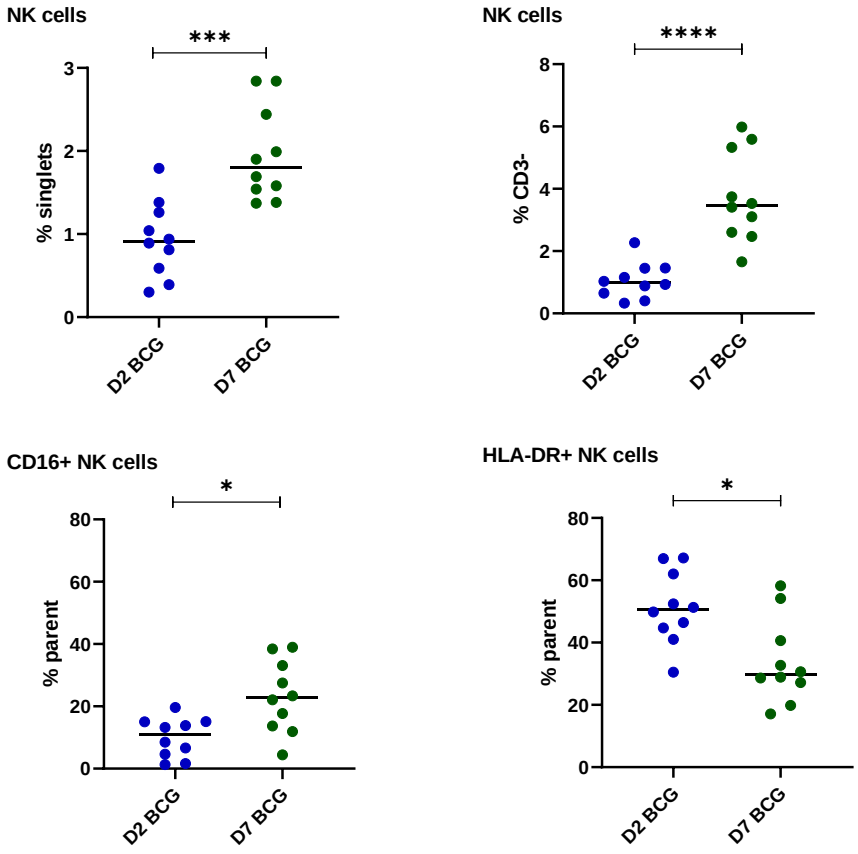


Figure 4.30. Frequency of cells using the innate flow cytometry panel. NK cells % singlets: p=0.0004, NK cells%CD3-: p<0.0001; CD16+ NK cells: p=0.0147; HLA-DR+ NK cells: p=0.0115; using Mann Whitney. Lines show median. Live singlets excludes CD19+ cells.

As the total proportion of NK cells increased at D7, while the proportion of HLA-DR+ NK cells reduced in the NK cell population, there was an overall increase in this cell subset at D7 compared with D2 in total CD3- cells (Figure 4.31).

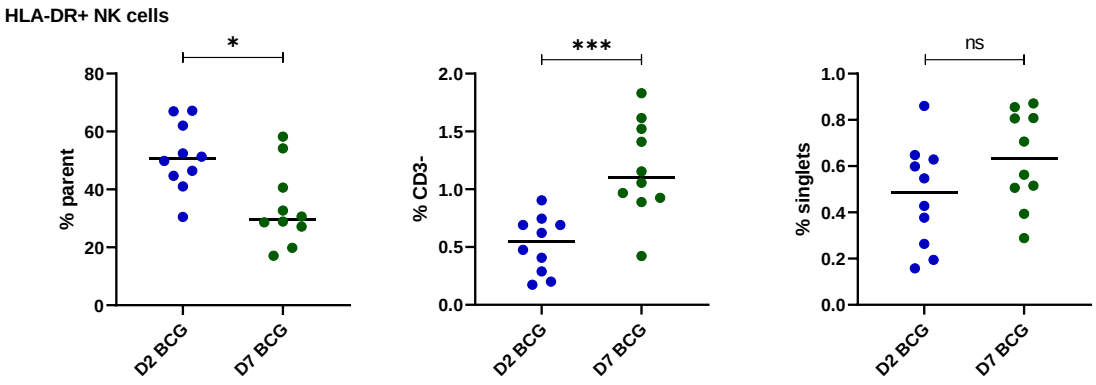


Figure 4.31. HLA-DR+ NK cells as total of NK cells, CD3- or total live singlets (minus CD19+ cells). % parent: p=0.0115; % CD3-: p=0.0005; % singlets: ns: not significant**,** using Mann Whitney. Lines show median.

There was a middle population on HLA-DR gating which varied by sample as to whether it came within the HLA-DR- or HLA-DR+ populations, based on FMO gating. To be conservative I have only included the clear HLA-DR+^{hi} population results throughout my thesis.

There was a trend for increased IFN-y producing NK cells in D7 v D2 on *ex-vivo* overnight stimulation (Figure 4.32).

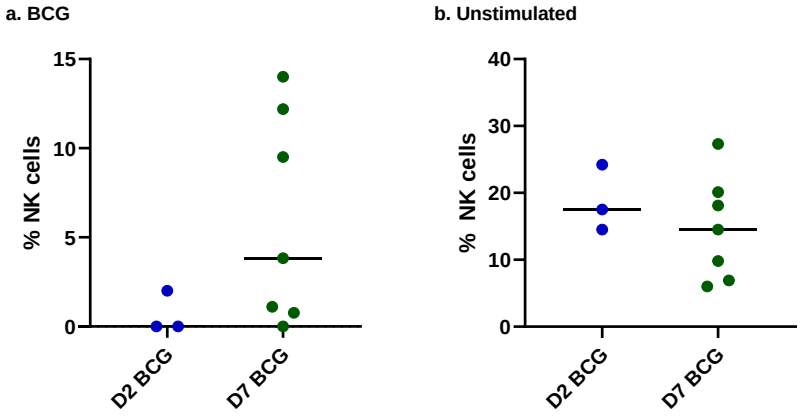


Figure 4.32. IFN-y+ cells using the homing flow cytometry panel after (a) overnight incubation with BCG, CD49d and CD29 or (b) CD49d and CD29 only ("unstim"/*in-vivo* stimulation only). (a) Calculated as IFN-y+ cells minus IFN-y+ unstimulated cells. Note different axes. Line: median.

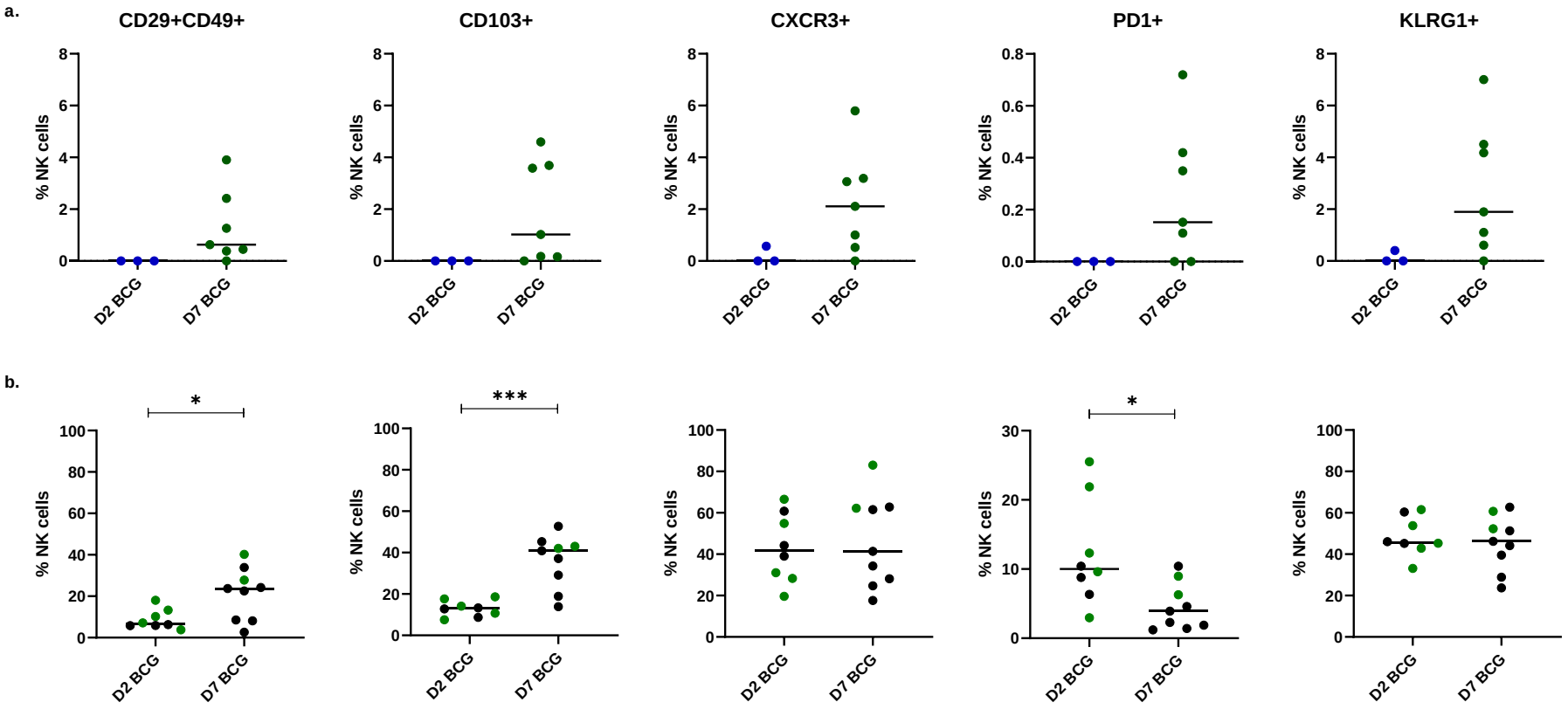


Figure 4.33. Proportion of NK cells subsets using the homing flow cytometry panel. (a) Antigen-specific IFN-y+ NK cells after overnight incubation with BCG, CD49d and CD29. Calculated as IFN-y+ cells minus IFN-y+ unstimulated cells. (b) Unstimulated NK cells stained same day (green) or incubated overnight with CD49d and CD29 and stained the following day (black). (a) No statistical analysis performed due to low sample size of D2 BCG volunteers. (b) CD29+CD49+ p=0.036; CD103+ p=0.0006; PD1+ p=0.0118 on Mann Whitney. Line: Median. Note different y-axis for PD1+ cells.

Non-specific NK cells expressing homing markers CD29+CD49+ and CD103+ increased at D7 (Figure 4.33). There was also a trend for antigen specific NK cells expressing homing markers and activation markers to increase at D7. Conversely, non-specific NK cells expressing the activation exhaustion marker PD1+ increased at D2, though this was not seen in the gene expression data.

Using 10x scRNA-seq, I identified NK cells as Cluster 32; CD3- NCAM+ FCGR3A/CD16+ (Figure 4.34).

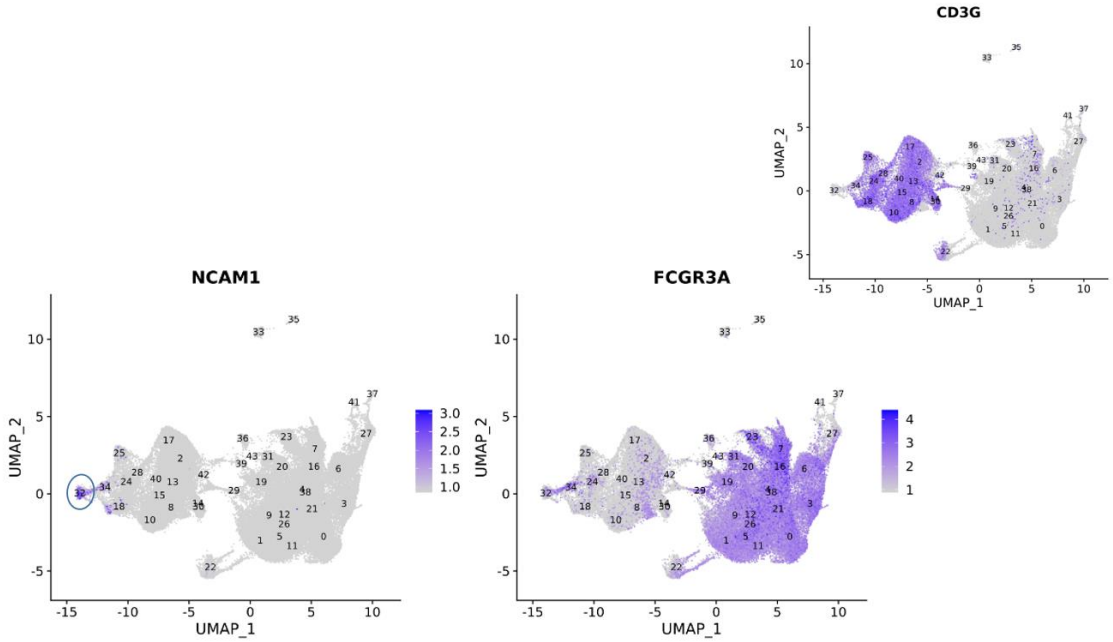


Figure 4.34. UMAP projection, showing RNA expression of canonical NK cell markers, predicts NK cell population as Cluster 32 (circled in NCAM1 plot). NCAM1 (CD56), FCGR3A (CD16), CD3G (CD3).

There was a difference in number of genes upregulated between saline controls and BCG volunteers on DGE at both D2 and D7 BAL (Table 4.2). Metascape pathway analysis is shown in Figure 4.35.

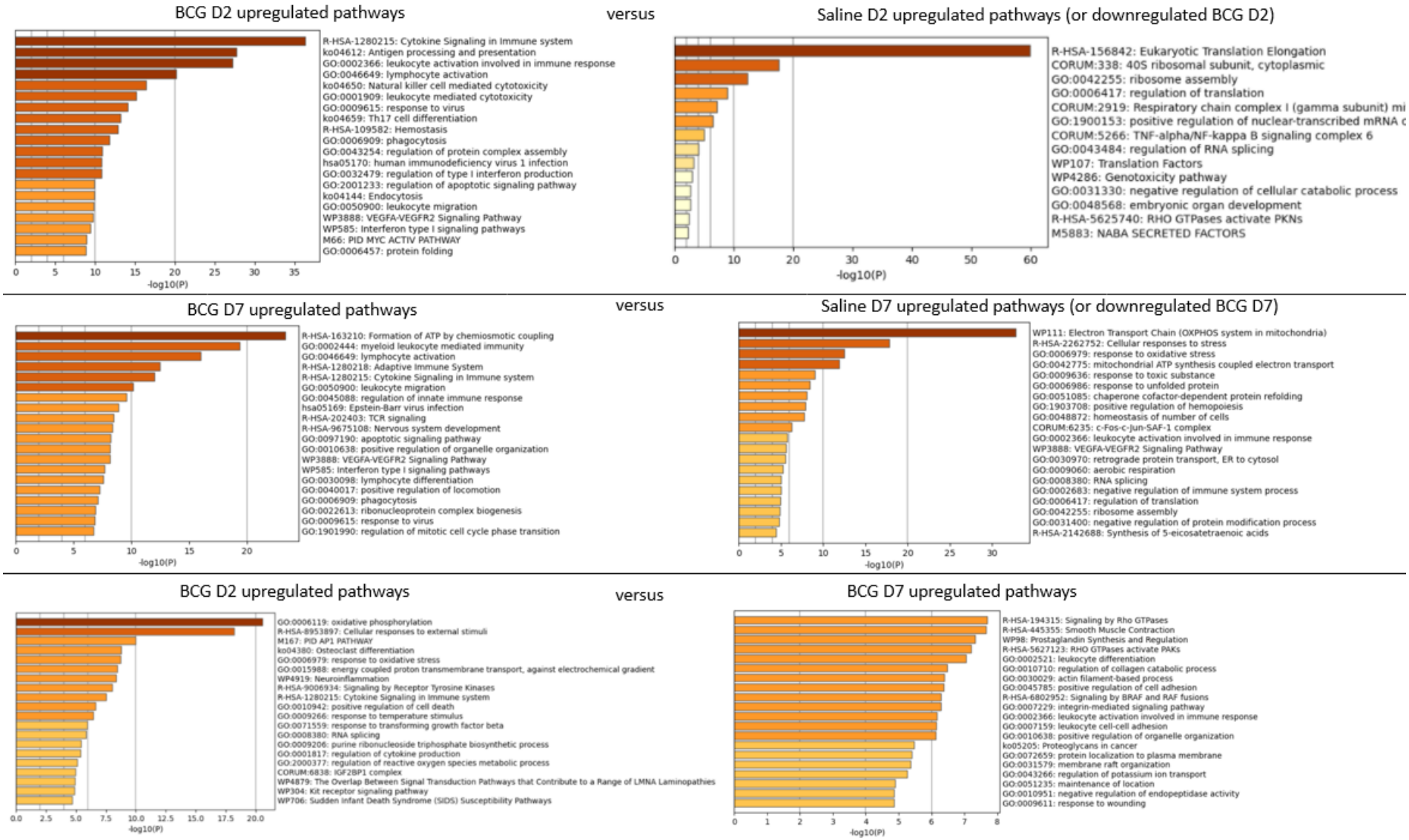


Figure 4.35. NK cell Metascape pathway enrichment analysis[568] of DGE by group. DGE criteria: Logfc ≥ 0.25 and expression ≥ 0.25 of the cells in at least one of the 2 groups.

Compared with saline controls, NK cells had upregulated pathways involved in cytokine signalling, antigen presentation and leucocyte activation, NK cell cytotoxicity, Th17 and Type 1 interferon pathways on D2 and D7. Comparing D7 with D2, there was upregulation of genes associated with leucocyte activation pathways and downregulation of genes associated with cytokine signalling pathways.

Figure 4.23 shows NK cell RNA expression in 100 pre-selected genes by group. Following BCG infection, NK cells in the BAL expressed more genes encoding homing and tissue residency markers CD29, CD40d, CXCR3 and CD69 compared with saline controls, suggesting an influx of these cells into the compartment. NK cells from BCG infected volunteers had a cytotoxic and activation signature of CD16, Granzyme and Perforin, particularly at D2 where there was also expression of HLA-DR, IL-12R and NOSIP (nitric oxide synthase interacting protein). Post BCG-infection, genes associated with cell recruitment were also upregulated (IL-16, CCR1: monocyte recruitment. CCR7: DCs and T cells recruitment). Inhibitory and activation KIRs were upregulated post-BCG, including IL-10 and CD94/NKG2B which recognises HLA-E.[233]

4.3.7 Donor Unrestricted T cells (DURTs)

4.3.7.1 MAIT cells

As the MAIT/NKT flow cytometry panel was third priority, there were only three D2 BCG and four D7 BCG BAL samples stained (Figure 4.2). Hence no statistical analysis was performed.

I used four different gating methods to identify MAIT cells,[249] which for our BAL cells, seemed to detect the same cell population, though CD161+TEM+ detected the fewest cells, which is consistent with literature (Figure 4.36a).

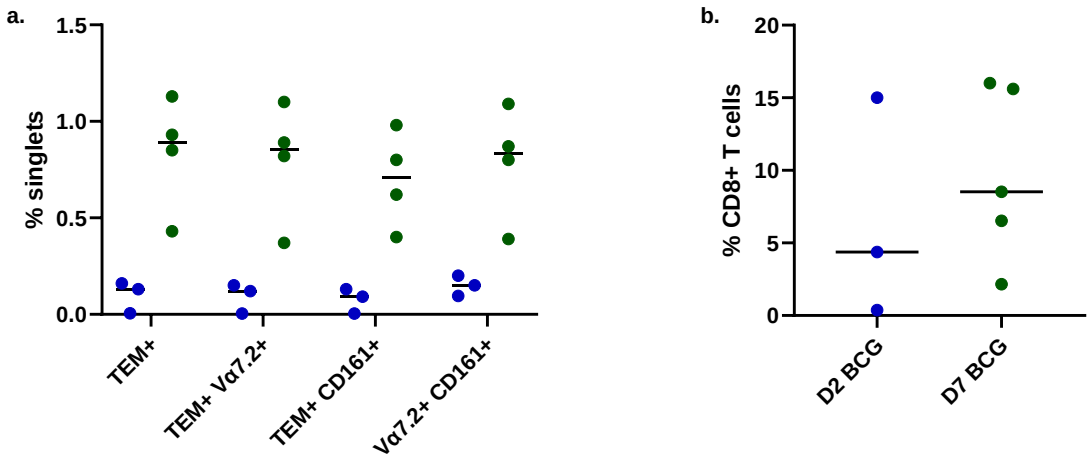


Figure 4.36. Frequency of CD8+ MAIT cells using the MAIT/NKT flow cytometry panel. (a) Identified using four gating strategies. Shown as frequency of live CD14-CD19-singlets. TEM: 5-OP-RU MR1 tetramer. Blue: D2, Green: D7. (b) Frequency of CD8+ MAIT cells out of total CD8+ T cells.

This similarity between gating strategies also held for total MAITs and CD4+ or double negative (DN) MAITs (data not shown). For all further MAIT data, MAIT cells were identified using TEM+ alone.

There was no convincing trend for CD8+ MAITs to increase out of total CD8+ T cells, but sample size was limited (Figure 4.36b).

There was a trend for total MAITs to increase at D7 out of total singlets, but not as % CD3+ cells (Figure 4.37a). This was likely due to the influx of other T cells at the same time point. DN MAIT were predominant at D2, but CD4+ and CD8+ MAITs increased at D7, with CD4+ MAITs accounting for the largest change in this small dataset (Figure 4.37b-d).

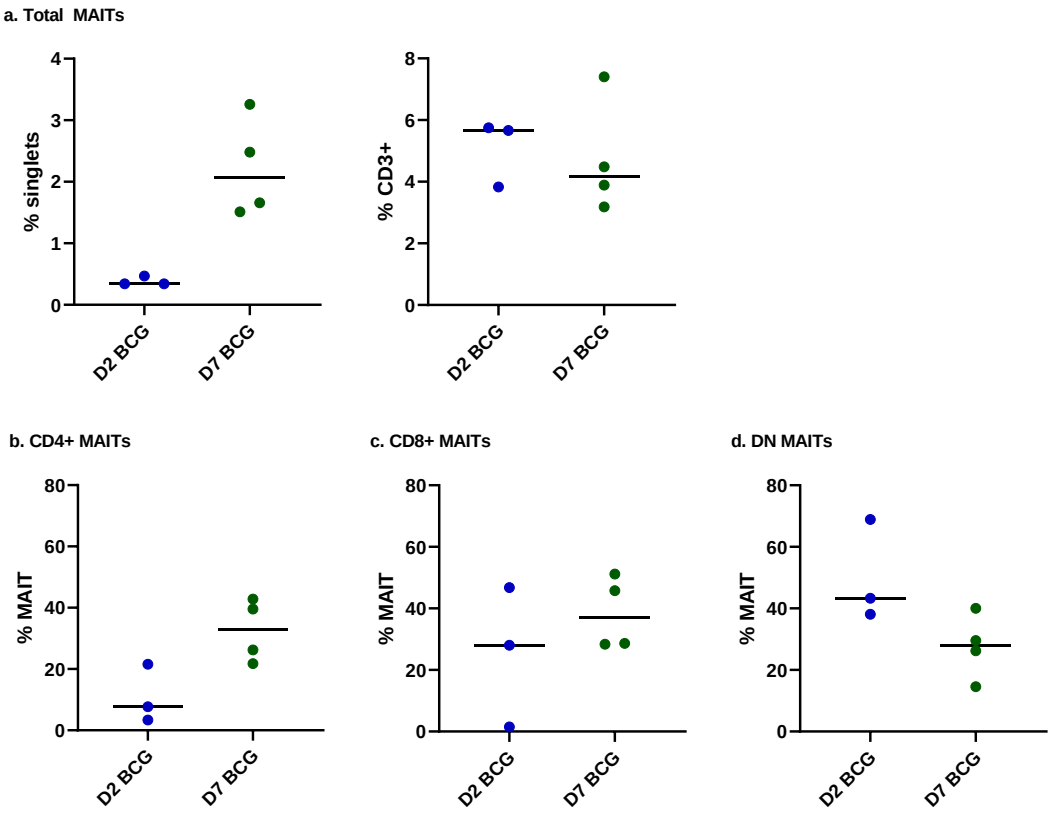


Figure 4.37. (a) Total MAITs, (b) CD4+ (c) CD8+ or (d) DN MAITs (CD4-CD8-) in the BAL D2 or D7 post-BCG aerosol infection. MAITs identified using 5-OP-RU MR1 tetramer using the MAIT/NKT flow cytometry panel.

Using 10x scRNA-seq, I identified MAIT cells as cluster 25 (Figure 4.38).

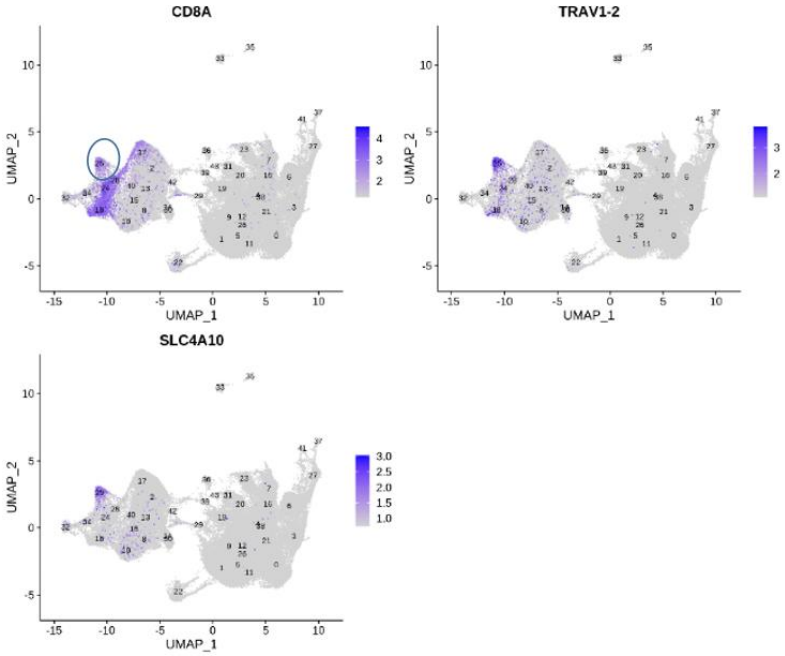


Figure 4.38. UMAP projection of canonical markers predict MAIT cells as Cluster 25.[587] TRAV1-2 (Vα7.2), SLC4A10 (Solute Carrier Family 4 Member 10).

There was a difference in number of genes upregulated between saline controls and BCG volunteers at both D2 and D7, with the greatest difference seen at D7 compared with saline (Table 4.2). However, when the two groups of BCG volunteers were compared, there were more genes upregulated after D2 than D7 (146 compared to 74 genes).

DGE pathway enrichment analysis is shown in Figure 4.39. Post-BCG, MAIT cells increased expression of pathways associated with Th1 cytokines, TCR signalling, leucocyte degranulation, lymphocyte activation, Th17 differentiation and response of *M.tb* to phagocytosis. There was also increased negative regulation of the immune system at both D2 and D7. At D2, IL-17 signalling pathway genes were increased compared with D7 and at D7, MAIT cells increased genes associated with T cell activation compared with D2.

MAIT cell expression of 100 pre-selected genes is shown in Figure 4.23. Compared with saline, MAIT cells post-BCG had increased expression of the lung homing marker CXCR6, cytotoxicity genes PRF1, NOSIP and GZM; and IL-16. IL-16 is a chemokine for CD4+ T cells, particularly Th1.[588]

In keeping with the pathway enrichment analysis, MAITs also expressed genes associated with negative immune regulation such as KLRG1 (co-inhibitory check point on late differentiated cells) and IL-10R.

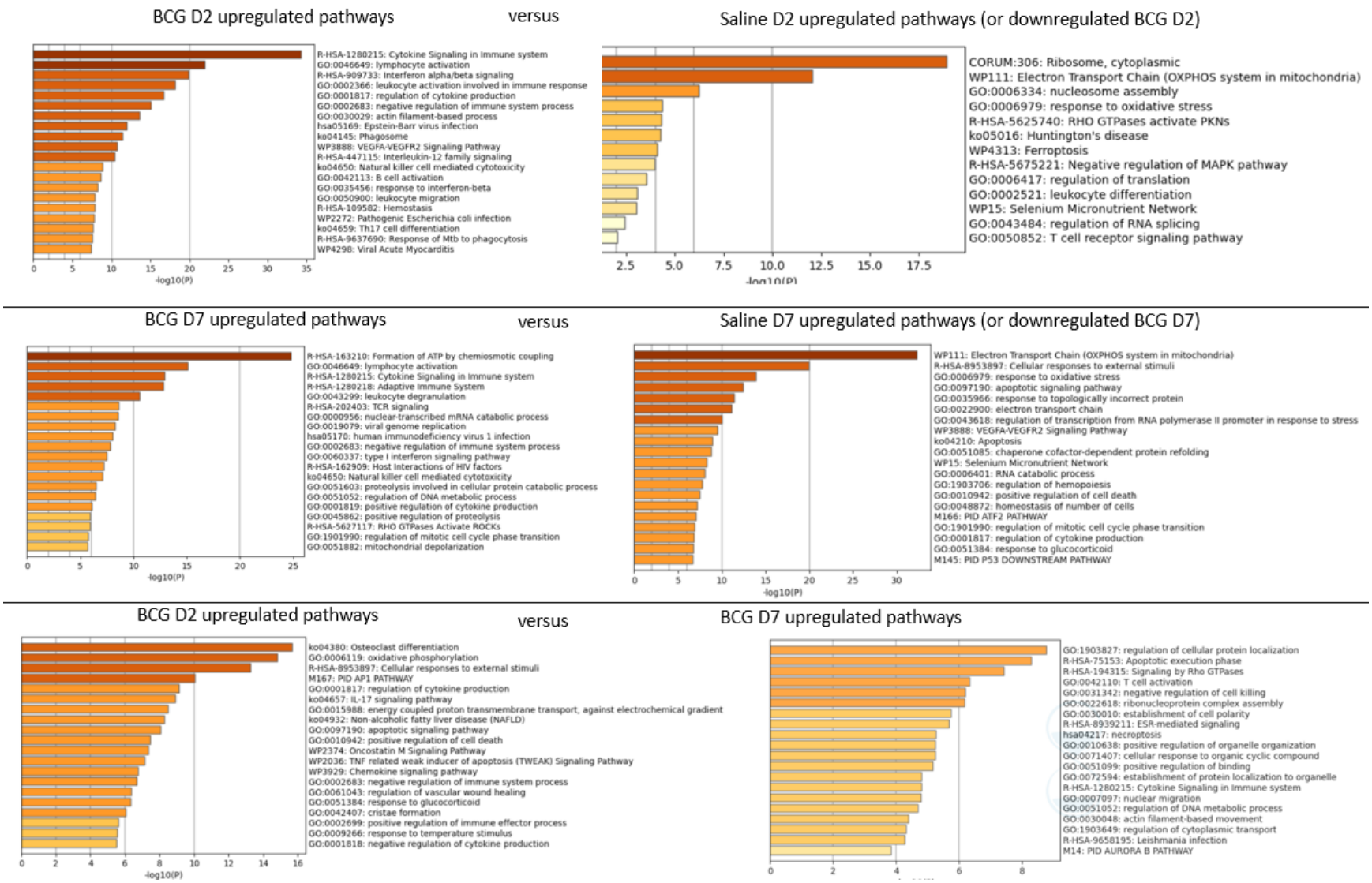


Figure 4.39. MAIT cell metascape pathway enrichment analysis[568] of DGE by group. DGE criteria: Logfc ≥ 0.25 and expression ≥ 0.25 of the cells in at least one of the 2 groups.

4.3.7.2 Natural Killer T cells (NKTs)

There was a trend for an increase in invariant Natural Killer T cells (iNKT) at D7 compared with D2, driven by an increase in the CD4+ subset (Figure 4.40a; Figure 4.41). CD56+ CD3+ cells also increased in the airway at D7 compared with D2 but encompassed a less specific group of cells, as indicated by the greater proportion of these cells compared with iNKTs (Figure 4.40b).

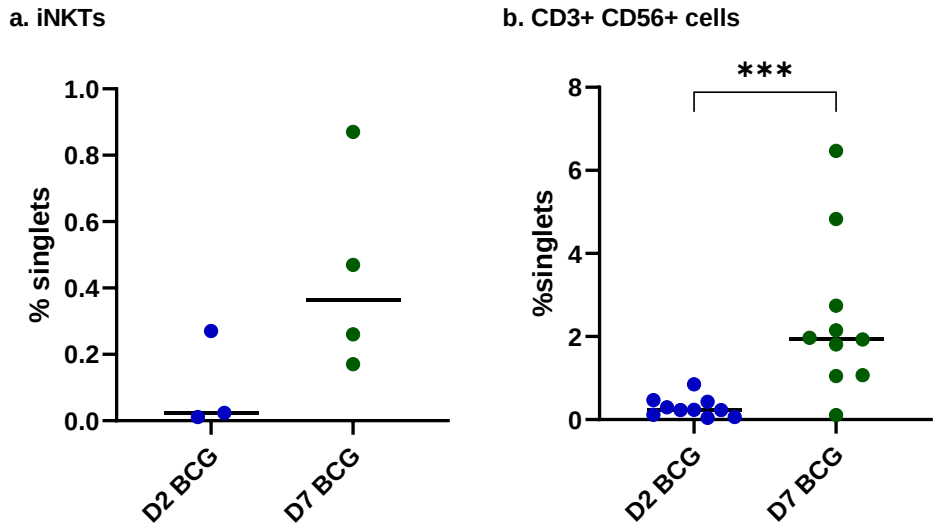


Figure 4.40. Frequency of (a) iNKT cells defined as Vα24+ Vβ11+ CD56+ CD3+ cells using the MAIT/NKT flow cytometry panel; or (b)"NKT" cells using the innate flow cytometry panel, defined as CD56+ CD3+ CD14- cells. p=0.0007 on Mann Whitney. Shown as frequency of live CD14-CD19-singlets. Note different y-axis between the graphs.

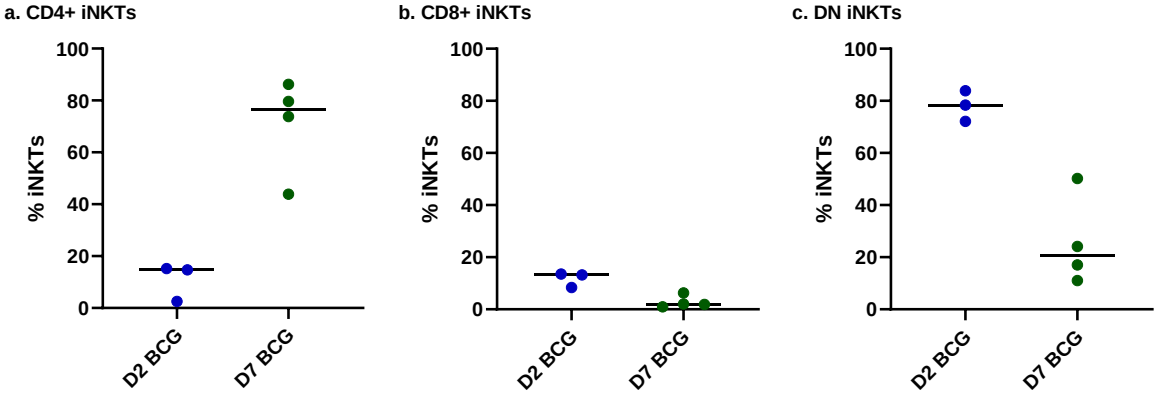


Figure 4.41. (a) CD4+ iNKTs, (b) CD8+ iNKTs (c) DN iNKTS (CD4-CD8- "double negative") in the BAL D2 and D7 following BCG aerosol infection. iNKT cells defined as Vα24+ Vβ11+ CD56+ CD3+ cells using the MAIT/NKT flow cytometry panel.

iNKTs were not identified on 10x (Figure 4.42). This could be due to low cell numbers. On flow cytometry, iNKTs made up only 0.4% singlets, compared with 0.8% MAITs and 2% γδ2 T cells.

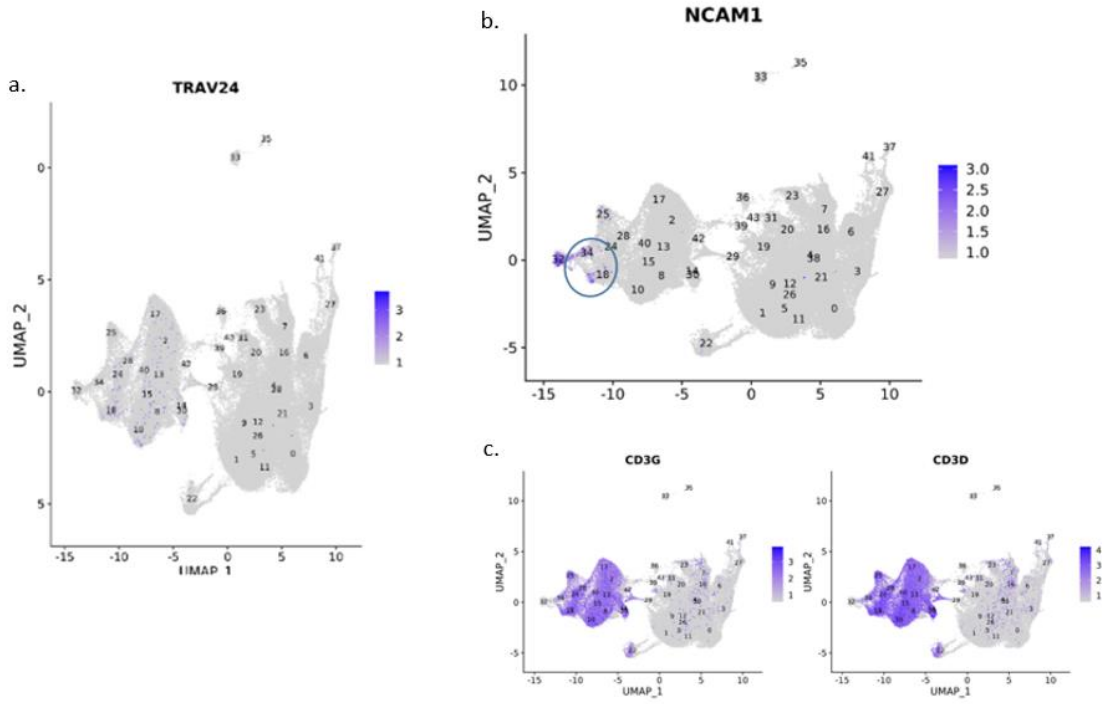


Figure 4.42. UMAP projection showing RNA expression of iNKT markers. Clusters 34 and 18 (CD3+ clusters, (c)) show partial expression of CD56 ("NCAM1") –circled (b). However there was very minimal TRAV24 (Vα24) expression in these clusters (a) and no TRBV11 (Vβ11) expression in any cluster.

4.3.7.3 γδ T cells

γδ cells BAL cells identified by flow cytometry are shown in Figure 4.43. The γδpan antibody stained poorly on γδ2+ cells (see Figure 4.44). This was consistent across different batches of γδpan antibody and also between BAL and whole blood cells. Hence true γδ2 cells (defined as γδ2+ γδpan+) were not able to be identified. γδ2 cells weakly binding γδpan+ have been reported.[285]

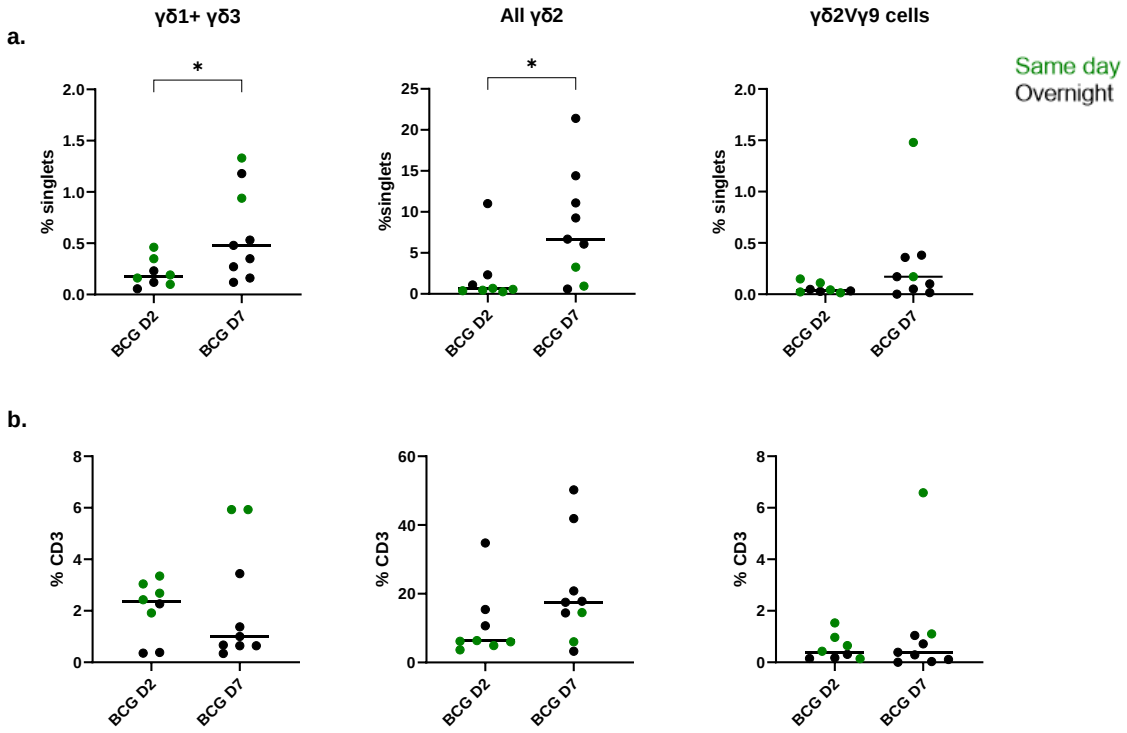


Figure 4.43. Proportion of γδ T cells in the BAL fluid identified on unstimulated cells from the homing flow cytometry panel. Proportion of (a) singlets (live+CD19-CD14-) or (b) CD3+ cells. Green dots: cells stained and fixed on same day as BAL collection. Black dots: cells incubated overnight before staining and fixing. "γδ1+ γδ3": CD3+ γδpan+ γδ2- (p=0.0292); "All γδ2": CD3+ γδ2+ (p=0.0152); "γδ2Vγ9": CD3+ γδ2+ γδpan+. All p-values calculated using Mann Whitney. Lines median.

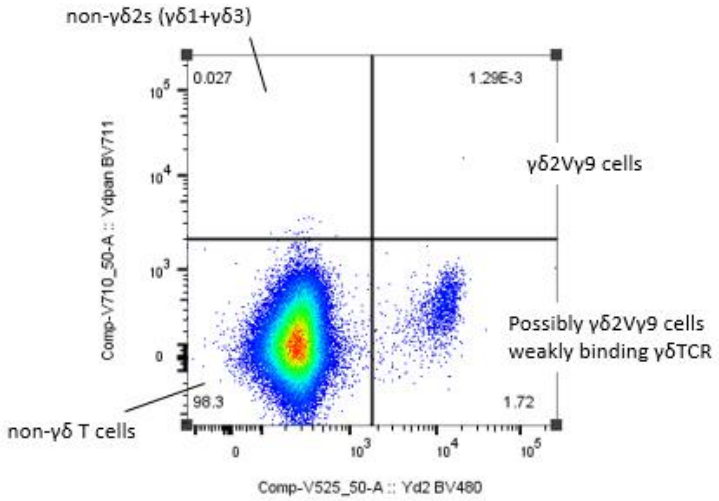


Figure 4.44. Typical flow cytometry plot showing CD3+cells by γδpan BV711 staining (y-axis) and γδ2 BV480 staining (x-axis). % of each quadrant shown

Both non-γδ2 and total γδ2 T cells increased at D7 post-BCG infection compared with D2, as proportion of total singlets (Figure 4.43). This effect was lost as % CD3+ cells.

There was a trend for an increase in antigen-specific IFN-γ+ γδ2 T cells at D7 compared with D2 (Figure 4.45a).

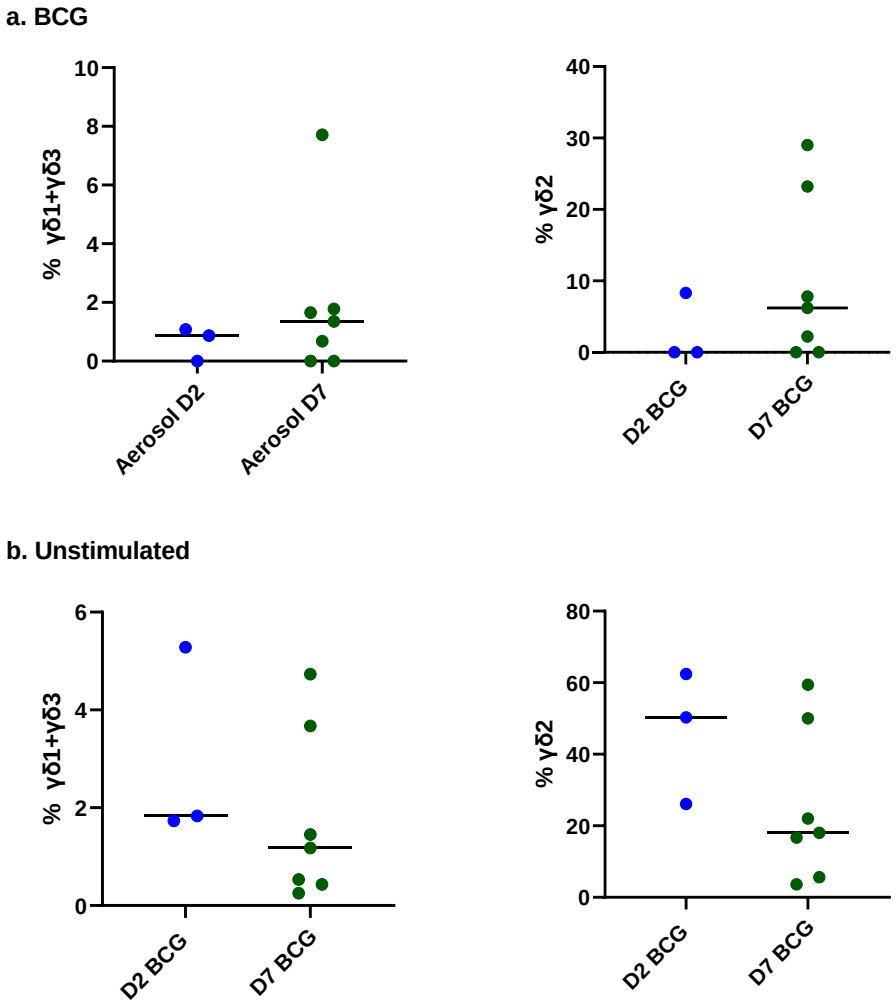


Figure 4.45. IFN-γ+ γδ cells using the homing flow cytometry panel after (a) overnight incubation with BCG, CD49d and CD29 or (b) CD49d and CD29 only ("unstim"/*in-vivo* stimulation only). "γδ1+ γδ3": CD3+ γδpan+ γδ2-; "γδ2": CD3+ γδ2+ (a) calculated as IFN-γ+ cells minus IFN-γ+ unstimulated cells. Note different axes. Line: Median.

Using single cell sequencing, I identified a clear γδ2 T cell population as Cluster 34 (Figure 4.46). γδ1 T cells were more widely distributed and clustered partially with Cluster 18 (identified as CD8+ T cells). This is likely due to the low levels of non-γδ2 T cells in BAL. There was an increase in genes expressed in γδ2 cells following BCG compared with saline at both timepoints (Table 4.2).

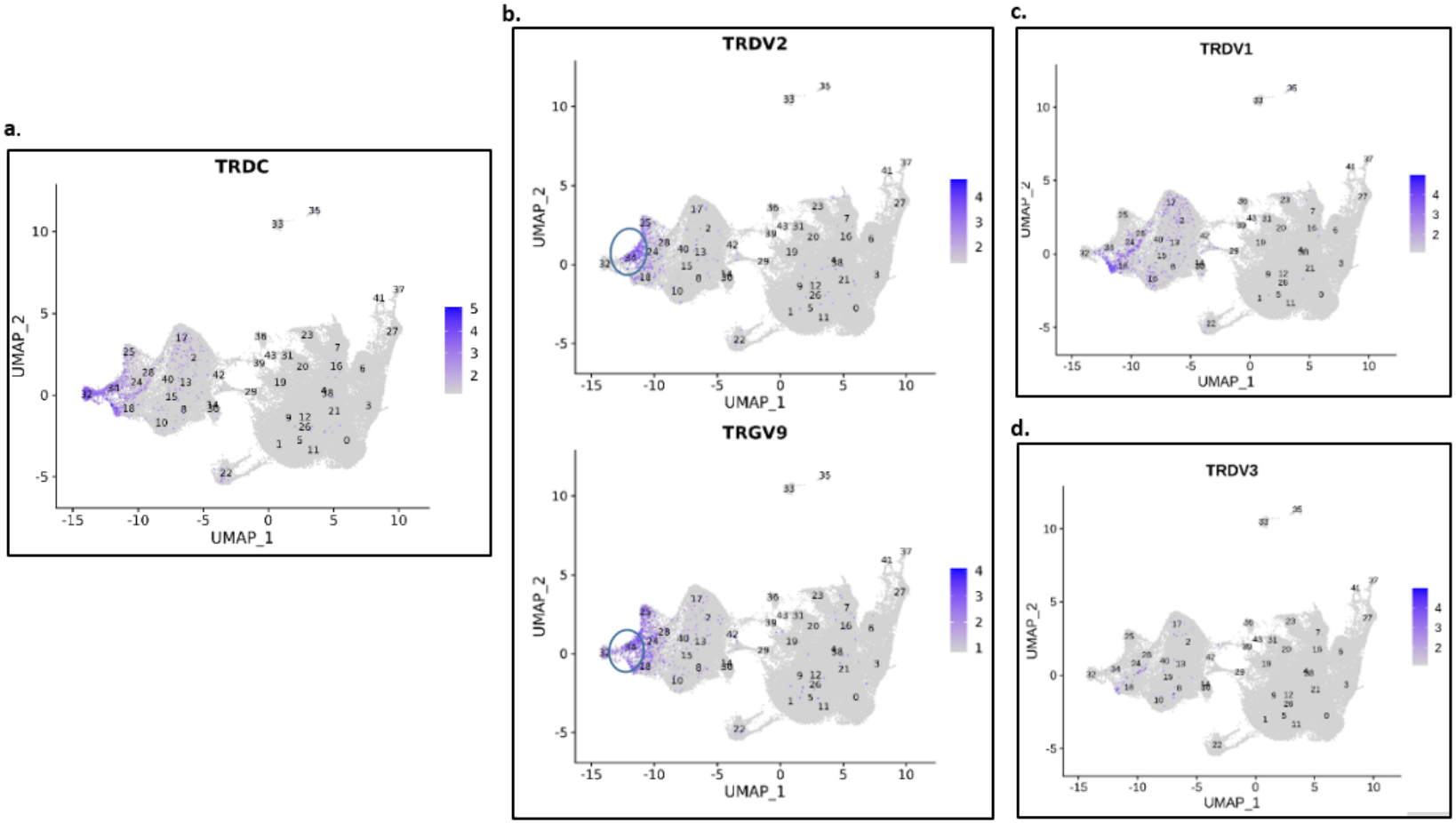


Figure 4.46. UMAP projection showing RNA expression of γδ T cell markers. (a) TRDC: TCR delta constant shows spread of γδ T cells. (b) TRDV2 "T cell receptor variable 2" (γδ2) and TRGV9 "T cell receptor gamma variable 9" (Vγ9) predicts γδ2 T cell population as Cluster 34 (circled). (c) Expression of TRDV1 "T cell receptor variable 2" shows variable distribution of γδ1 T cells, with partial expression around Cluster 18; (d) TRDV3 "T cell receptor variable 3" shows no clear γδ3 T cell population.

Figure 4.47 shows the Metascape analysis of γδ2 cells. At D2 and D7 post-BCG infection, γδ2 cells upregulated genes associated with cytokine, leucocyte activation, adaptive immune system, IFN-α/β, phagocytosis, migration, cytotoxicity and cellular defence pathways. In addition, at D7 there was upregulation of TCR signalling and negative regulation of the immune system.

On analysis of 100 pre-selected genes (Figure 4.23), at D2 and D7 post-BCG exposure, γδ2 cells expressed cytotoxic genes and the Th1 cytokine IL-16. At D2 there was increased expression of CD16 and HLA-DR; and homing markers CD29/ ITGB1 and CD49/ ITGA4. By D7 there was upregulation of homing markers CXCR3 and CXCR6 and evidence of negative regulation with an increase in IL-10, the terminal maturity marker KLRG1 and downregulation of the activation marker CD69.

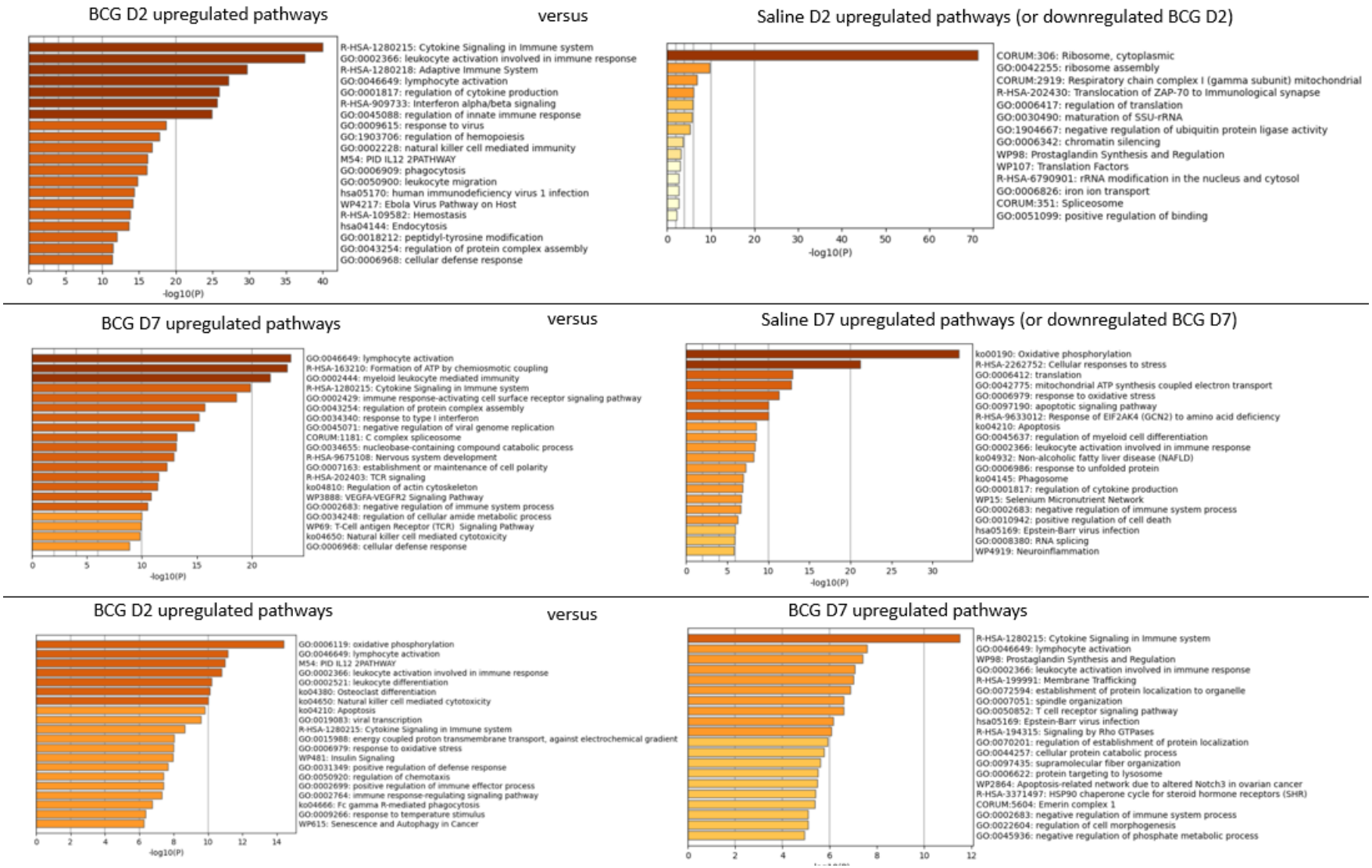


Figure 4.47. γδ2 Metascape pathway enrichment analysis[568] of DGE by group. DGE criteria: Logfc ≥ 0.25 and expression ≥ 0.25 of the cells in at least one of the 2 groups.

4.3.8 T cells

There was an increase in T cells at D7 post-BCG compared with D2 (Figure 4.48). The trend was the same across all flow cytometry panels (innate panel and homing panel shown; no difference in T cell proportions on Wilcoxon between flow cytometry panels at matched timepoints).

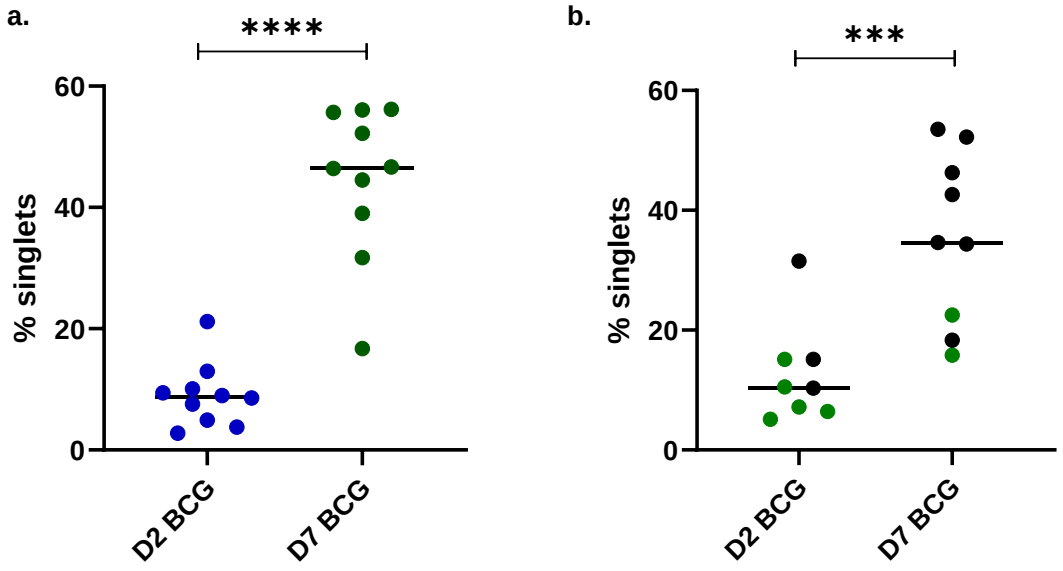


Figure 4.48. Frequency of T cells using the (a) innate or (b) homing flow cytometry panel. (a) "singlets": live CD19- singlets p=<0.0001; (b) "singlets": live CD19- CD14-singlets p=0.0005. Calculated using Mann Whitney, lines show median. Colours in (b) indicate differences in processing. Green: same day staining, black: cells incubated overnight before staining the following day.

All T cell subsets (CD4+, CD8+, DN) increased at D7 post-BCG compared with at D2 (Figure 4.49a-c) as % total singlets. As the total T cell (CD3+) population also increased at D7, CD4+ T cell proportions did not change, and CD8+ T cell proportions decreased as a percent of total T cells (Figure 4.49a-b). The ratio of CD4+ T cells/CD8+ T cells significantly increased at D7 as percentage of total T cells Figure 4.49f) compared to D2. There was a trend for total DN T cells to increase at D7 as % singlets and also decrease as a total proportion of T cells, but this did not reach significance (Figure 4.49c). This was a similar trend to that seen

with DN MAIT cells (Figure 4.37). The T cell population, which did increase as a proportion of total T cells was a double positive population (DP T cells), due to a few high expressing volunteers (Figure 4.49d). However, as CD4+ T cells made up the largest T cell population, despite no significant increase as a proportion of T cells, they were the main source of T cells in the BAL at D7 (Figure 4.49e).

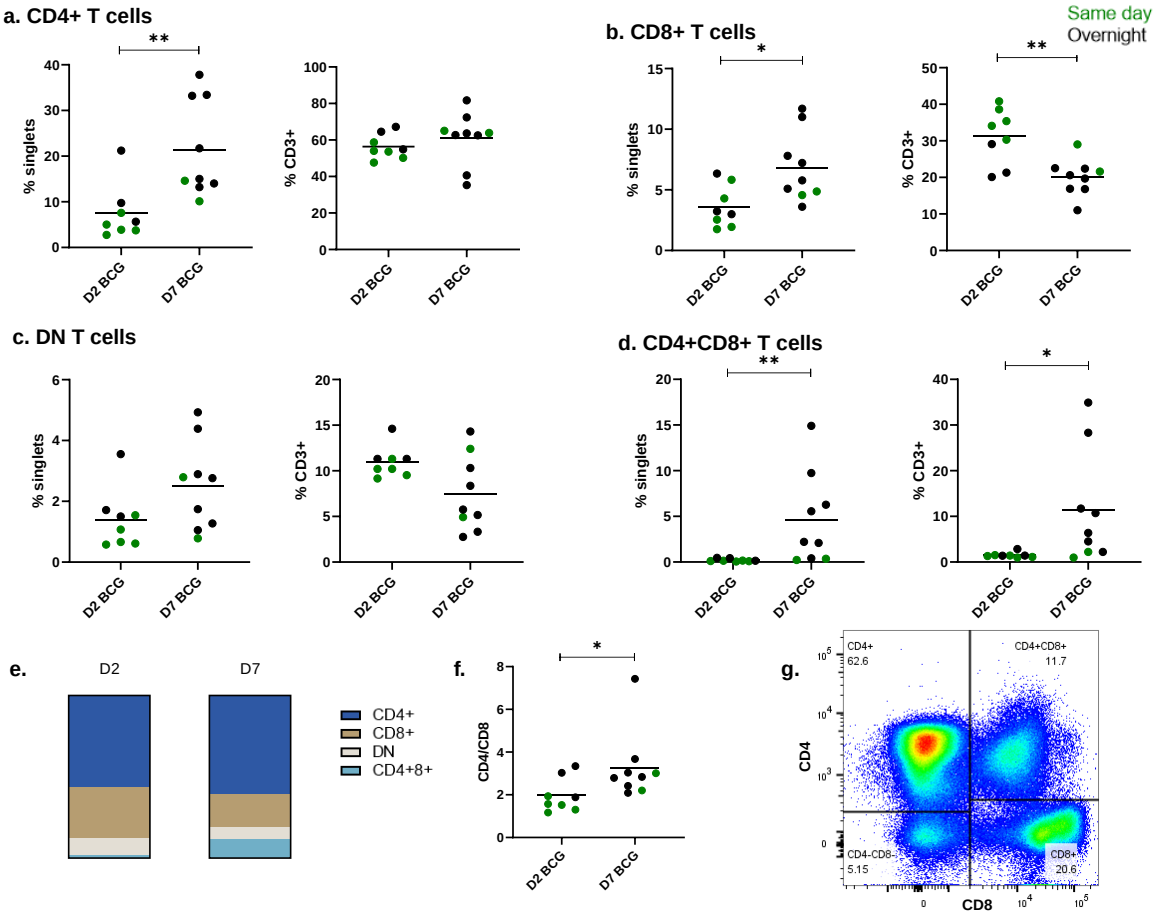


Figure 4.49. Frequency of (a) CD4+ (b) CD8+ (c) DN or (d) CD4+CD8+ T cells using the homing flow cytometry panel. "singlets": live CD19- CD14-singlets. (a) CD4+T cells %singlets p<0.0048. (b) CD8+ T cells %singlets p<0.0142, %CD3 p=0.0024. (d) CD4+CD8+ T cells % singlets p=0.0023, %CD3+ p=0.033 (e) Parts of whole, means. (f) Ratio CD4+/CD8+ T cells (%CD3+ cells) p=0.027. (g) Typical flow cytometry plot from a moderate CD4+CD8+ volunteer (unstimulated sample). Differences in cell frequency between groups calculated using unpaired T-test, after normality testing. Lines show mean. Coloured dots indicate differences in processing. Green: Same day staining, black: cells incubated overnight before staining the following day.

HLA-DR+ total T cells increased at D7 compared with D2 (Figure 4.50).

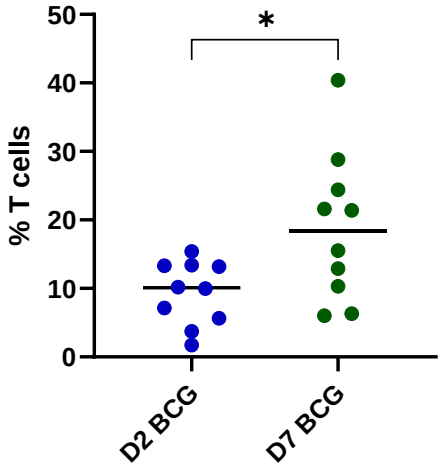


Figure 4.50. Frequency of HLA-DR+ T cells on the innate flow cytometry panel. p=0.0355 on Mann Whitney after normality testing. Line: Median.

Figure 4.51 shows the strategy I employed to identify T cells in the BAL 10x data set, following an extensive literature search.[408,507-513]

Figure 4.52 shows further investigation into the identity of Cluster 40 which revealed this was not a unique CD161+ CD4+ CD8+ cell but likely a cluster of doublets. The low number of cells in this cluster (see Figure 4.53) further suggests that this is not a true cell type. Alternatively, as only one of the three samples at D7 included a volunteer with high levels of CD4+ CD8+ cells on flow cytometry, it may be that numbers of DP T cells were too low to be detected and fully described on 10x.

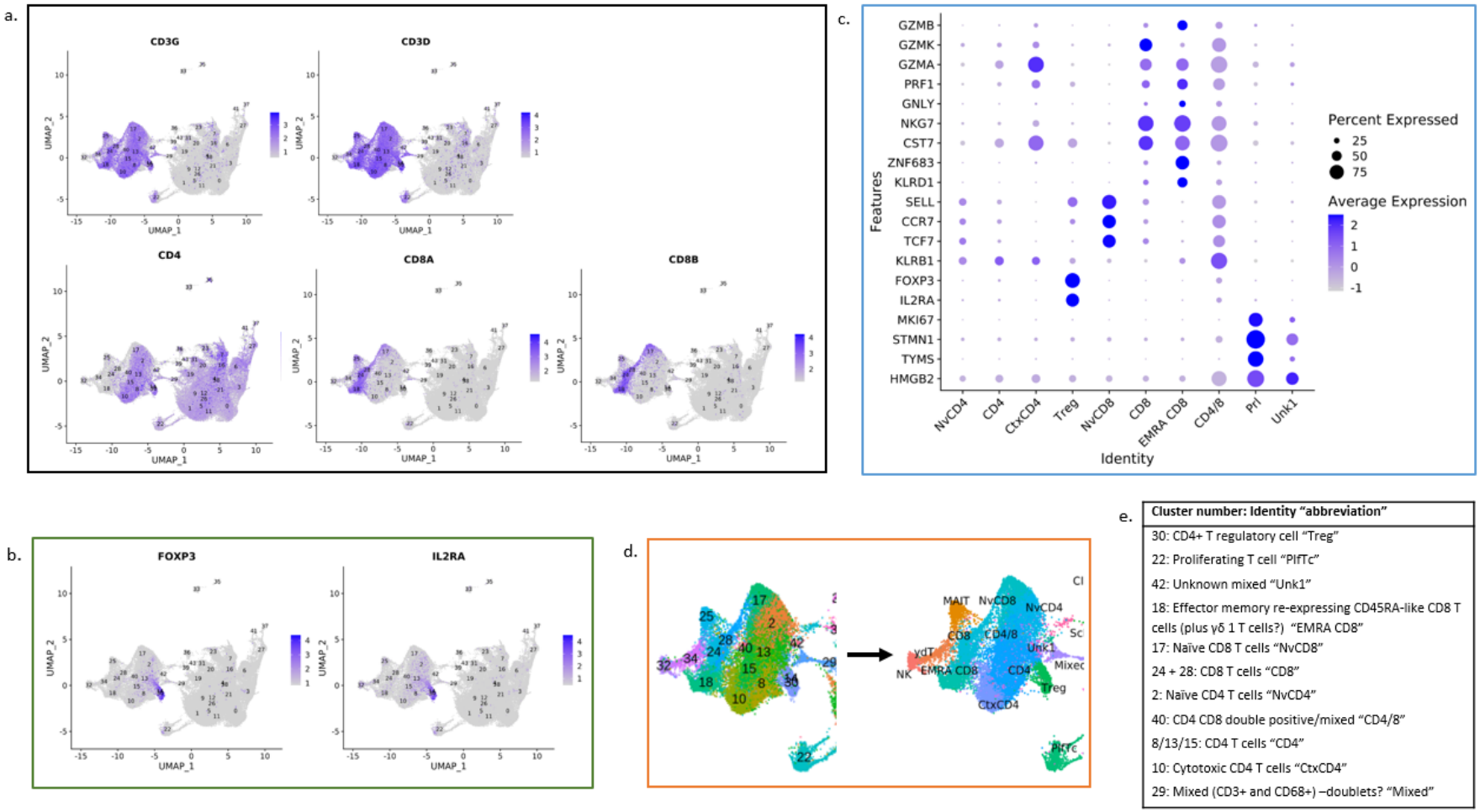


Figure 4.51. T cell subsets identified on single-cell sequencing. (a) UMAP projection showing expression of CD3, CD4, and CD8. (b) Canonical markers for T regs predict Cluster 30 as CD4+ T regulatory cell. (c) Dotplot of gene expression used to identify T cell subset phenotypes. (d) Modified UMAP plot showing association between named and numbered clusters. (Cluster numbers are assigned automatically by the Seurat programme and is inversely proportionate to the number of cells in each cluster, where Cluster 1 has the most cells). (e) Association between cluster number, identity and name/abbreviation.

Gene abbreviations (from the Genecard database)[584]: “CD3G” and “CD3D”: Part of TCR-CD3 complex (transmembrane chains).”GZM”: Granzyme. “PRF” Perforin. “GNL”: Granulysin. “NKG7”: Natural Killer Cell Granule Protein. “CST7”: Cystatin F. “ZNF683”: Zinc finger protein 683. “KLRD1”: Killer Cell Lectin Like Receptor 1/CD94. “SELL”: L-Selectin. “CCR7”: Chemokine receptor 7. “TCF7”: Transcription factor 7. “KLRB1”: CD161. “FOXP3”: Forkhead Box P3.“IL2RA”: Interleukin 2 receptor. “MK167”: Marker of Proliferation Ki-67. “STMN1”: Oncoprotein. “TYMS”: Thymidylate Synthase. “HMGB2”: High mobility group box 2.

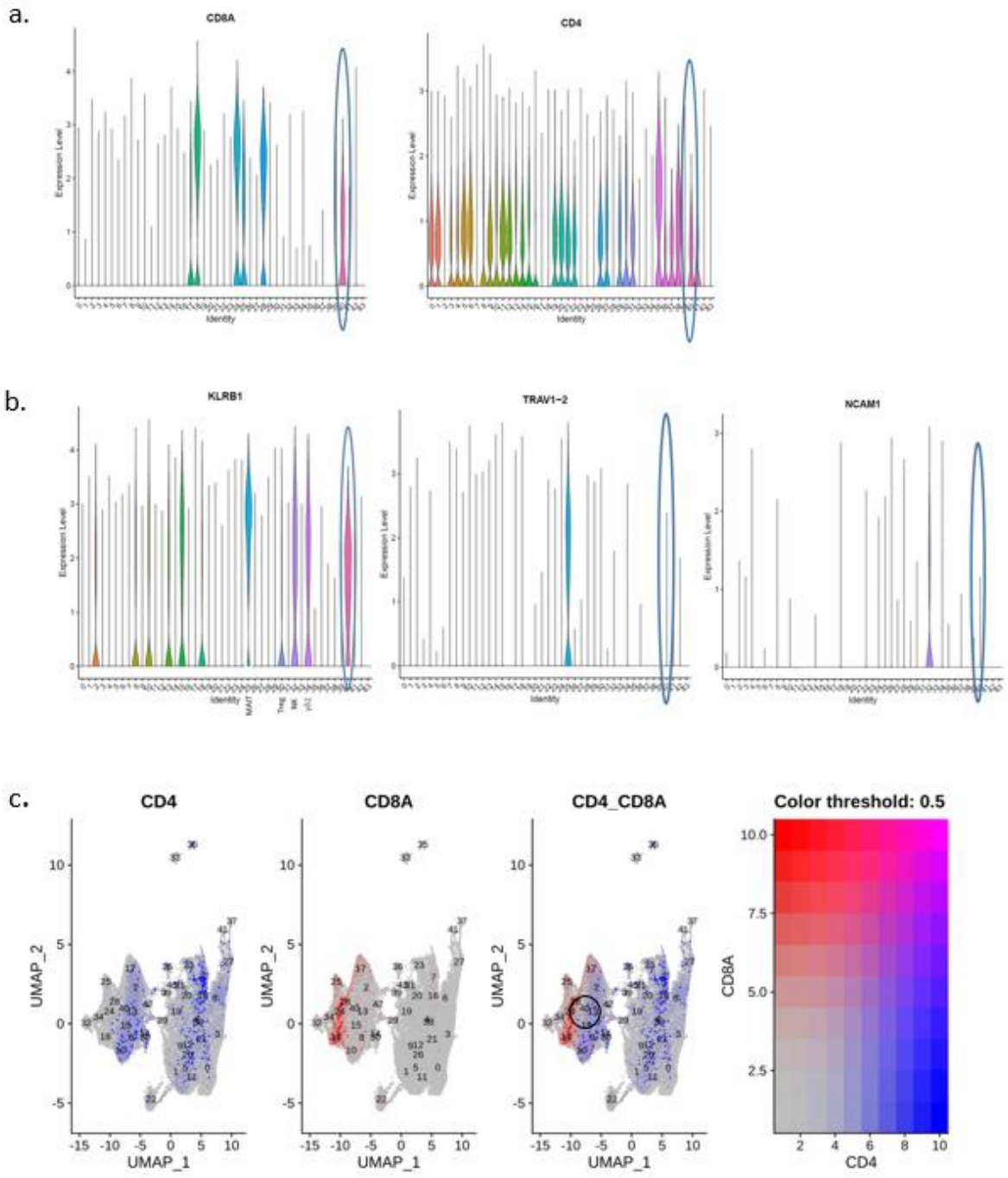


Figure 4.52. Further investigating identity of Cluster 40, identifies likely doublets not double positive cells. (a+b) Violin plots showing that Cluster 40 cells express CD8, CD4 and KLRB1 (CD161) (Cluster 40 circled) and are negative for CD56 (NCAM) and TRAV1-2 (canonical marker for MAIT). (c) No purple expression on UMAP plots shows Cluster 40 cells are not double positive for CD4 and CD8 (Cluster 40 circled)

Figure 4.53 shows numbers of each T cell subtype out of total T cells defined by 10x. Using this method to detect cell numbers there was no trend for difference between groups.

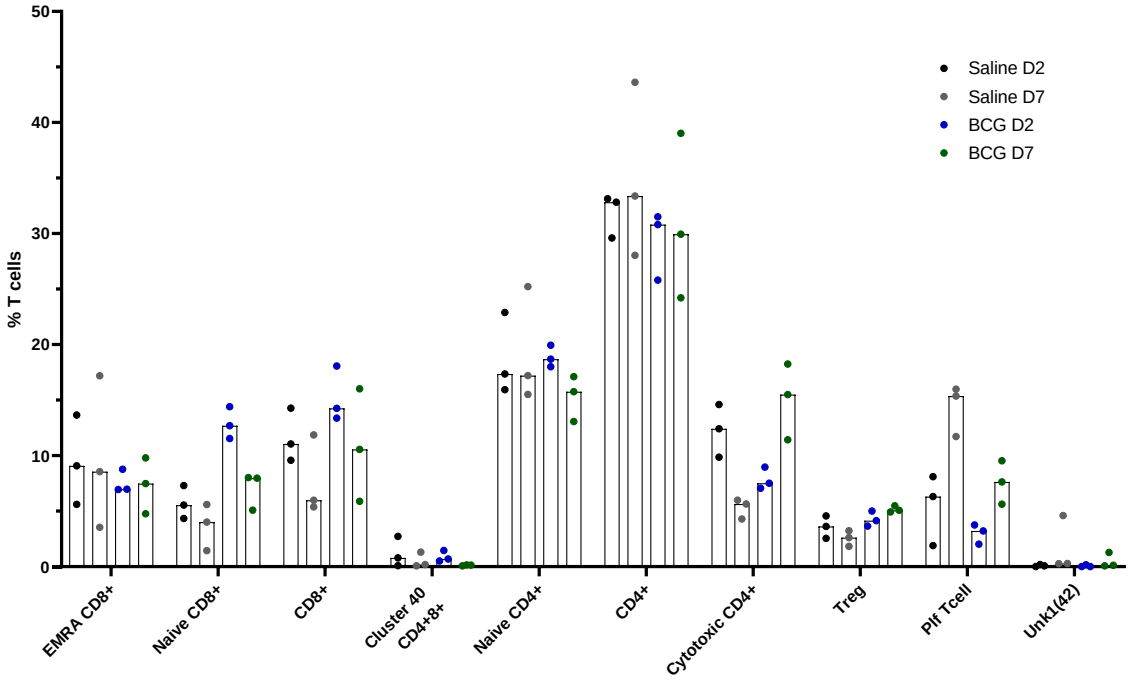


Figure 4.53. Percent of T cell subtypes by group. No statistical analysis due to small sample size. Bar: median. ”Plf T cell” proliferating T cell.

The AM to T cell ratio (as defined by 10x) showed a trend to decrease post-BCG compared with saline controls (Figure 4.54).

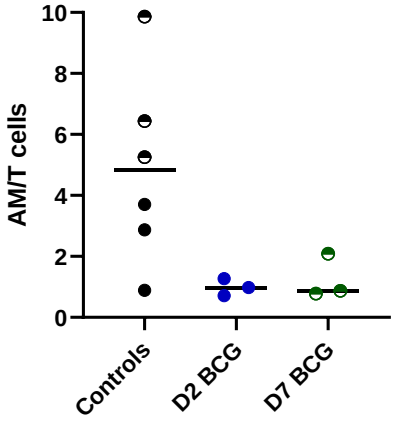


Figure 4.54. Ratio of AMs to T cells in the BAL fluid D2 or D7 post-BCG or saline (control) identified from 10x. Open circles show D7 samples, lines show median.

For DGE, I combined T cell subsets as follows. CD4+ T cells: “CtxCD4”, “CD4”, “NvCD4” and “Treg”. CD8+ T cells: “NvCD8”, “CD8” and EMRA CD8”. There was a difference in number of genes upregulated between saline controls and BCG volunteers at both D2 and D7 BAL in CD4+ T cells and CD8+ T cells, with the most difference seen in CD4+ T cells at D7 post-BCG compared with saline (Table 4.2).

Pathway enrichment analysis for CD4+ T cells is shown in Figure 4.55. At D2 and D7 post-BCG, CD4+ T cells increase expression of genes associated with cytokine signalling, T cell activation, IFN-α and IFN-β, *M.tb* phagocytosis, activation of nuclear factor-κB (NF-κB) pathways and also negative regulation of the immune system pathways. At D7 upregulated gene pathways also include migration (T cell migration, actin, leucocyte migration).

There were similar patterns seen with the CD8+ T cells, with upregulation of gene pathways at D2 and D7 post-BCG associated with Th1 cytokines (TNF-α and IL-12), Th17 (IL-21), T cell differentiation, negative regulation of the immune system and, additionally at D7, cell migration (Figure 4.56).

There was also evidence of γδ T cell interaction, possibly due to mixed cell types in Cluster 18. There was no upregulation of cytotoxic pathways detected in CD8+ T cells using this method of analysis.

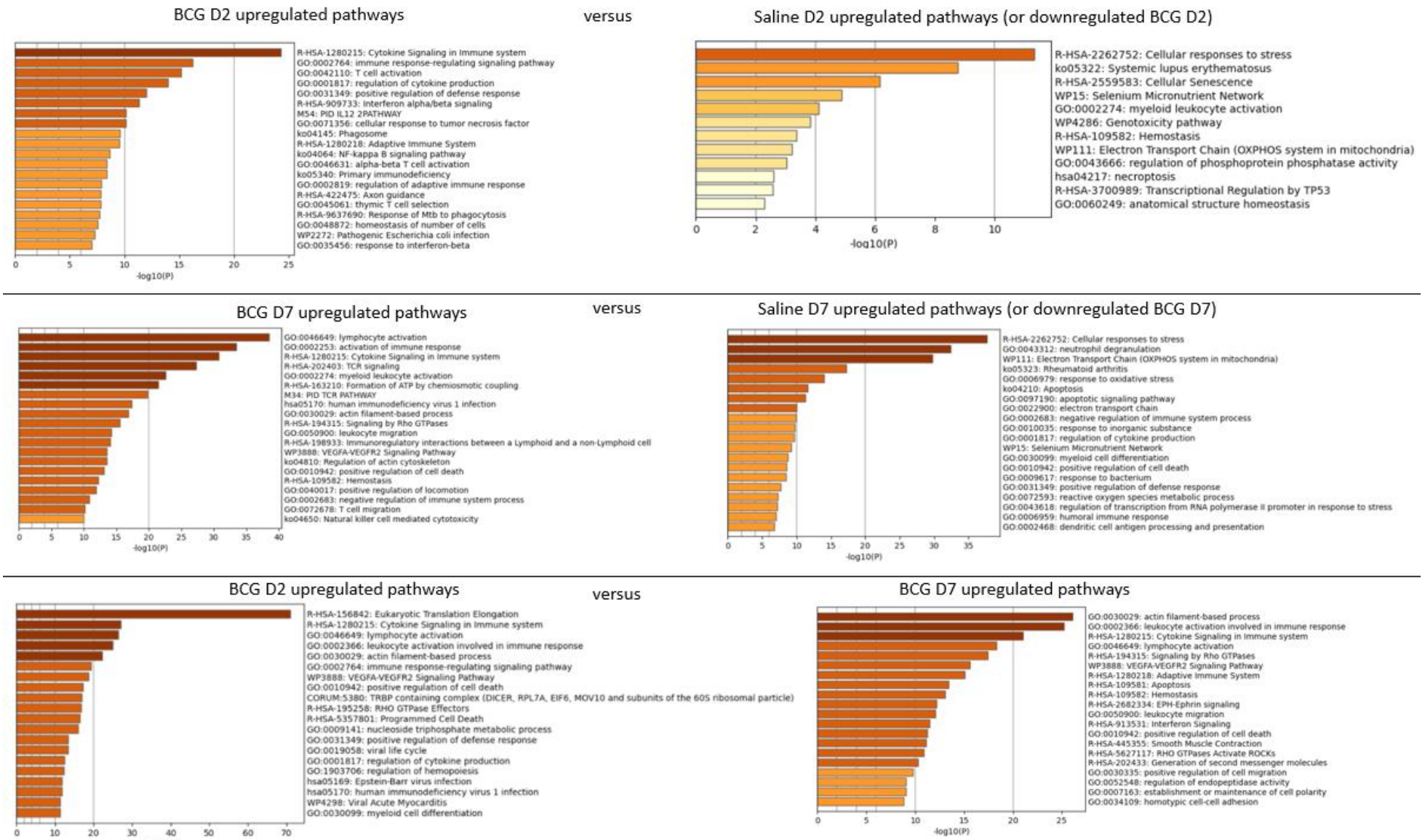


Figure 4.55. CD4+ T cell Metascape pathway enrichment analysis of DGE by group.

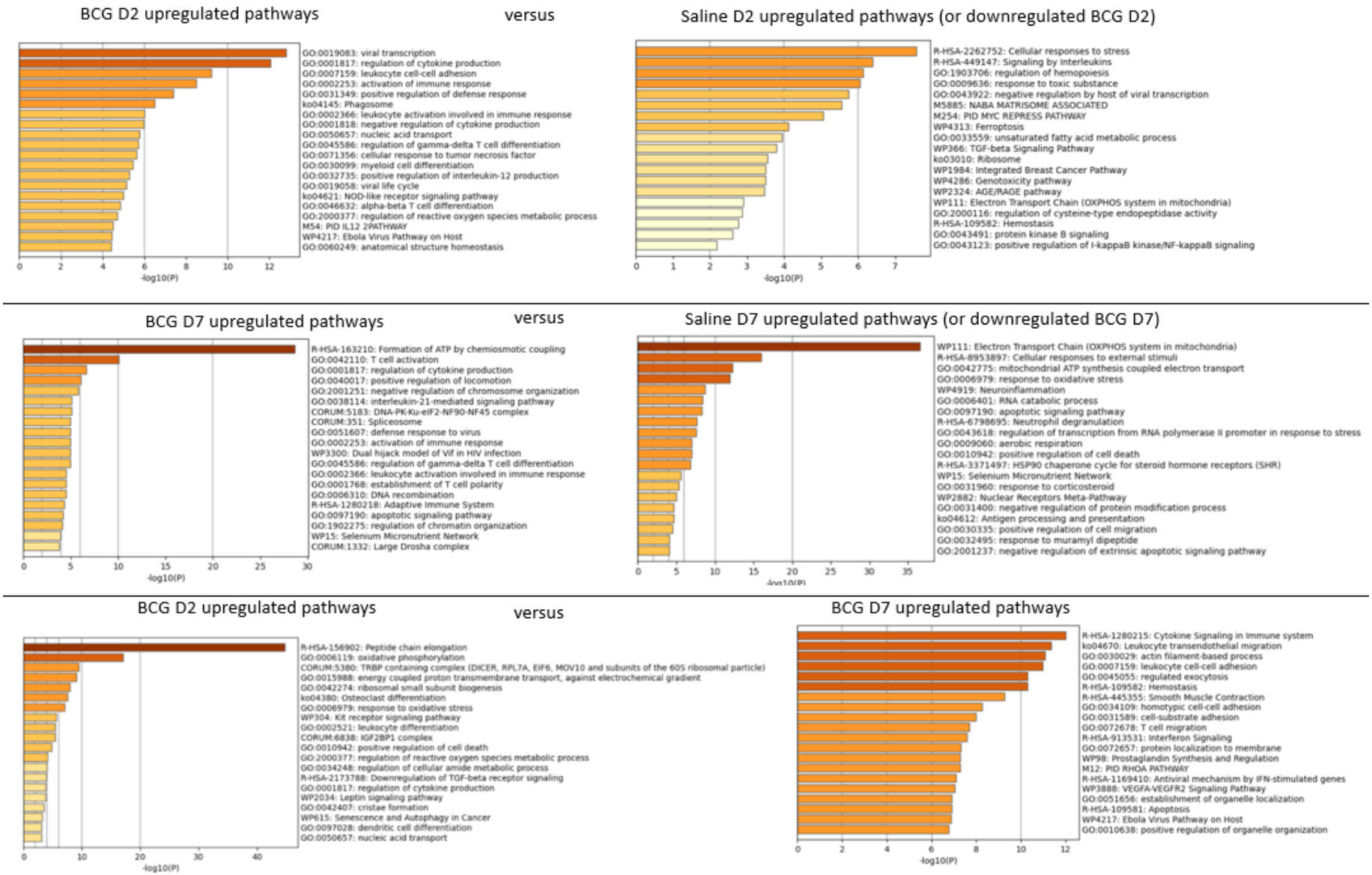


Figure 4.56. CD8+ T cell Metascape pathway enrichment analysis of DGE by group.

However, when looking at expression of the 100 pre-selected genes (Figure 4.23), BCG-exposed CD8+ T cells expressed a clear cytotoxic gene signature at both D2 and D7 compared to saline. In both CD4+ and CD8+ T cells there was an increase in the LN tracking marker CCR7 and an increase in the Th1 cytokine IL-16. By D7 there was an upregulation of lung homing genes, particularly in CD4+ T cells (ITGB1/CD29, ITGA4/CD49), CCL5, CXCR3, CXCR6). There was no increase in the lung residency marker CD103, and, in keeping with the DGE pathway analysis, there was a downregulation in the activation marker CD69. There was also a downregulation of the lung homing ligand CXCL16 at D7.
In the proliferative T cell cluster there was an increase in CD69, CD103, and lung homing integrin α4β7 at D2 and D7. This cluster also increased expression of TNF, IFN-γ and MHC-II and cytotoxic markers. More in depth analysis to define this cell cluster is warranted and will form part of future work.

I then confirmed the 10x DGE analysis using flow cytometry, showing that cells were not only upregulating genes related to Th1 cytokine pathways, but that IFN-γ was being produced in both CD4+ and CD8+ T cells post-BCG (Figure 4.57). Further, the source of this cytokine was from antigen specific cells. While no statistical analysis was performed due to the low volunteer numbers at D2, there was a trend for increased IFN-γ expression at D7 compared with D2 of antigen-specific cells, with no trend for *ex-vivo* unstimulated cells, despite the fact that the BAL fluid contained BCG, and therefore all cells would have been stimulated *in-vivo.*

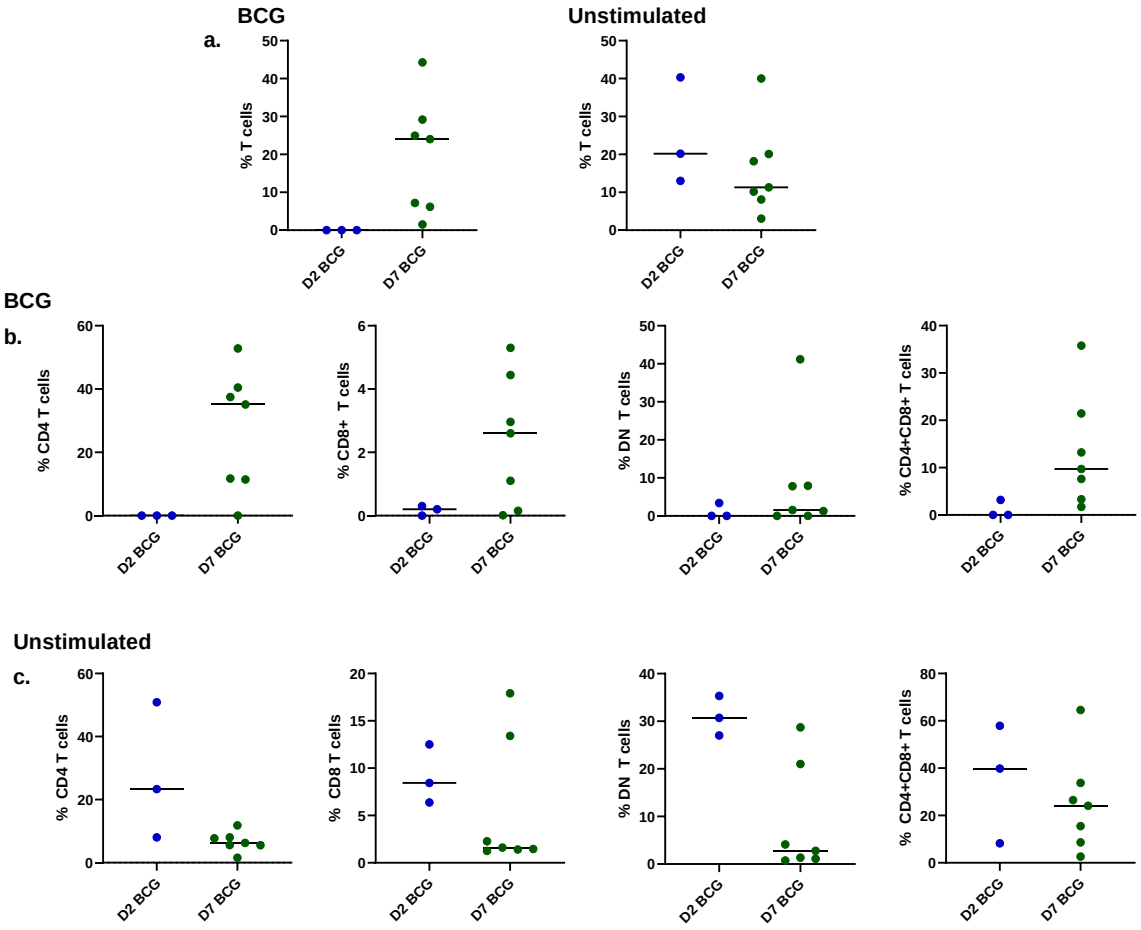


Figure 4.57. IFN-γ+ T cells on the homing flow cytometry panel. "BCG": overnight incubation with BCG, CD49d and CD29 calculated as IFN-γ+ cells minus IFN-γ from unstimulated cells) or "Unstimulated": Incubated overnight with CD49d and CD29 only. (a) Total T cells; (b) BCG T cell subsets or (c) Unstimulated T cell subsets. Line: Median. DN: Double negative.

BCG stimulated antigen specific total T cells, CD4+, CD8+ and DP T cells all increased production of IFN-γ at D7 post-BCG compared with D2 (Figure 4.57). There was only one high responder in the double negative population. A median of 35.1% (IQR 11.4-40.5) of CD4+ T cells were producing antigen specific IFN-γ at D7.

I stained two volunteer samples with the polycytokine flow cytometry panel (Figure 4.58), both D7 post-BCG. 8.7% monocytes produced IFN-γ.

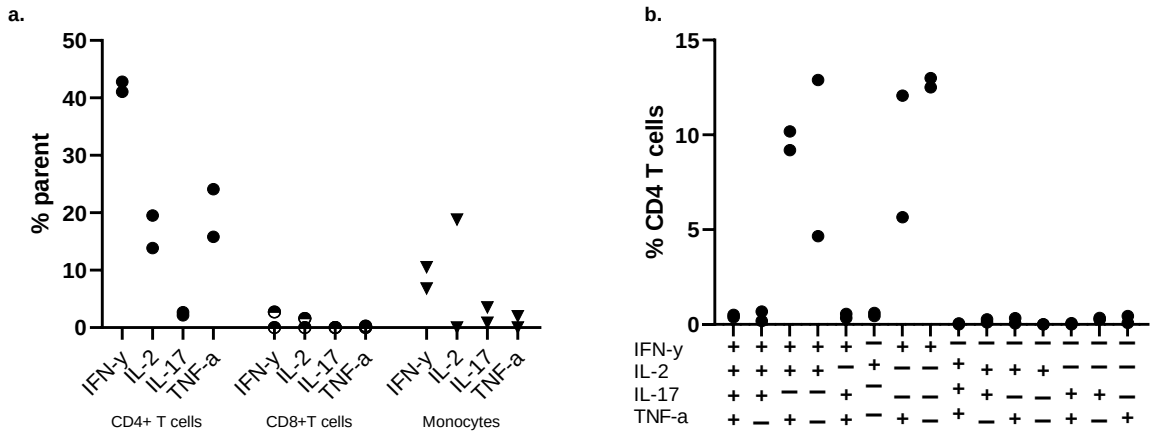


Figure 4.58. Antigen-specific cytokine producing cells using the polycytokine flow cytometry panel. (a) Cytokines by cell type, (b) polyfunctional CD4+ T cells. Two BCG infected D7 bronchoscopy volunteers. Monocytes defined as Live singlet, CD19- CD3- CD14+ cells. CD4+ T cells: Live singlet, CD19- CD3+ CD14- CD4+ CD8-; CD8+ T cells: Live singlet, CD19- CD3+ CD14- CD4- CD8+

CD4+ T cells, were the highest sources of antigen specific cytokines in the two D7 BCG volunteers. There was poor expression of IL-17 in these two volunteers, which is interesting given the upregulation of the IL-21 cytokine pathway at D7 in the Metascape pathway analysis, which is associated with Th17 polarisation (though IL-21 is also associated with Th2 and inhibition of Th1 polarisation).[589] Unfortunately there was no difference in expression of IL-17 RNA between cell types (hence it is missing from the gene expression Figure 4.23).

I then confirmed the increase in homing marker gene expression seen in the 10x by detecting protein expression using flow cytometry. I was able to determine that the upregulation of the homing markers CD29, CD49 and CXCR3, was in antigen-specific CD4+ T cells (Figure 4.59a), though this did not reach statistical significance. There was also a trend for CD4+ antigen specific T cells expressing proliferation and terminally differentiated, PD-1 and KLRG1[590] to increase in the airways D7 post-BCG, compared with D2. There was also a significant increase in CD103+ CD4+ T cells at D7 compared to D2 in unstimulated (*ex-vivo*) cells (Figure 4.59b) which was not detected in the unstimulated gene expression data.

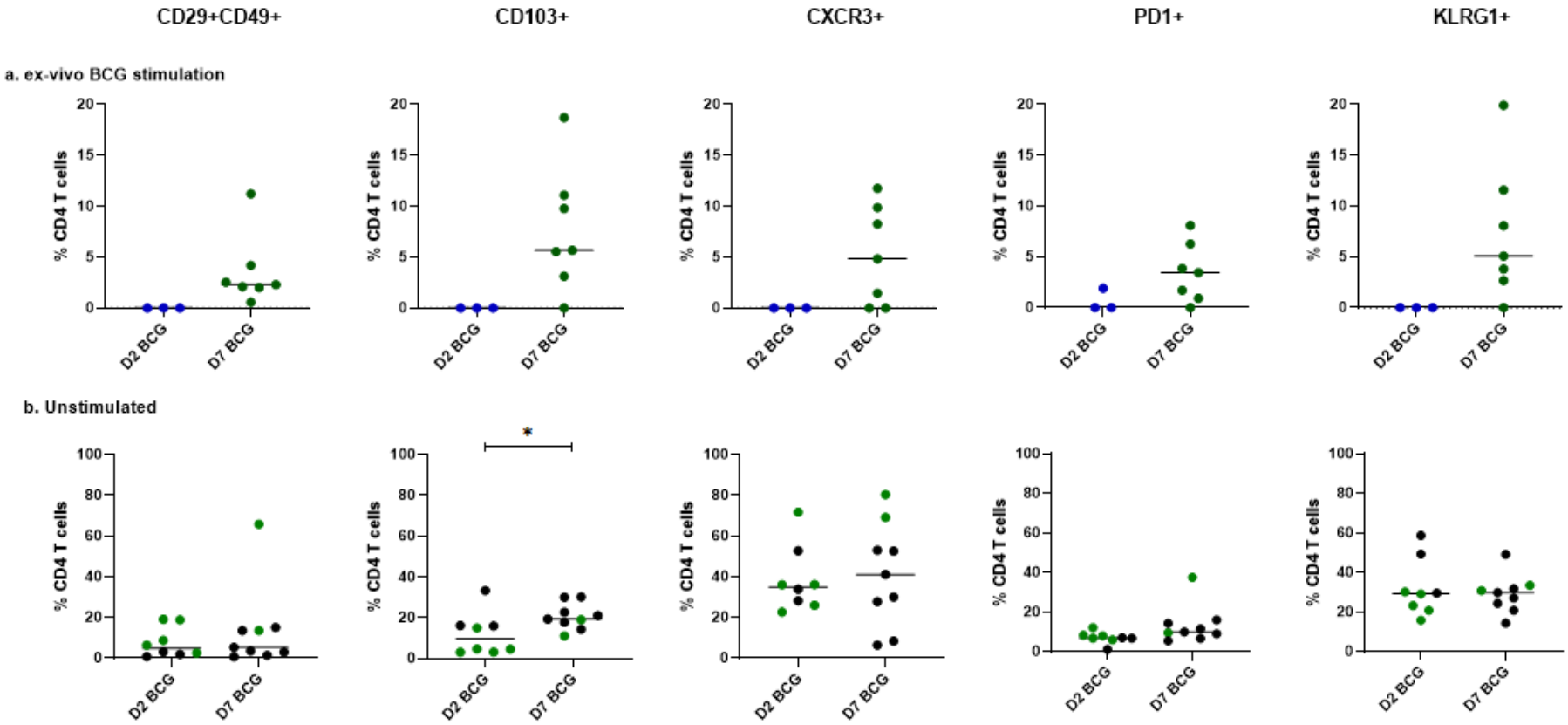


Figure 4.59. CD4+ T cell subsets from the homing flow cytometry panel. (a) Antigen-specific IFN-y+ CD4+ T cell subsets after overnight incubation with BCG, CD49d and CD29**.** Calculated as IFN-y+ cells minus IFN-y+ unstimulated cells. (b) Unstimulated CD4+ cells, stained same day (green) or incubated overnight with CD49d and CD29 and stained the following day (black). (a) No statistical analysis performed on plots due to low numbers of D2 BCG volunteers. (b) CD103+ unstimulated CD4+ T cells p= 0.046 on Mann Whitney after normality testing. All others not significant. Lines show media

There was no clear positive CD153 population (Figure 4.60).

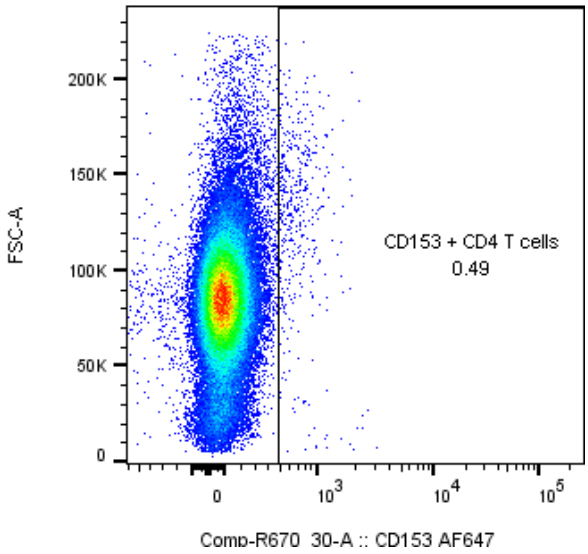


Figure 4.60. Representative plot showing CD153+ CD4+ T cells

I investigated the proportion of antigen specific double or triple positive PD1, KLRG1, CXCR3 CD4+ T cells. However, numbers were too small for any valuable interpretation.

There was a trend for CD8+ antigen specific T cells expressing the lung homing marker CD103 or exhaustion/maturation markers KLRG1 and PD-1 to increase in the airways D7 post-BCG compared with D2, but numbers were small (Figure 4.61). This expression was not seen in the 10x data.

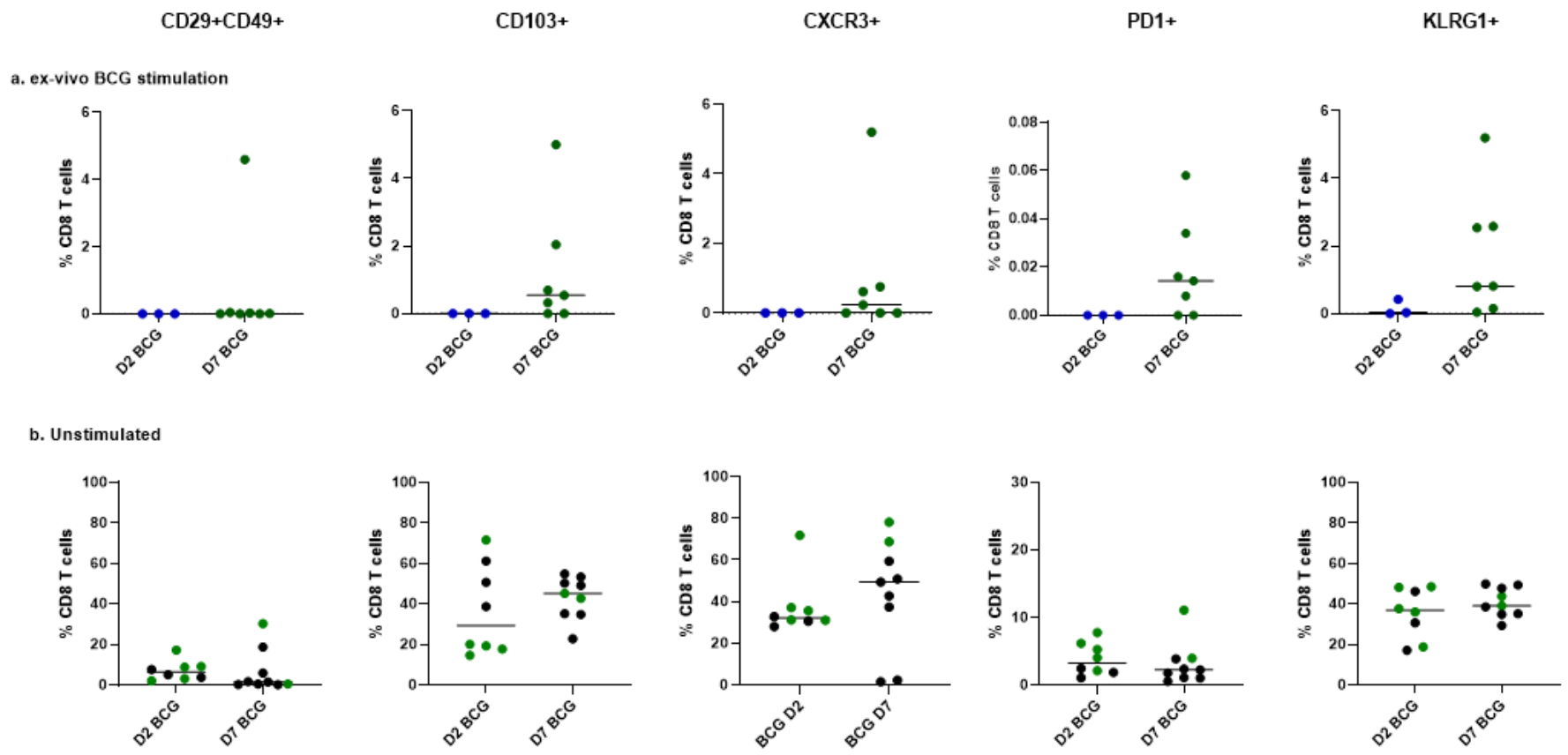


Figure 4.61. CD8+T cell subsets from the homing flow cytometry panel. (a) Antigen-specific IFN-y+ CD8+ T cell subsets after overnight incubation with BCG, CD49d and CD29**.** Calculated as IFN-y+ cells minus IFN-y+ unstimulated cells. (b) Unstimulated CD8+ cells, stained same day (green) or incubated overnight with CD49d and CD29 and stained the following day (black). (a) No statistical analysis performed on plots due to low numbers of D2 BCG volunteers. (b) nil significant on Mann Whitney after normality testing. Note different y-axis for PD1+ cells. Lines show median

4.3.9 Non-immune and B cells

Bronchial epithelial cells were identified on single cell sequencing 10x (Figure 4.62) and were present at a frequency of 0.6% total cells (IQR 0.36-0.82%). Metascape pathway enrichment analysis was unremarkable (data not shown) as was expression for 100 pre-selected genes (Figure 4.23).

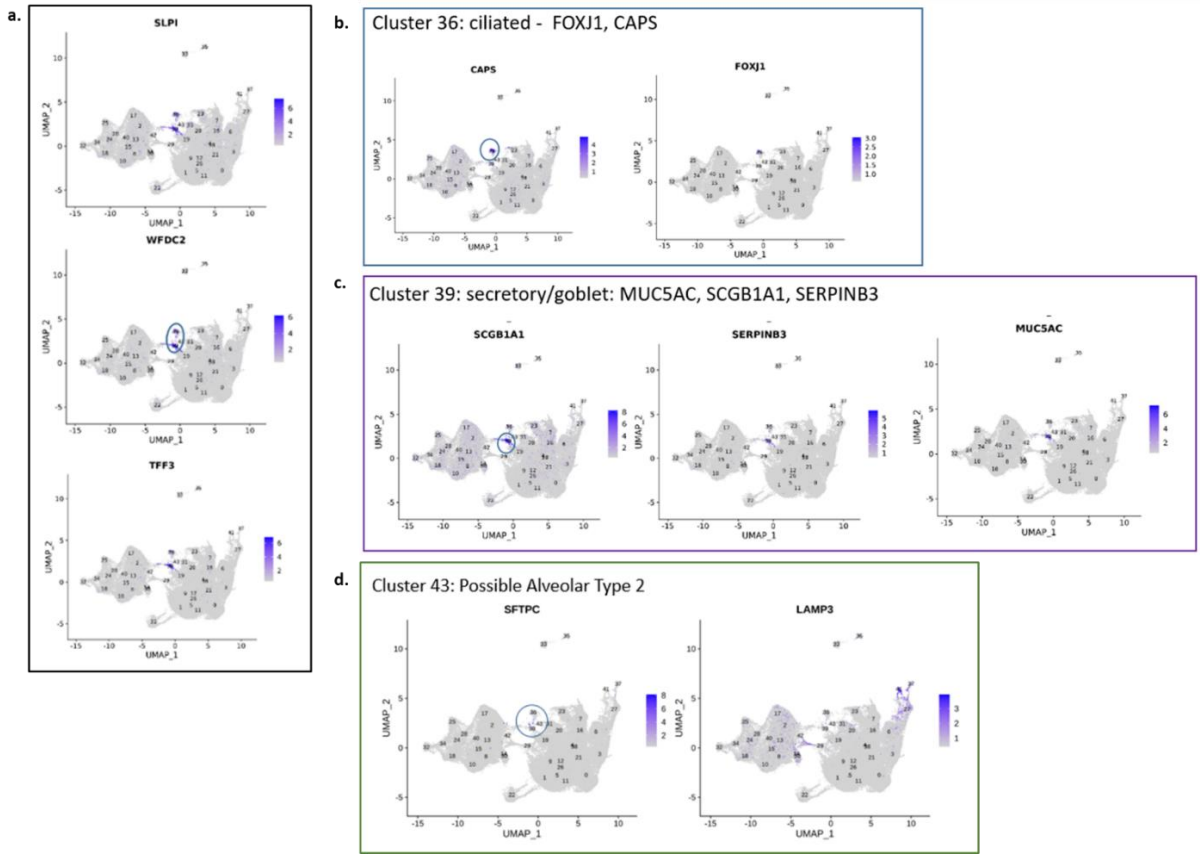


Figure 4.62. UMAP projection of canonical bronchial epithelial cell markers predicts Clusters 36 and 39. (a) Canonical bronchial epithelial cell markers (b) FOXJ1 and CAPS gene expression predicts Cluster 36 as ciliated bronchial epithelial cells. (c) MUC5AC, SCGB1A1 and SERPINB3 expression predicts cluster 39 as secretory and/or goblet cells. (d) Cluster 43 unknown, possibly Alveolar Type 2 cells due to increased gene expression in some cells for SFTPC (surfactant protein) but poor expression of LAMP3 which is expressed in Type II pneumocytes [507,508,512] Gene abbreviations (from the Genecard database):[584] "CAPS": Calcyphosine, "FOXJ1": Forkhead Box J1, "LAMP3": Lysosomal Associated Membrane Protein 3, "MUC5AC": Mucin 5AC Oligomeric Mucus/Gel-Forming, "SCGB1A1": Secretoglobin Family 1A Member 1, "SERPINB3": Serpin Family B Member 3, "SFTPC": Surfactant Protein C, "SLPI": Secretory Leukocyte Peptidase Inhibitor, "TFF3": Trefoil Factor 3, "WFDC2": WAP Four-Disulfide Core Domain 2.

While outside the scope of this thesis, I identified B cells by flow cytometry and 10x and present results here for completeness (Figure 4.63).

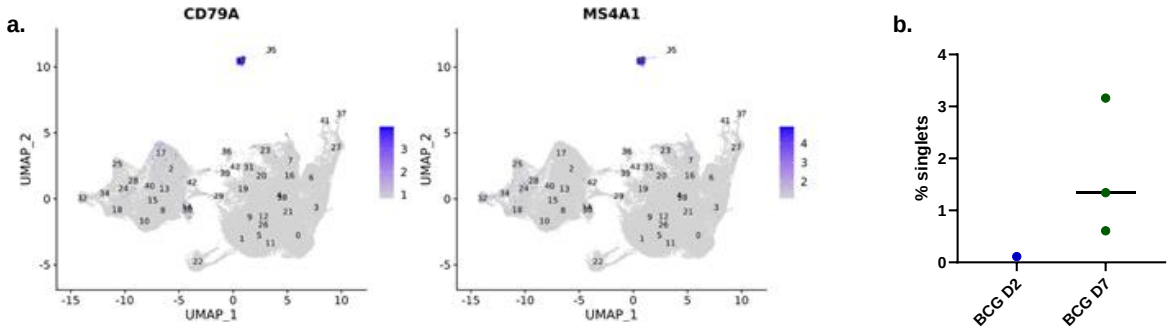


Figure 4.63. B cells. (a) UMAP projection predicts B cells as Cluster 33 using canonical markers CD79A (part of B cell receptor) and MS4A1 (CD20). (b) Frequency of B cells identified on the B cell flow cytometry panel defined by CD3-CD19+.

A selection of other cell types were investigated but not found in my cell set (Figure 4.64)

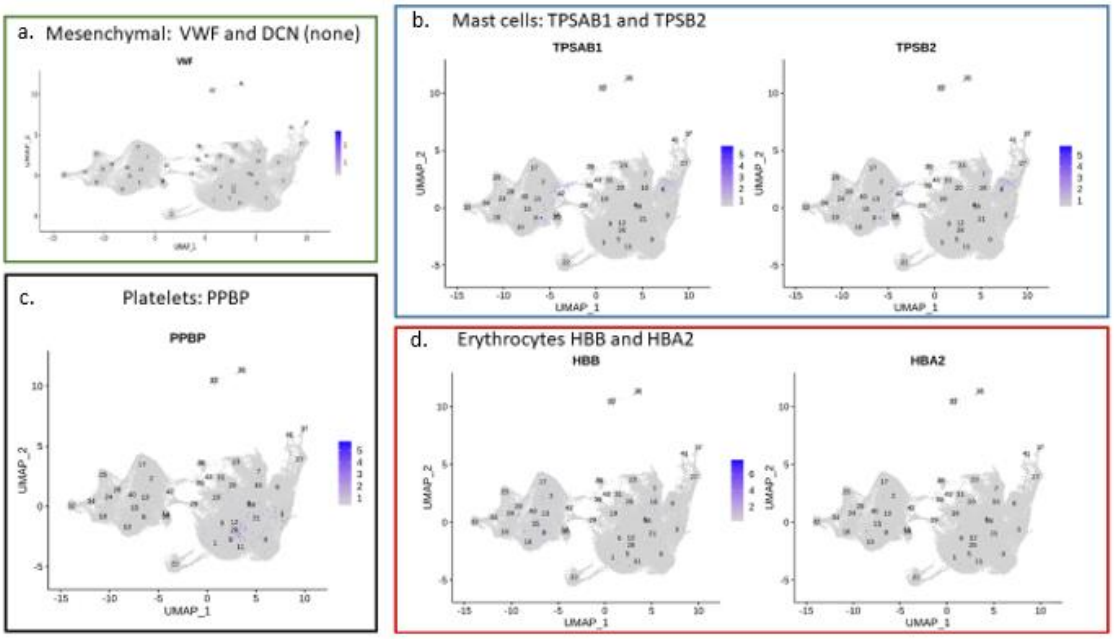


Figure 4.64. UMAP projection of canonical markers for (a) mesenchymal tissue, (b) mast cells, (c) platelets and (d) erythrocytes.[507] Gene abbreviations (from the Genecard database):[584] "VWF": von Willebrand factor, "DCN": Decorin, "TPSAB1": Tryptase Alpha/Beta 1, "TPSB2": Tryptase Beta 2, "PPBP": Pro-Platelet Basic Protein, "HBB": Hemoglobin Subunit Beta, "HBA2": Hemoglobin Subunit Alpha 2.

4.4 Discussion

As the airway and alveolar spaces are the first immune contact with the mycobacteria, and the gateway to the parenchyma, understanding these early local mucosal responses is important. Through phenotypic detection of proteins and glycoproteins using flow cytometry, antigen specific responses from ex-vivo overnight stimulation with BCG and transcriptomic analysis and DGE, I was able to detect and describe components of the innate and adaptive cellular immune response in the airway and alveolar spaces. I have demonstrated temporal changes in the local mucosal milieu and significant changes in gene expression following a mycobacterial aerosol infection.

As early as 2 days after aerosol infection in naïve volunteers, BCG induced significant increases in gene pathways associated with cytokine signalling, including interferon, NOD-like pathway, apoptosis, leucocyte activation and mycobacteria phagocytosis, and, by D7, leucocyte migration and adaptive immune system pathways. Further, there was a lymphocytosis in the airways at D7.

4.4.1 Cell counts

D2 BCG samples had more total cells and live cells than D7 BCG samples, per ml of BAL fluid. This may be biological. Dheda and Silver's groups saw an increase in total BAL cell counts at 2 or 3 days versus baseline following lobar PPD or BCG instillation but only in mycobacteria-exposed patients.[206,409]

Where possible, I used the flow cytometry results as the more accurate predictor of cell proportion changes in the lung compartment. Cells defined by gene expression may detect slightly different cell populations than those defined by protein and glycoprotein expression on flow cytometry, particularly where there is gene expression overlap such as

between CD8+ T cells and NK cells.[591] In addition, 10x samples only had 3 volunteers per condition and included both live and dead cells. D2 10x samples were also processed differently to D7 (CD3+ MACs beads) which means 10x cell counts between D2 and D7 may not have comparable.

Normal BAL composition consists of 85% macrophages (IQR 65.3–95.4) and 12% lymphocytes (IQR 3.0–32.4)[503] which is similar to the levels seen in the saline controls in my samples, as defined by 10x. Post-BCG I saw an influx of T cells at D7 compared with D2, defined by flow cytometry, to more than 40% of total BAL cells, with a corresponding fall in proportion of MPs. This was also reflected in the reduction in AM to lymphocyte ratio at D7 post-BCG (10x).

Lobar PPD or BCG instillation at D2 or D3 led to an influx of lymphocytes and reduction in AMs, in *M.tb* exposed patients but not naïve patients.[206,409] These changes were only seen at the highest BCG dose of $1x10^4$ cfu. My volunteers likely inhaled $1\text{-}5x10^5$ cfu. This would suggest, that while I did not have baseline or control samples in flow for comparison, that my results do indicate a true influx at D7, as opposed to a fall at D2 and return to baseline at D7 (though on 10x there are less obvious differences in T cell count between the two timepoints).

There was also a reduction in the AM:L ratio (defined by 10x only) post-BCG. In LTBI volunteers after PPD lobar installation, AMs increased 2.35-fold.[409] However, similar to what I observed, AMs decreased as a proportion from 88 ± 2% in control segments to 77 ± 7% in PPD-exposed patients, due to the dominant influx of lymphocytes.[409] A low AM:T cell ratio may be a marker of protection. In NHPs, following protective IV BCG vaccination, there was an influx of lymphocytes (largely classical αβ T cells) into the BAL with sustained low AM:T cell ratio at 6 months.[91] TB patients with less advanced disease had a higher Th1

lymphocytosis on BAL and lower macrophages and/or neutrophils than patients with advanced disease.[219]

Whether this local lymphocytosis and low AM:L ratio corresponds to an increase or decrease of the monocyte:lymphocyte ratio in the blood will be shown in the next chapter.

4.4.2 BAL versus parenchyma

Whether the cellular composition in the BAL fluid reflects the lung parenchyma is unclear. In mice, the proportion of mycobacteria-specific CD4+ T cells and resident memory cells was comparable between BAL and lung parenchyma and differed from lung vasculature.[391,458] However, at four weeks following aerosol BCG infection, frequency of PPD-specific cytokine producing CD4+ and CD8+ T cells was higher in the BAL than in the lung tissues of macaques.[91] Studies in humans seem to vary depending on the condition.[592,593] BAL fluid is unable to reflect local immune variation, as has been described between TB granulomas in humans.[594] Both Silver and Dheda demonstrated regional differences in immune responses in their BCG and PPD instillation studies in humans.[409,206] However, in one study of five HIV-uninfected TB patients, lung biopsy tissue from granulomas reflected similar lymphocyte subset frequencies to the BAL fluid.[595] As I delivered the BCG globally into the airway, and my study aims to capture the initial immune responses before any regional infection is established, it is possible that the cellular responses detected in this chapter reflect changes in the underlying parenchyma. A parallel study is underway in NHPs investigating responses in BAL, lung parenchyma and draining LNs, with the aim of comparing results from this human study across compartments. Further, work on the endobronchial IHC staining from TB043 is ongoing. Together, these will yield important insights into this question.

4.4.3 Saline controls in flow cytometry analysis

The analysis of my flow cytometry data was significantly impacted by the lack of saline control samples as a comparator. This was a result of chance, with all D7 saline volunteers randomised on the same day. The original study was designed for all 15 controls (three from each bronchoscopy timepoint) to be combined for analysis, if post-hoc analysis showed no significant difference between the samples. This was deemed likely, as there was no difference seen in cell composition following saline lobar instillation on bronchoscopy at D3 compared with baseline in Dheda's study[206] and hence I hypothesised that nebulised saline inhalation should not significantly alter the immune milieu. However, due to delays, the trial is still ongoing and so the other control groups (with bronchoscopy done at D14, D28 and D56) could not be included in this analysis. It was therefore not possible to conclude if the differences seen in the D2 and D7 saline samples were biological or due to technical error. As the D7 saline processing time was longer, the difference was likely technical. An error in staining is unlikely as a number of different panels were used over the course of two days. The 10x results show that D7 saline sample cell proportions correspond to saline controls in the literature.[567] As the cells sent for 10x were processed first, it is feasible that 10x D7 saline samples are reliable, but that the prolonged time to flow cytometry staining could have led to aberrant results. The reason for this delay in processing was due to logistical issues with our cell counter and insufficient staff to help in sample processing.

4.4.4 Combining homing panel samples

I combined homing panel samples which had been processed differently (same day stain versus overnight incubation), to increase power and sample size. While incubating cells

overnight shouldn't change cell composition, delay to cell staining and fixing could result in certain cell types that are more susceptible to cell death being under-represented. Similarly, some markers used to identify cell type, or activation, could be upregulated or downregulated during incubation, particularly if taken out of the *in-vivo* system which is driving that particular phenotype. While I detected no difference in marker detection between methods, there may have been differences only detectable in a larger sample size. Therefore, in all combined data I clearly outlined the different processing conditions between samples. In hindsight I should have processed all samples the same way. The homing surface stain samples should have been incubated overnight with CD49a and CD29, and stained the following day with the identical panel. We have made this change for the remaining TB043 groups.

4.4.5 Granulocytes

Granulocyte cell counts in the BAL samples were consistent with proportions described in human BAL.[503] Median neutrophil count at D7 was 5.8% (IQR 3.1-8.2), which might indicate a mild neutrophilia (defined as >4-5% total BAL cells).[503]

Given neutrophils are amongst the first responders, it is interesting that neutrophils increased in the airway at D7 compared with D2. I could not determine from flow cytometry if neutrophils had increased at D2 compared with baseline, though there was no increase in expression of the neutrophil chemokine CXCL8[219,220] at either timepoint from any cell type on 10x. In mycobacteria-exposed patients there was no change in neutrophil count in the BAL following PPD lobar instillation at D2 or D3, but there was an increase seen following BCG at D3 (defined as CD66b+NGAL+; BCG vaccinated, *M.tb* exposed patients).[206,409] A BCG ID vaccination site was characterised by a neutrophilia on suction blister analysis at D7 in humans.[480] It is unclear whether a neutrophilia is protective

against mycobacterial infection and it may depend on timing.[205,209,210,596] In a mouse *M.tb* aerosol challenge study, neutrophilia peaked just before peak DC migration.[207] Here I show that there was an increase in neutrophils and a decrease in DCs (potentially due to migration away to the LNs) at D7 compared with D2.

Eosinophils increased at D7 compared with D2, on flow cytometry. Levels were still within the normal range, with no overt eosinophilia (median 0.63% D7, IQR 0.45-0.88; normal range in BAL 0.1-3.5).[503] An early influx of BAL eosinophils following mycobacterial aerosol infection also occurs in NHP and guinea pig models.[226,225] I defined eosinophils on flow cytometry as Siglec8+, but this population may also include basophils, as a subset in BAL and blood express Siglec8.[597] Given eosinophil's association with granulomas,[225,598] their presence here early in infection is unclear.

I was unable to identify granulocytes using RNA-sequencing analysis. Granulocytes are difficult to capture on RNA-seq as they have low RNA content and high RNA inhibitory factors.[599] For this analysis, I used a lower than standard threshold for RNA detection[600] but granulocytes were still undetectable. Neutrophils are short lived and highly susceptible to degradation, particularly if placed on ice (part of the RNA processing).[601,602] This, combined with the increased friability of BAL cells, likely explains why granulocytes weren't detected on 10x. A focussed study using conditions optimised for BAL granulocytes, such as using RNase inhibitors, minimising processing time or optimising temperature, would be required.

4.4.6 Macrophages

There was a higher proportion of AMs in controls than following BCG infection, at both D2 and D7 by 10x. This may be a result of the influx of lymphocytes seen at these timepoints. Alternatively, it may reflect a "macrophage disappearance reaction", a loss of resident

macrophages due to death, tissue adherence or relocation,[603] such as infected AMs moving from the airway and alveoli into the parenchyma, or being destroyed by BCG infection. In their murine model, Cohen et al. demonstrated that airway resident AMs were the primary *M.tb* infection targets, and that only infected AMs migrated into the interstitium.[103] However, as this was dependent, in mice, on ESX-1 which BCG does not express,[485] it is unclear if the same process may be occurring here. BAL may contain cells derived from the airway and the parenchyma, further complicating the picture.

I identified three subsets on 10x, showing an influx of non-resident macrophages into the airways at D2 and D7 post-BCG, an increase in effector macrophages at D2. This is consistent with the known physiological response in animal models following an alveolar macrophage disappearance reaction, where the resident tissue population self-renews with macrophages upregulating effector function and is replenished with monocyte-derived macrophages.[172] An influx of non-resident AMs following PPD lobar instillation occurred in LTBI patients at D2, which were more effective at controlling intracellular *M.tb* growth *ex-vivo*.[604]

4.4.6.1 Effector function

DGE pathway results of D2 and D7 post-BCG AM effector function were consistent with the putative mechanisms of AM responses to aerosol mycobacterial infection in animal models; BCG phagocytosis, phagolysosome fusion, MHC-II processing, apoptosis; antigen presentation; Th1 polarisation and lung homing.[57,121,125-127] To my knowledge this is the first time these AM responses have been demonstrated following aerosol mycobacterial infection in humans. This first line defence occurs by D2. By contrast, naïve mice AMs do not upregulate effector function until 10 days following *M.tb* aerosol infection.[146]

CD11c gene expression increased in the AMs in my dataset at D2. In mice CD11c is used as a marker of *M.tb* infection.[224] Mycobacteria bind to CD11c to enter the AM, but increased expression also improves MPs ability to stimulate T cells.[605]

There was also increased IL-17RA gene expression at D2 post-BCG. IL-17RA activation inhibits apoptosis of BCG-infected AMs, prolonging mycobacterial survival.[606]

The increase in chemokine expression, such as CXCL9, suggests AMs are contributing to influx of non-resident macrophages, and other immune cells. BAL AMs derived from ID BCG adults produced more chemokines, such as CXCL9, expressed genes associated with IFN-y and lysosome pathways, and displayed enhanced *M.tb* growth control *in-vitro* than BCG-naïve infant BAL AMs.[607]

Upregulation of HLA-DR/MHC-II by AMs and other MPs is thought to be critical in *M.tb* defence by facilitating antigen presentation and activation of CD4+ T cells.[50,137,138] Vaccination with a recombinant adenoviral vector in mice, produced activated AMs with increased expression of MHC-II, which protected against aerosol *M.tb* infection in the absence of T cells.[144] AMs in my dataset upregulated genes associated with antigen processing, presentation and MHC-II presentation at D2 and D7 post-BCG. However, there was no difference in MP HLA-DR expression between D2 and D7 post-BCG on flow cytometry, nor between D2 and D7 post-BCG and saline controls in AM HLA-DRA gene expression on 10x. (Other HLA-DR subtypes were not detected in my data set). It may be that only a subset of cells are upregulating MHC-II. Mycobacteria evade the immune system by inhibiting MHC-II expression and antigen loading,[608] a putative mechanism here for some of the MPs/AMs in my samples. There is conflicting evidence for the protective effector function of AMs in animal models, where IFN-y+ AM aids CD4+ T cell activation and recruitment and is associated with protection,[398] and yet AM-depleted mice improved recruitment of cytotoxic cells leading to *M.tb* clearance.[145,399] This mixed picture is likely

because AMs are the preferred niche for mycobacteria, but also required for antigen presentation and adaptive immune system activation. Hence variable effector functions of AM and MP subsets may explain the mixed picture seen in my dataset.

4.4.6.2 Trained immunity

The nuclear factor-κB (NF-κB) pathway was downregulated and IL-1β gene expression upregulated at D2 post-BCG compared with saline. The NF-κB/NOD-receptor activation pathway then increased at D7. Both are associated with monocyte training and protection against mycobacterial infection.[122,299,609] Certain AM subsets may be upregulating or downregulating these pathways simultaneously. Other biomarkers associated with trained monocytes such as IL-6 and GM-CSF, were not upregulated in my dataset.[304,395,610,611]

4.4.7 DCs

The proportion of DCs reduced in the BAL at D7 post-BCG compared to D2. This would fit with the hypothesis that DCs migrate to the LNs for T cell priming.[181] Though DCs as % total singlets may fall simply due to an influx of lymphocytes into the compartment. Dheda's group saw no change in BAL DC proportion at D3 following BCG lobar instillation in mycobacteria-exposed patients (DCs were CD83+HLADR/DQ/DP+).[206]

cDC pathway enrichment analysis was consistent with DC mechanisms described in mycobacterial animal or *in-vitro* human infection studies.[178,181,183] cDCs exposed to aerosol BCG infection upregulated pathways responding to BCG cell wall-derived lipopolysaccharides and other antigens, through TLRs, phagocytosing, processing and upregulating antigen on MHC-II, and promotion of and response to Th1 cytokines. Follow-up flow cytometry work is required to confirm these upregulated genes are being translated to expression. In addition, at D2, cDCs increase expression of CXCL10, CXCL9, IL-

1β and IL-6, associated with early clearance and training.[395,404] However in a mouse model, *M.tb* infection of PBMC-derived DCs, led to IL-1β-induced Th2 polarisation, and worse bacterial control.[402] This effect was likely dependent on *M.tb* virulence factors RD-1/ESAT-6 however, as BCG infected cells nor RD-1 and ESAT-6 *M.tb* mutants did not induce this Th2 response. This is consistent with the data shown here where BCG exposed DCs express a Th1 rather than Th2 RNA phenotype. Associations with protection are explored in chapter 6.

The BCG exposed pDCs in my study display a cytotoxic phenotype. *Ex-vivo,* pDCs from human PBMCs cultured with BCG show upregulation of maturation markers and granzyme B in response to cDC activation.[404] pDCs in turn promoted cDC IL-1β production, (as seen in my study) which improved growth control.[404] In this *in-vitro* model by Lozza et al., pDCs did not produce IFN-y, however, my pathway enrichment results suggests pDCs are producing cytokines. These could be a different subset than the pDCs expressing GZMB.[612,613]

M.tb mouse challenge models display delayed DC tracking or poor DC antigen presentation (without affecting MHC-II expression),[48,192] neither of which seem to be occurring in my dataset, where DCs are upregulating genes associated with antigen presentation and migration by D7. Follow up studies are required to determine whether gene upregulation translates to actual antigen presentation. Molecules associated with DC activation in mouse models such as co-stimulatory CD86, CD209 (DC-SIGN) and inhibitory PDL1 were either not seen or not investigated in my study and warrant further investigation into DC effector function.[614]

4.4.8 NK cells

There was an enrichment of NK cells at D7 compared with D2 post-BCG. Despite this, NK cell counts at D7 were still within the normal range for healthy controls in BAL (median 3.47% of CD3- cells IQR 2.6-5.4; normal 4.5% range 1.8-12).[615,616]

The influx of NK cells is much sooner than in naïve mouse models where NK cells appear 10-15 days post-mycobacterial aerosol infection.[143,239] In the human lobar instillation studies, BAL NK cell count did not change after PPD instillation at D2 or D3 in mycobacteria-exposed or naive patients.[206,409,410] However, there was a trend for increased NK cells post- BCG instillation at D3 (mycobacteria-exposed patients).[206] There was no change in NK cell frequency in BAL following aerosol or IV BCG infection in NHPs, but the earliest timepoint investigated was from 2 weeks.[91]

NK cells in my samples produced antigen-specific IFN-y at both timepoints (highest at D7), and on gene expression, displayed a lung homing, Th1/Th17 cytokine and cytotoxic degranulation signature. The NK cell gene effector signature seen here is consistent with the known biological mechanisms of NK cells against TB,[230,231,241,244,248] however, this is the first time these functions have been demonstrated in humans immediately following an aerosol mycobacterial infection.

HLA-DR+ NK cells in my data set increased in the BAL at D2 compared to D7 on flow cytometry and in the gene expression data, compared to saline. *In-vitro*, HLA-DR+ NK cells derived from human PBMCs express CXCR3, have a degranulation effector function, expand in response to BCG and augment the BCG-specific IFN-y response.[617]

Whether the NK cell effector functions in my dataset are protective is unclear. NK cells from human PBMCs produce perforin, granulysin and IFN-y, and induce apoptosis of mycobacteria-infected cells.[230,231,618] In mice, aerosol *M.tb* expands IFN-y+ NK cell BAL populations, though depletion of these cells has variable impact on survival.[239,619] NK cells

producing IFN-y are increased in the lung of active TB patients.[235,240,245] Hence the implications of increased IFN-y+ NK cells in my dataset are unclear. Conversely, KLRB1 (CD161) was reduced at D2 post-BCG compared with saline on 10x in my samples. This pro-inflammatory subset is associated with a less favourable outcome in TB patients, suggesting there may be a protective effect in my dataset.[620,621]

The correlation between these NK cell effector functions and BCG growth control will be explored in Chapter 6.

Due to the inherent limitations in the number of markers available on flow cytometry panels, I was unable to fully characterise the NK cell subsets seen here. A further study, using CD56 and CD57 markers to define the common subsets, would be helpful and link these to the RNA effector mechanisms described.[228,229] Further work, confirming the cytotoxic gene expression signature phenotypically, and investigating antigen-specific homing markers in a larger sample size, is also warranted.

4.4.9 MAIT

Using the MR1 tetramer, and/or the classic MAIT phenotype markers, I demonstrated that MAIT cells could be identified in the BAL and, consistent with previous research in the blood, using the TEM with the CD161 marker identified a smaller subset.[249] I chose to identify MAITs using the TEM alone strategy to ensure all MAITs were identified.

There was a trend for MAITs to increase in the airway at D7 compared to D2, though this trend was lost when looking at total T cells, likely due to the increase in other T cells at the same time point. Numbers seen here were consistent with the expected 4% MAITs in BAL out of total T cells.[622]

DN MAITs were the predominant subtype at D2, with 43.3% total MAITs, which is consistent with previous reports of proportions in healthy humans (40-50% total MAITs).[623] However by D7 post-BCG, CD4+ and, to a lesser extent, CD8+ MAITs increased. In blood, CD4+ MAITs have reduced functional capacity (lower expression of CD69, reduced Th1 cytokine production and less cytotoxic potential than the other subsets) even after *in-vitro* stimulation.[624] By contrast, DN+ and CD8+ MAITs have reduced capacity to produce Th2 cytokines. DN MAITs are considered to be a terminally differentiated MAIT with less IFN-y, more IL-17 capacity and increased propensity to undergo apoptosis than CD8+ MAITs.[625] I was unable to explore the different effector functions between the subsets on flow cytometry due to low sample size. However, despite a large proportion of CD4+ MAITs in the BAL, there was a robust effector function on gene expression, though a downregulation of CD69 was noted.

Post-BCG, MAITs upregulated Th1 and Th17 polarisation and cytotoxicity pathways, and also upregulated some negative immune regulatory pathways. I also saw an indication on gene expression that MAITs might be directly attracting CD4+ T cells, through expression of IL-16.[588] All are consistent with known effector functions of MAITs.[249] In *ex-vivo* studies, human MAIT cells inhibit BCG growth within AMs in an MR1 and Th1 cytokine dependent manner.[249] MAITs also display direct cytolytic affect by lysing *M.tb* -infected cells, including bronchial epithelial cells *in-vivo* via perforin and granzyme.[105]

MAITs accumulate in the airway of mice from 10-15 days after intranasal BCG or *M.tb* infection and express tissue homing markers α4β1 and CXCR3.[253] The BAL of active TB patients are enriched for MAIT cells but these exhibit a different, blunted phenotype to that seen here, with less IFN-y, IL-17 and granzyme production and a higher expression of PD-1 compared with healthy controls.[257] However in another study of TB patients, pleura-

derived MAITs showed increased production of IFN-γ, IL-17 and granzyme, consistent with the MAIT gene expression data here.[459]

There was also a Th17 signature in the BAL MAITs in my study. Following intranasal treatment with an 5-OP-RU and TLR agonist, MAIT cells accumulated in the lungs of *M.tb* or BCG-infected mice.[626] These mouse MAITs produced IL-17A and low levels of IFN-γ and TNFα at D7-D14, and inhibited BCG but not *M.tb* growth.[626] The downregulation of CD69 seen in my data was also reported in exposed but uninfected household contacts of active TB patients, presumed early clearers. These subjects had an expansion of CD4+ MAIT cells in their blood, and MAITs with CD25 granzyme B upregulation and downregulation of CD69 and IFN-γ, though lung-derived MAITs were not investigated.[263] In an NHP model of aerosol infection with the CDC1551 low virulence *M.tb*, GranzymeB+IFN-γ+IL-17+TNFα+ MAITs at 3 weeks in the airway were associated with control to latency (as opposed to progression to active disease).[254] Further analysis of my gene expression data is warranted to detect co-expression patterns and to also confirm that gene expression is reflected in protein expression data.

4.4.10 iNKTs

There was a trend for iNKTs to increase in the airway D7 post-BCG compared with D2. I identified iNKTs using conventional Vα24$^+$/Vβ11$^+$ staining. I used the C15 Vα24 clone which binds regardless of the Jα gene.[627] There is no significant difference in the frequency of iNKT cells (or their subsets) detected in blood using Vα24C15$^+$/Vβ11$^+$, a more specific 6B11 clone (which binds Vα24-Jα18) or using the α-GalCer/PBS-57–CD1 TEM.[271,627,628] However there are rare subtypes of non-invariant or non-CD1d-restricted Vα24$^+$ cells which may be able to pair with Vβ11 and hence my population of iNKTs defined by Vα24/Vβ11 markers may also include some non-iNKT cells.[271]

I further identified a CD3+ CD56+ group of cells which I labelled here as NKT-like cells. These cells are likely CD1d restricted T cells with more diverse TCRs than the iNKTs.[628] They may also include activated classically restricted αβ T cells.[629] Conversely, not all iNKTs express CD56, but rather, CD56+ iNKTs may be a cytotoxic subset.[630] CD3+ CD56+ cells in my BAL samples also increased post-BCG at D7 compared with D2. Activated iNKTs have been shown to be enriched in the pleura of TB patients, however, there is a paucity of other evidence for lung mucosal iNKTs and the association with TB in humans.[278,631] My dataset is the first description of NKTs in the airways of humans immediately following a mycobacterial aerosol infection.

Further discussion of NKT cells will occur in Chapter 5.

4.4.11 γδ T cells

The proportion of T cells staining positive for γδ2 in the BAL at D2 are consistent with that described in healthy individuals and those with chronic infection,[632 633]

Median frequency at D2 post-BCG of "all" γδ2+ T cells was 6.2% CD3+ cells (IQR 5.2-14.2%) and γδ1+3 T cells were 2.35% (IQR 0.8-2.9%). This fits with the prevalence described in the literature, where non-γδ2 T cells should be 30% of γδ2 in BAL in healthy individuals and γδ2 T cells ~4-5% BAL CD3+ T cells in health and chronic lung disease (range 1-16%).[632,633] However "all" γδ2 T cells increased to a median 17.5% CD3+ T cells at D7, either indicating an acute physiological influx at this timepoint, or that the "all" γδ2 T cell population is detecting more than γδ2 T cells, as I didn't use γδpan co-staining.

I identified γδ2 T cells, but not γδ1+3 T cells, as a discrete cluster on 10x. Post-BCG these cells were expressing genes consistent with γδ2 mechanisms described in mouse and NHP models following *M.tb* infection, including Th1 polarisation, cytotoxic pathways and lung homing markers.[289,292] In addition, there was a trend for increased antigen-specific IFN-γ+

γδ2 at D7 compared with D2 on flow cytometry. *In-vivo*, γδ2 T cells recognise mycobacteria-infected DCs and induce their maturation through Th1 cytokine production.[634,635] *M.tb*-infected DCs in turn rapidly activate γδ2 T cells. Cytotoxic γδ2 T cells kill extracellular and intracellular mycobacteria *in-vivo* through granulysin and perforin.[634]

However, γδ2 T cells in my dataset also increased gene expression of Type 1 interferon pathways post-BCG, which are associated with poor outcome.[218,358] In addition, there was no Th17 gene expression signature detected. In murine models, IL-17+ γδ T cells rapidly track to lung following *M.tb* aerosol infection and Th17 cytokines are associated with protection.[289,337] Though whether IL-17+ γδ T cells are protective is unclear, as circulating IL-17+ γδ T cells are increased in active TB patients.[292,294]

The increase in the immune regulatory signature at D7 is also interesting. Whether downregulation of immune mechanisms as early as D7 is a result of BCG manipulation of γδ2 T cells to enhance intracellular survival in MPs or whether it has a protective local anti-inflammatory role warrants further investigation. [345,347]

4.4.12 T cells

Here I show that following an aerosol BCG infection, there is a lymphocytosis into the airways with an influx of both CD4 and CD8+ T cells by D7, with CD4+ T cells accounting for the majority of T cells. This is consistent with the CD4+ T cell lymphocytosis seen in both mouse and NHP studies following BCG or *M.tb* aerosol infection[391,422,423,460] and fits with the understanding in the field of the primary importance of CD4+ T cells for protection against mycobacterial infection.[316,317,636] However in protective C57BL/6 mouse models, influx of T cells into the BAL is not seen until at least 10 days following infection, and up to

3 weeks in susceptible strains,[637] and in NHPs this can be as late as 5-6 weeks,[422,423] which is much later than seen here.

In the human lobar instillation studies in mycobacteria-exposed patients, total BAL cells contained 19% T cells D2 post-PPD (compared with 10% post-saline), and 40-60% T cells at D3 post-PPD or BCG instillation.[206,409] In my naïve volunteers, post-BCG, total BAL cells contained 8.76% T cells at D2 (IQR 4.6-10.8%), with an increase to 37.19% by D7 (46.6-55.8% IQR, defined by the innate panel). This might indicate a slight delay in T cell influx into the mucosa compared to these mycobacteria-primed patients, though bronchoscopy at D3 would be needed to confirm this.

4.4.12.1 Homing and residency

At D7 T cells were expressing migration gene pathways, and upregulation of the central residency LN homing marker CCR7, and lung homing markers CCL4 and CCL5.

While there is no definitive phenotype for human lung tissue resident CD4+ T cells, a number of markers associated with lung homing and residency have been investigated in relation to protection against *M.tb*.[638] CD4+ T cells upregulated gene expression of lung homing markers α4β1 (CD29/CD49d), CXCR3 and CXCR6. I demonstrated that at least for some of these markers the source of expression was from antigen-specific CD4+ T cells (α4β1 and CXCR3).

α4β1 binds to VCAM, and homes to inflammation sites, and has been shown to be important in recruitment of *M.tb*-specific Th1 cells the lung.[410,639] In mycobacteria-primed patients following PPD lobar instillation, 83% of PPD-specific IFN-γ+ CD4+ T cells expressed this lung homing marker.[410] Only 5.37% of the BCG-specific CD4 cells in my study expressed this integrin at D7 (IQR 2.23-14.35).

CXCR3 is an important chemokine receptor, upregulated in activated Th1 cells and associated with protection in TB.[640] In mice, CXCR3+ CD4+ T cells migrate into the lung and suppress *M.tb* growth.[641] Lymphocytes expressing CXCR3 migrate down IFN-y-induced chemokine gradients CXCL9, CXCL10 and CXCL11, produced by bronchoepithelial cells and MPs.[640,642] $CD14^{dim}$ cells from healthy contacts were associated with the ability to control BCG in an MGIA, which was reliant on CXCL10/CXCR3 homing and dependent on CD4+ TCMs. This suggests that CD4+ TCMs were involved in a clearing immune response.[395] There was an upregulation of CXCL10 and CXCL9 seen in the AM and cDC populations at D2 in my dataset.

Recently it has been shown in mouse models that the correlation of antigen-specific CD4+ T cells with protection relates to the ability of CD4+ T cells to enter the parenchyma, rather than to produce IFN-y.[643] The source of the influx of CD4+ T cells in the BAL following intranasal BCG infection, is reflective of tissue resident cells in the parenchyma as opposed to a transient circulating CD4+ T cells from lung vasculature.[458] These cells are antigen-specific CD4+ T cells co-expressing CXCR3 and PD-1 with reduced KLRG1 expression and are associated with protection in this model.[458,467,644] In my antigen-specific CD4+ T cell population, I saw a trend for an increase in both PD1 and KLRG1. PD1+ CD4+ T cells are less differentiated cells capable of proliferating and exhibit greater protection than PD1-KLRG1+ cells, which are thought to represent terminally differentiated, short lived cells which lack the ability to migrate into the lung parenchyma (though these cells do still produce IFN-y and TNFα).[644] It may be that I am detecting two separate cell populations and that the cells in the BAL fluid reflect cells from both the lung parenchyma and the lung vasculature. Though, of note, in mice, PD-1- KLRG1+ subsets were also found in the parenchyma following IN BCG administration.[458] Alternatively, human effector resident T cells may have a different phenotype to that seen in mice. A further explanation could be

the trend for an increase at D7 may have been confounded by a low sample size and I might see a different pattern with larger numbers.

CD69+ CD103+ T cells are also thought to represent a lung resident population.[308] Aerosol BCG infection in NHPs leads to more CD69+CD103+ T cells in the BAL and lung tissue than in the periphery.[91] However, in my cells, while I detected a trend for an increase in antigen-specific CD103+ cells, on gene expression analysis, cells downregulated CD69. The exception to this was a proliferating, ill-defined T cell subset. Unfortunately, due to panel size limitations I did not include CD69 on my flow cytometry panels. Further, when defining my T cell populations by 10x, I didn't see a clear tissue residency signature, though certain canonical markers used to define these cell populations on flow cytometry don't translate to single cell sequencing results.[308,591]

Taken together, the expansion of the T cell population, upregulation of homing markers and some tissue residency markers, as well as migratory gene pathway signatures indicates that T cells, particularly CD4+ T cells, are migrating into the airway, and possibly also the parenchyma, by 7 days following BCG infection in my volunteers. This is likely facilitated by IFN-y expression and chemokine secretion from AMs and other MPs.

4.4.12.2 Effector function and IFN-y

Post-BCG, the CD4+ and CD8+ T cells in my dataset were upregulating genes associated with Th1 polarisation and cytotoxicity pathways. These effector functions are in keeping with the putative immune mechanisms following mycobacterial infection defined by animal models and *in-vitro* human studies.[378,379,381,382]

On flow cytometry at D7 post-BCG there was a large proportion of BCG-specific IFN-y+ CD4+ T cells (35.1%; IQR 11.4-40.5). This was much higher than frequencies seen following

PPD instillation in mycobacteria-exposed volunteers, with less than 5% antigen-specific IFN-y+ CD4+ T cells at D3.[206] Frequency of CCR6+ IL-17+ PPD-specific CD4+T cells fell at the same timepoint in that study.[206] In NHPs, giving the same loaded aerosol BCG dose as in TB043 led to PPD-specific IFN-y+ CD4+ and CD8+ T cells in the BAL of ~15% and 5% respectively but not until 13 weeks post-BCG.[426] In B6 resistant mice, *M.tb* aerosol infection leads to an influx of IFN-y+ CD4+ T cells by D14, with peak of 69% IFN-y+ CD4+ T cells at D21.[637] This is a stronger and earlier response than in susceptible mice. IFN-y, especially from circulating CD4+ T cells, is an important player in *M.tb* protection in humans and mouse models,[360,362,372,645] but whether mucosal IFN-y+ CD4+ T cell responses are protective in humans is unclear. BAL from *M.tb* infected patients are enriched for IFN-y+ antigen specific CD4+ T cells though most of these also produced IL-10, suggesting a negative correlation with protection.[439] However in another study, TB patients switched to a higher IFN-y producing, Th1 CD4+ T cell signature in the BAL following treatment.[219]
I saw lower proportions of CD8+ T cells in the BAL and they produced less IFN-y then CD4+ T cells (2.6% BCG-specific IFN-y+ CD8+ T cells at D7 IQR 0.2-4.4%). While DCs have been shown to cross-present mycobacterial antigen to CD8+ T cells in human *in-vitro* models,[646] as MPs are the preferred niched for mycobacteria, BCG likely evades most loading onto MHC-I and may be a poor inducer of CD8+ T cells. Further, the role, if any, of CD8+ T cells in the early control of infection is much debated.[383,385,388] However the fact that here we see an early cytotoxic and Th1 effector response from CD8+ T cells following aerosol BCG infection is worthy of further investigation.

4.4.12.3 Cytotoxic HLA-DR+ CD4+ T cells

On *in-vitro* stimulation with *M.tb*, human CD4+ T cells upregulate granzymes and perforin and lyse *M.tb, and M.tb*-specific CD4+ T cells derived from the circulation of IGRA positive patients, demonstrating antigen-specific cytotoxic potential.[374,647,648] This is in keeping with the gene expression data I saw here. However, this effector function may be associated with worse control of mycobacterial growth. HLA-DR+ T cells from the BAL of active TB patients exhibit a cytotoxic phenotype and CD4+ HLA-DR+ T cells in the periphery have been correlated with an increased risk of developing TB disease in infants and adolescents.[372-374] HLA-DR+ T cells increased at D7 compared with D2 in my dataset. Conversely, TRMs which have been associated with protection have an activated and multifunctional phenotype, including HLA-DR in NHPs.[91] In my study I was unable to determine which T cell subsets were responsible for upregulating HLA-DR and this warrants further investigation.

4.4.12.4 DP T cells

A few volunteers had a large proportion of double positive T cells (DPs), especially D7 post-BCG. DPs are an understudied T cell populations which produce pro-inflammatory and anti-inflammatory cytokines and have cytolytic effector function.[649,650] They are believed to be an activated MHC-II CD4+ T cell population that have acquired the expression of CD8.[649,650] In humans they make up 1-6% of T cells in the blood.[650] Here I show that DPs accounted for 6.37% (IQR 2.2-20) of BAL T cells (with one volunteer having 34.9% DPs), and that they produce antigen-specific IFN-y by D7 post-BCG. In NHPs, DPs increased in both blood and airways after *M.tb* aerosol infection, with airway frequencies positively associated with *M.tb* control.[651] These NHP cells produced more IL-17 and IL-10 compared

to either CD4+ and CD8+ T cells and more Th1 cytokines than classic CD8+ T cells.[651] As the high DP volunteers samples were not processed for 10x scRNA-seq analysis I was unable to definitively identify this cell population on single cell sequencing. Further analysis of this population and its role in *M.tb* infection is warranted.

4.4.12.5 Th17 responses

Dijikman and colleagues showed increased polyfunctional Th17 T cells (IL-17, IL-2, TNFα and IFN-y positive) 8 weeks following intratracheal administration of $5x10^5$ cfu BCG Intervax to NHPs.[304] In my samples, there was no definitive upregulation of the Th17 gene expression pathway. Flow cytometry IL-17 expression was poor in the two D7 post-BCG volunteer samples. This is in keeping with our previous aerosol MVA-85A viral vector studies.[386,387] BCG is thought to be a poor inducer of IL-17 compared with *M.tb*, possibly due to the lack of the RD1-encoded ESAT-6 protein, which promotes a protective Th17 response in mice.[652]

On gene expression data, I saw mixed Th1 and cytotoxic CD4+ T cell profiles alongside an increase in negative regulation. For this initial exploratory analysis, I combined T cell subsets for the DGE analysis. However, these subsets have different effector phenotypes, so further planned work analysing DGE by subset may shed further light on specific effector functions.

4.5 Conclusions

- Aerosol BCG infection led to an influx of granulocytes, a lymphocytosis and reduction in AM/L ratio in the airways and alveolar spaces by D7. This is earlier than predicted from animal models.
- There was an enrichment of lung homing cytotoxic NK cells capable of producing antigen-specific IFN-y, which is earlier than predicted by animal models.
- The proportion of total alveolar macrophages (defined by RNA expression) in the BAL decreased following aerosol BCG, which is possibly due to cell death induced by BCG infection. There was an influx of non-resident macrophages.
- There was a reduction in DCs in the airway and expression of genes associated with migration at D7 compared with D2. This is consistent with mechanisms from animal models where mature antigen-primed DCs migrate out of the airways to draining LNs.
- There was a trend for CD4+ and CD8+ MAITs to accumulate in the airway by D7. These cells had a cytotoxic phenotype, and exhibited Th1 and Th17 effector function, which may indicate MAITs play a role in BCG control. However, MAITs also increased negative immune regulation gene expression.
- There was an influx of T cells, particularly CD4+ but also CD8+ T cells, into the BAL at D7, characterised by homing markers and a Th1 cytokine profile. T cells had upregulated a Th1 gene expression phenotype on D2 post-BCG, and CD8+ T cells were expressing genes consistent with a cytotoxic signature.

- Antigen-specific T cells exposed to BCG produced more IFN-y at D7 than at D2, with 35% of all antigen-specific CD4+ T cells taken from the BAL producing IFN-y. This is consistent with the significant role antigen-specific Th1 CD4+ T cells are predicted to play in mycobacterial control. This is the first time these responses have been described following an aerosol mycobacterial infection in *M.tb* naïve humans.

A summary of the novel findings in this chapter are outlined in Chapter 7, Table 7.1.

Chapter 5.

Systemic immune responses

5 Systemic Immune Responses

5.1 Introduction

Peripheral blood immune responses have been well described in mycobacterial vaccine and field studies due to the relative ease of access. How relevant these responses are to control of an aerosol mycobacterial infection, particularly in early clearance, is unclear.[653] Further, the relationship between mycobacterial systemic responses and immune mechanisms at the site of infection is not well described in humans.[50,471] In this chapter I report the innate and systemic cellular peripheral blood responses following BCG aerosol infection and discuss how they relate to the early mucosal responses described in the previous chapter. ELISpot and flow cytometry assays (phenotype staining and overnight BCG stimulation for intracellular cytokine staining) and have been used to describe cellular immune responses.

5.2 Statement of Authorship

All assays were developed by J. Marshall unless otherwise stated. Blood processing and ELISpot was performed by J.Marshall, S.Harris and R.Wittenberg. All ELISpot counting and analysis was performed by J.Marshall. Additional hands were provided on PBMC thawing days as required by S.Harris, R.Wittenberg, R.Tanner, E.Hoogkamer and H.Morrison. J.Marshall performed all flow cytometry staining and analysis.

5.3 Results

Due to the large inter-individual variability on flow cytometry, only data from BCG aerosol volunteers, and not saline controls, is presented. In addition, as I had baseline samples available for all blood samples, this allowed me the power to do a paired analysis and compare responses within an individual over time. Group 1 and Group 2 volunteers have been combined as all volunteers inhaled the same dose of BCG. Paired analysis was performed where possible, and due to the multiple timepoints included, analysis has also been corrected for multiple comparisons. Volunteers in Group 1 and 2 had a bronchoscopy at different timepoints (either D2 or D7) where BCG was removed and where there may be a non-specific systemic response due to the bronchoscopic procedure. Therefore, for every analysis, prior to combining groups, I compared groups 1 and 2 at each timepoint. Significant results between groups are presented where relevant.

Table 5.1. Baseline counts, Group 1 (G1) and Group 2 (G2) BCG volunteers combined as % leucocytes. There was no difference between G1 and G2 at any timepoint

	Baseline mean %	95% Confidence Interval
Neutrophils	58.11	54.95-61.28
Eosinophils	5.12	4.67-5.58
Total MPs	9.91	9.04-10.78
Monocytes	8.23	8.02-9.64
DCs	4.23	3.79-4.67
NK cells	3.73	3.04-4.42
iNKTs*	0.03 (median)	0.01-0.05 (IQR)
MAITs*	1.13 (median)	0.67-1.72 (IQR)
All γδ T cells	1.56 (median)	0.80-2.01 (IQR)
Total T cells	19.68	17.36-22.00
CD161+ T cells*	2.27	1.69-2.84

*% Peripheral Blood Mononuclear Cells (PBMCs) not leucocytes

5.3.1 Granulocytes

Using flow cytometry, I identified neutrophils and eosinophils, which had a mean baseline percentage of 58.11% and 5.12% total live leucocytes respectively (Table 5.1). Following BCG aerosol infection, circulating neutrophils fell from D14 onwards (Figure 5.1). There was no significant difference in proportion of eosinophils over time. There was no difference between groups 1 or 2 at any timepoint for either cell type.

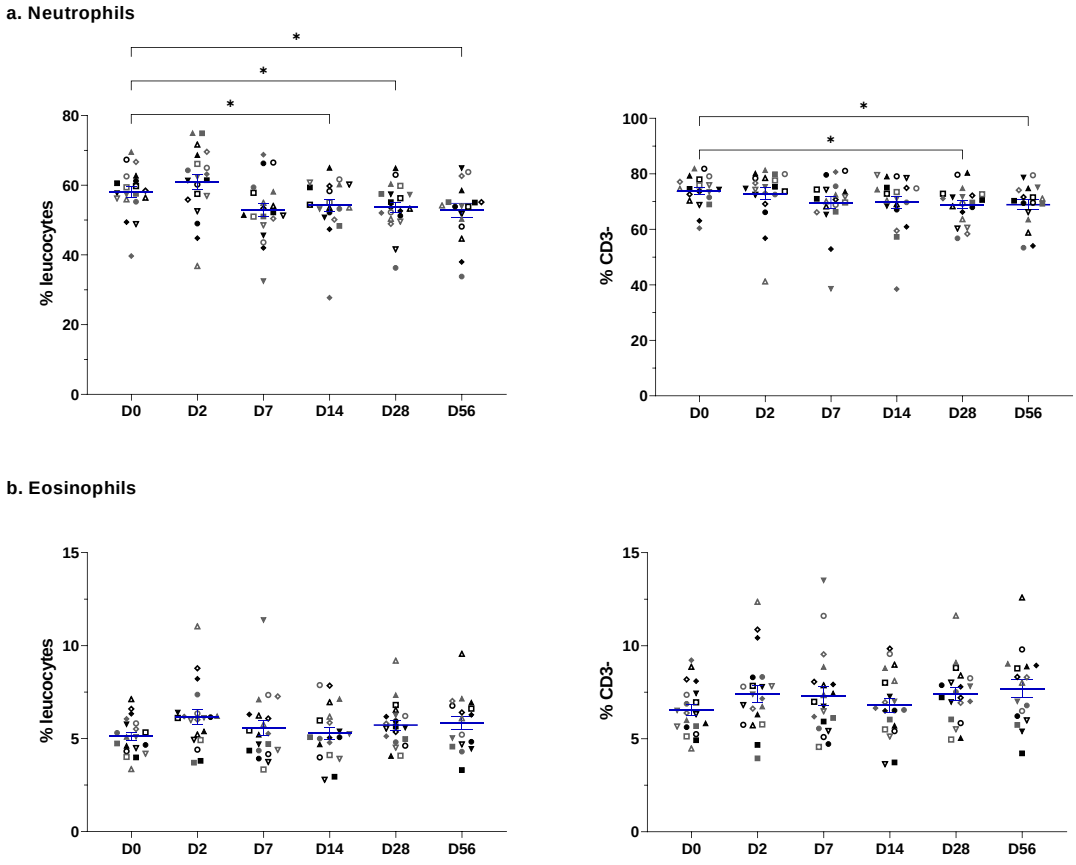


Figure 5.1. Frequency of Neutrophils or Eosinophils using the innate flow cytometry panel on unstimulated whole blood. Neutrophils defined as singlets, Lin- (CD19, CD56, CD3), CD16+, CD66b+. Eosinophils defined as singlets, Lin- (CD19, CD56, CD3), CD16- Siglec8+. "Leucocytes": Live, singlets, CD19-. Shapes represent each individual; black: D2 bronchoscopy, or grey: D7 bronchoscopy. D0: baseline sample taken before BCG aerosol infection. Pairwise comparisons using mixed model corrected with Dunnett's, following normality testing. Blue lines: Mean with SEM. *p<0.05,>0.01.

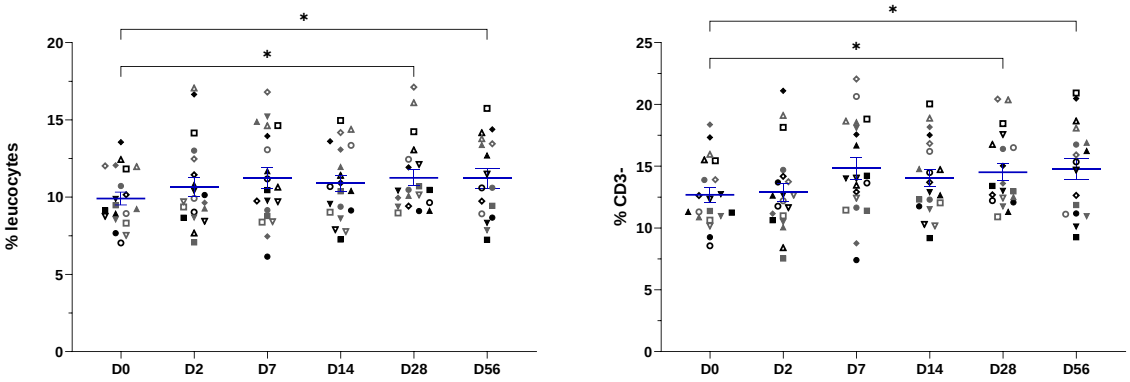
5.3.2 Mononuclear Phagocytes (MPs): macrophages, monocytes and dendritic cells

MPs in the PBMCs increased from D28 onwards after BCG aerosol infection (as percentage of total and/or CD3- cells; Figure 5.2a). There was no difference over time in DCs as

percentage of total leucocytes or CD3- cells (Figure 5.2b). However, DCs fell as percentage of total MPs at D14 compared with baseline.

The proportion of CD14+ cells ("monocytes") are shown in Figure 5.3a where they increased in the blood at D2 (10.46%; 95%CI 9.29-11.64). As outlined in the previous chapter, due to poor separation of CD206 and CD86 populations, a full analysis of macrophages and monocytes is not presented in this thesis. However, unlike the BAL samples, I was able to define CD14 CD16 populations in the blood. Hence classical, intermediate and non-classical monocyte populations are presented here (Figure 5.3c).[161] Classical CD14+ CD16- monocytes decreased at D7 and intermediate CD14+ CD16+ monocytes increased at the same timepoint, however this was not statistically significant when I corrected for multiple comparisons. There was no significant difference seen in the $CD14^{lo}$ CD16+ non-classical monocytes. There was a similar trend to classic monocytes when looking at total $CD14^{hi}$ monocytes; and a similar trend as that seen in intermediate monocytes when looking at total CD16+ monocytes or $CD14^{lo}$ monocytes (data not shown). This was likely due to the fact that the intermediate monocyte population was $CD14^{int}$ (Figure 5.3b).

a. Mononuclear Phagocytes



b. Dendritic cells

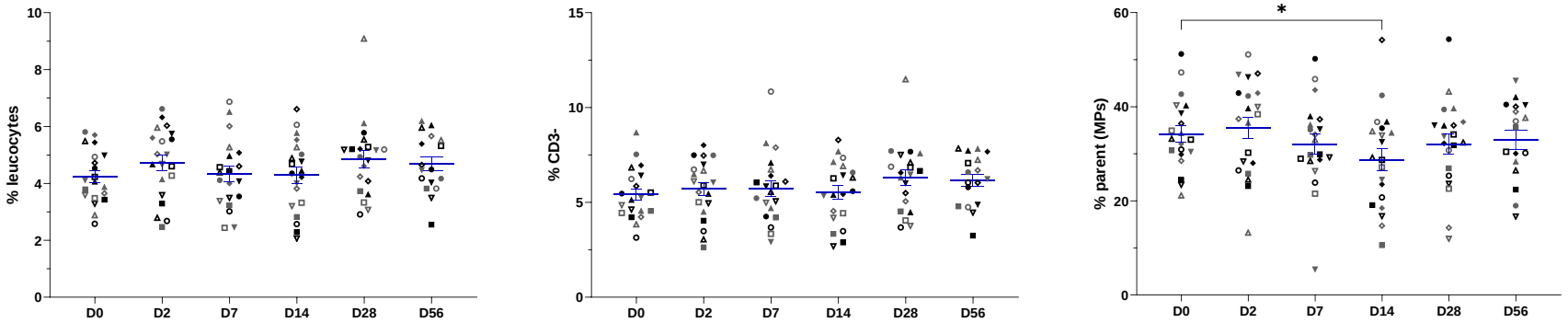


Figure 5.2 Frequency of Mononuclear Phagocytes (MPs) using the innate flow cytometry panel on unstimulated whole blood. (a) MPs defined as live singlets, CD19-CD3-CD56-CD66b-CD45+. "Leucocytes": Live, singlets, CD19-. MPs: % singlets D28 p=0.019, D56 p=0.043; % CD3- D28 p=0.024, D56 p=0.018; (b) DCs defined as live singlets, negative for CD19, CD3, CD56, CD66b and CD16; HLA-DR+ CD11c+. DC % parent D14 p=0.046. Shapes represent each individual; black: D2 bronchoscopy, or grey: D7 bronchoscopy. Normally distributed, pairwise comparisons using a mixed model with Dunnett's, blue line mean with SEM.

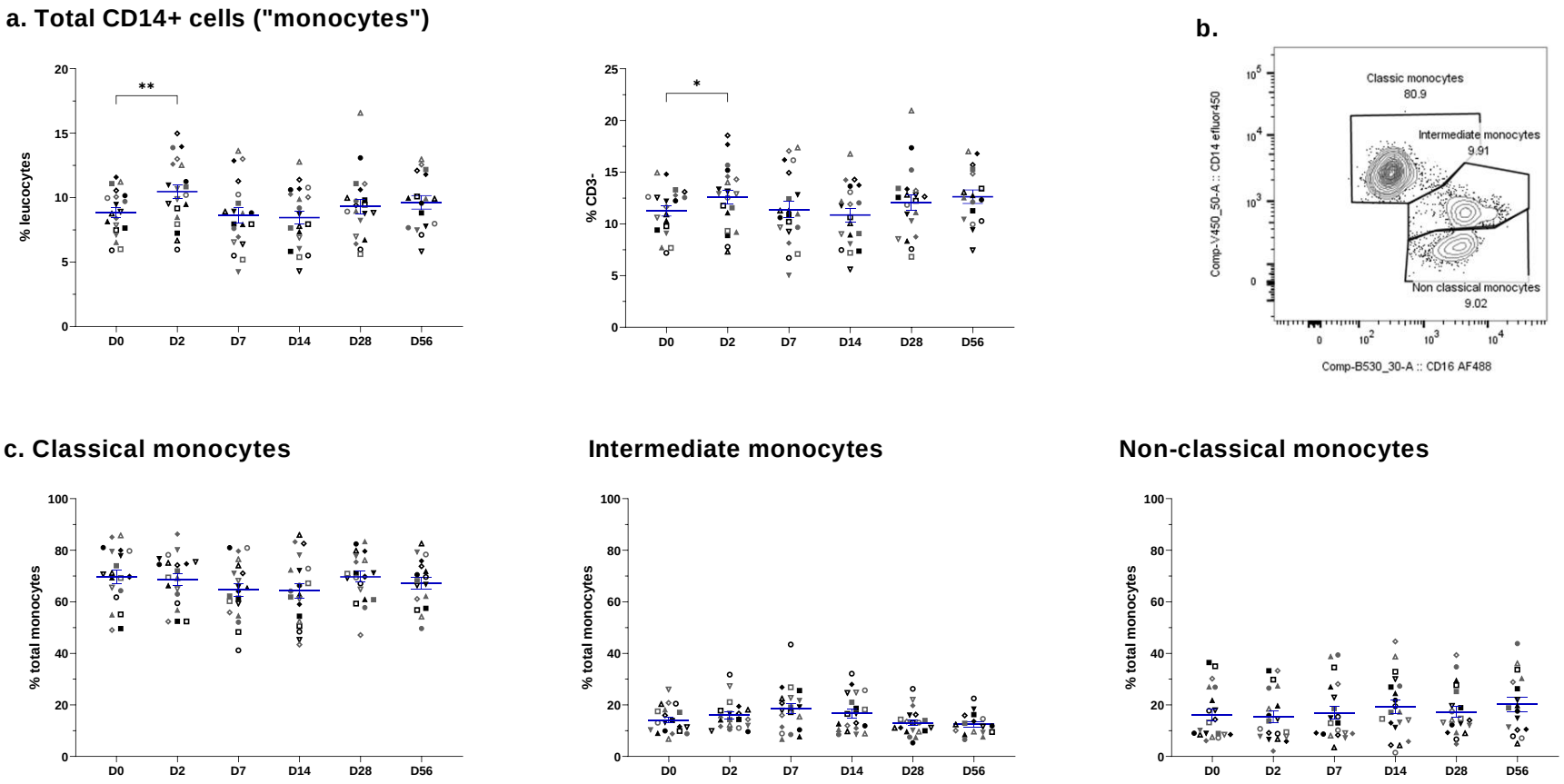


Figure 5.3. Frequency of monocytes using the innate flow cytometry panel on unstimulated whole blood. Monocytes defined as live singlets, CD19-, CD3-, CD14+. % singlets. (a) "Leucocytes": Live, singlets, CD19-. Total monocytes D2 p=0.002; % CD3- D2 p=0.040; (b) Representative flow cytometry plot for classical, intermediate and non-classical gating strategy. (c) Monocyte subsets defined as live singlets, CD19- CD3- CD56- CD66ba. Classical CD14+CD16-; Intermediate CD14+CD16+; Non-classical CD14lo CD16+. Shapes represent each individual; black: D2 bronchoscopy, or grey: D7 bronchoscopy. Normally distributed, pairwise comparisons using a mixed model with Dunnett's, blue line mean with SEM.

Activated (HLA-DR+) MPs fell at Day 14 compared with baseline (Figure 5.4a). There was an increase in HLA-DR+ total CD16+ monocytes at D2 but this lost significance on correction (Figure 5.4b). There wasn't a clear HLA-DR+ CD14+ population in most samples. Hence, I was not able to investigate HLA-DR+ in classical or CD14+ total cells.

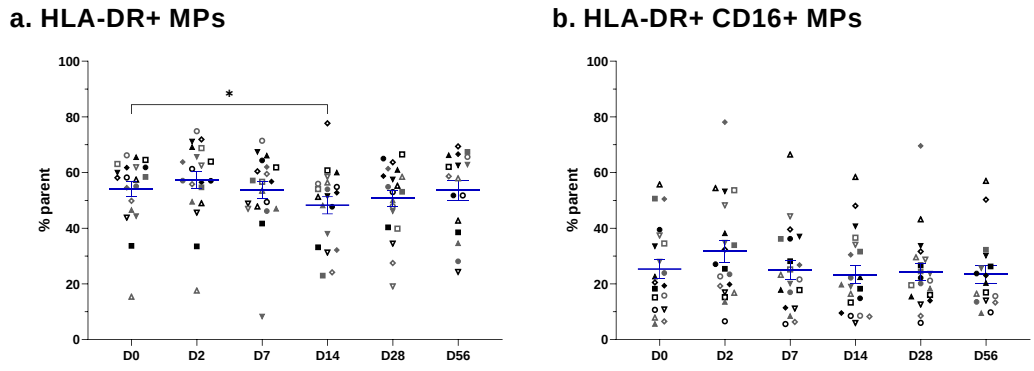


Figure 5.4. Frequency of HLA-DR+ MPs using the innate flow cytometry panel on unstimulated whole blood. (a) HLA-DR+ MPs as percent MPs: D14 p=0.049; (b) HLA-DR+ CD16+ MPs. Shapes represent each individual; black: D2 bronchoscopy, or grey: D7 bronchoscopy. Pairwise comparisons using Wilcoxon with Holm-Bonferroni. Blue line median with IQR.

5.3.3 NK cells

There was no difference in the proportion of NK cells at baseline and at any timepoint post BCG aerosol infection. NK cells significantly increased at D7 compared with D2 out of total cells (p=0.045) and CD3- cells (p=0.025). This significance was lost after correcting for multiple comparisons (Figure 5.5).

Compared with baseline, CD16+HLA-DR+ NK cells increased at D56 (Figure 5.5f). CD16+ NK cells increased at D2 and D7 and fell at D56, though significance was lost with correction. There was a mirroring effect in HLA-DR+ NK cells which decreased at D7, and increased at D56 compared with D0, but again significance was lost with correction (Figure 5.5c,e). Between groups, CD16+ NK cell frequencies were lower at D14 in volunteers who underwent a bronchoscopy at D7 (G2) compared with D2 (G1); (p=0.033, unpaired t-test). However, significance was lost on correction (Figure 5.5d).

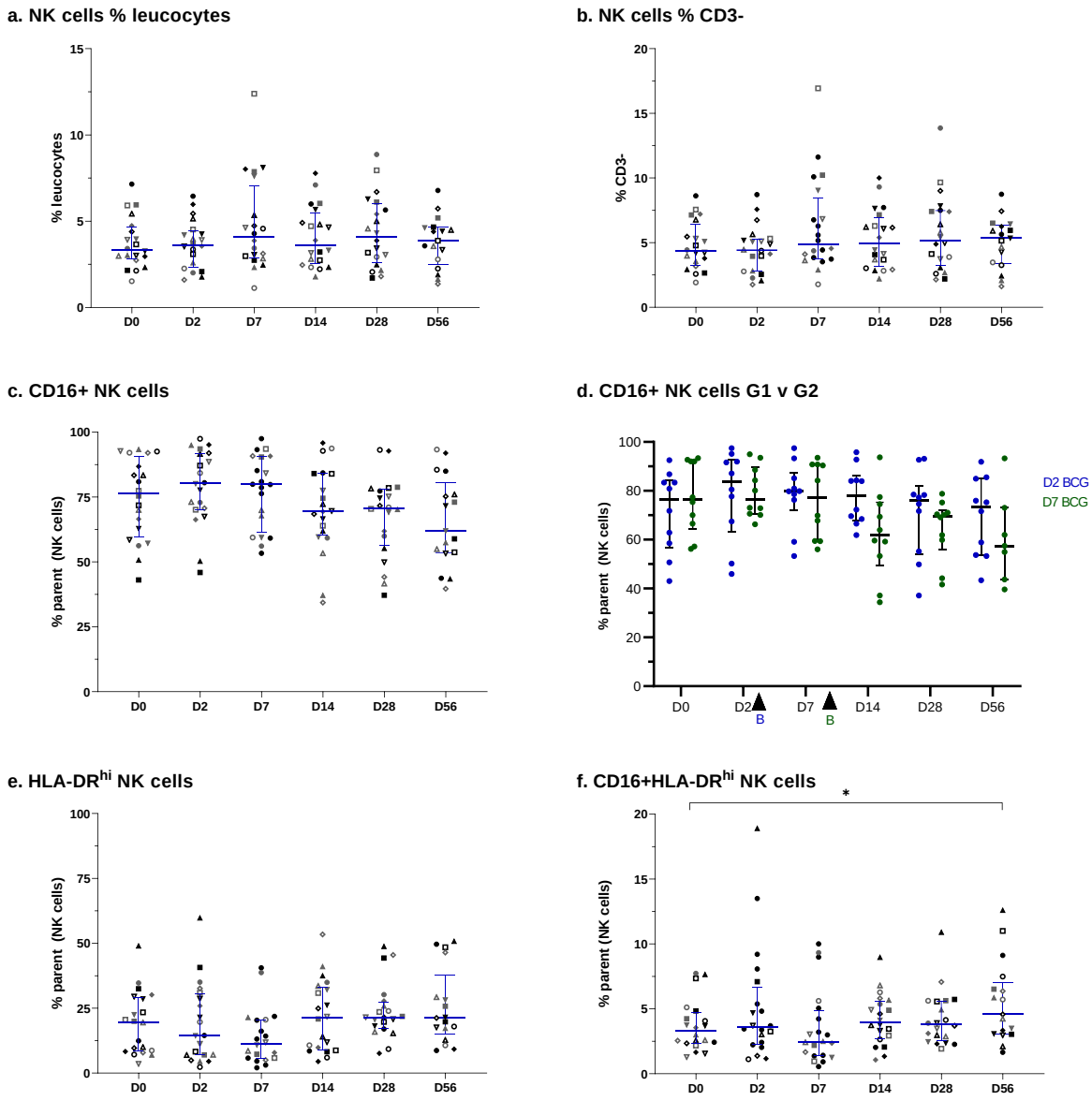


Figure 5.5. Frequency of NK cells and NK cell subsets using the "innate" flow cytometry panel on unstimulated whole blood. (a) "Leucocytes": Live, singlets, CD19-. (d) "B" indicates timing of bronchoscopy, following D2 or D7 blood collection. (f) D56 p=0.006 on pairwise comparison using Wilcoxon after correction for Holm-Bonferroni, following normality testing. Shapes represent each individual, black: Day 2 bronchoscopy, grey: Day 7 bronchoscopy. D0 sample taken before BCG aerosol infection. Blue/black line median with IQR.

5.3.4 Donor Unrestricted T cells (DURTs)

5.3.4.1 MAIT cells

Different methods were compared for identifying MAIT cells, using the 5-OP-RU MR1 tetramer (TEM), CD161+ and the T cell receptor (TCR) invariant variable α-chain 7.2

(Vα7.2). There was a significant difference between most methods at all time points (Figure 5.6), however, as with the BAL data, the differences were subtle. TEM+ CD161+ detected the fewest cells, though this was not significantly different to TEM+ Vα7.2+. There was no difference between TEM+ alone and the other two strategies. As with the BAL data, in this chapter I have identified MAITs using the TEM alone.

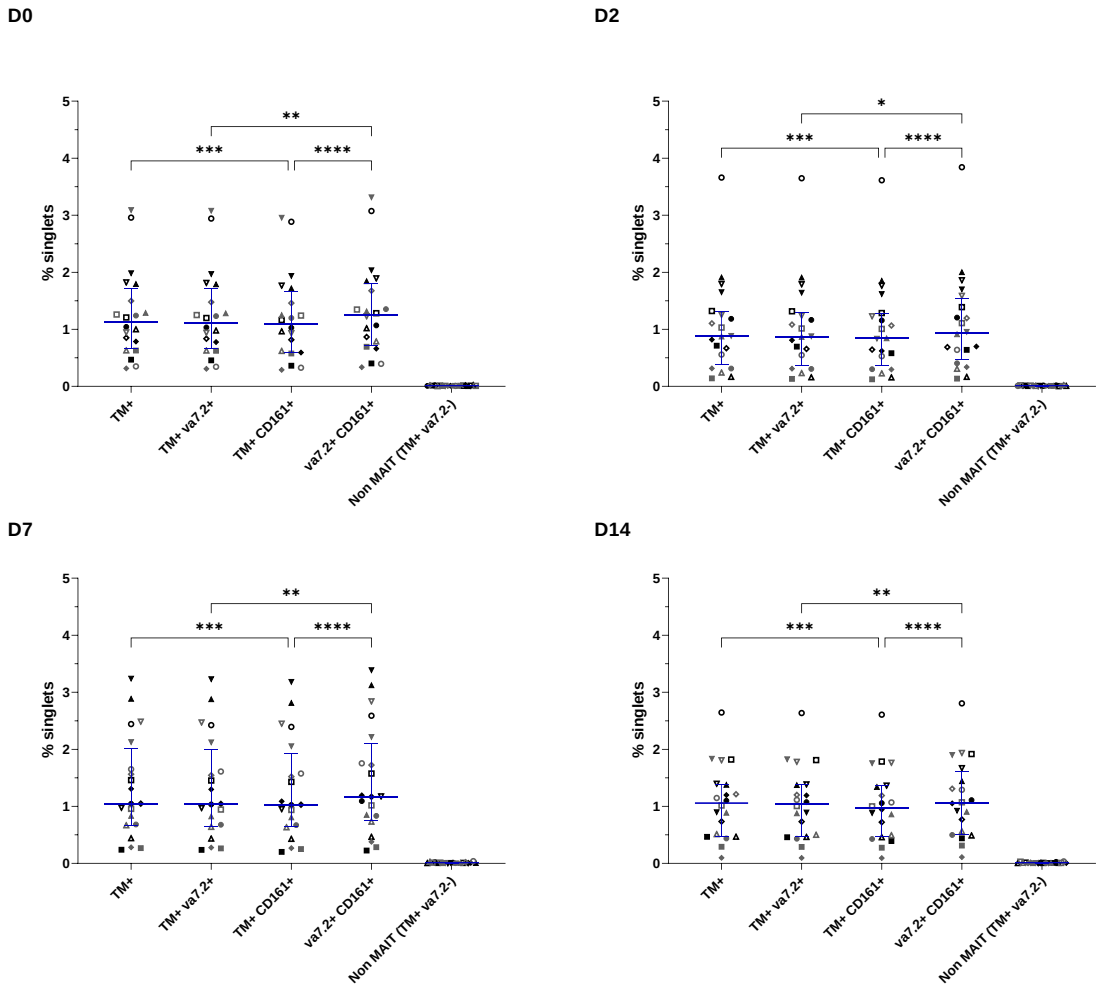


Figure 5.6. Frequency of MAIT cells (live, CD19-CD14- cells) out of "singlets" from PBMCs using the MAIT/NKT flow cytometry panel using four methods of identification (and a fifth non MAIT population TEM+ Vα7.2-). All cells are live singlets, CD14-, CD19-, CD3+. Shapes represent each individual, black: Day 2 bronchoscopy, grey: Day 7 bronchoscopy. D0: baseline sample taken before BCG aerosol infection. Non-parametric pairwise comparisons using Wilcoxon corrected with Holm-Bonferroni, following normality testing (non-MAITs excluded from this correction). Blue line median. ** p <0.01; ***p<0.001; ***p<0.0001. All methods significantly different to the non-MAITs (significance not shown).

There was no difference in total circulating MAIT cells as % singlets following BCG aerosol inhalation at any time point (Figure 5.7a). When looking at circulating subsets, CD8+ MAITs

increased at D2 then decreased at Day 7 but this effect was lost on multiple comparisons. In DN cells there was no difference on correction comparing only baseline, however, there was a significant fall at D2 and increase at D7 when comparing all time points (Figure 5.7c). In the BAL there was a trend for CD4+ MAITS to increase and DN MAITs to decrease at D7 compared with D2. In PBMCs, the MAIT cell population is dominated by the CD8+ subset across all time points, however the BAL is enriched with DN, with a shift to CD4+ and CD8+ MAITs at D7 (Figure 5.7d+e).

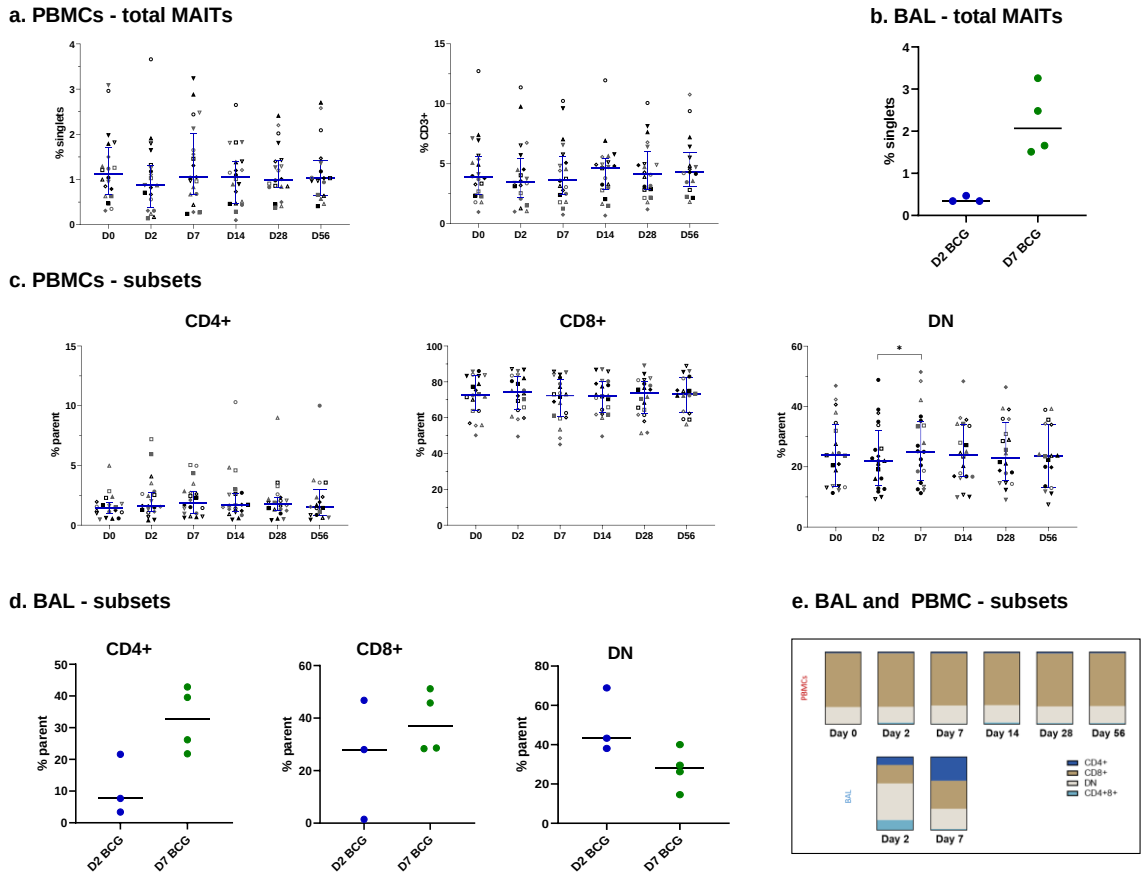


Figure 5.7. Frequency of MAIT cells in PBMCS or BAL using the MAIT/NKT flow cytometry panel. All cells are live singlets, CD14-, CD19-, CD3+, TEM+, CD161+. Blue/black line median and IQR; "singlets": live, CD19-CD14- cells. (a+c): Shapes represent each individual, black: D2 bronchoscopy, grey: D7 bronchoscopy. D0: baseline sample taken before BCG aerosol infection. Non-parametric pairwise comparisons using Wilcoxon corrected with Holm-Bonferroni, following normality testing. DN (Double negative): D2vD7 p=0.0067. (e) Parts of whole schema of same data from PBMCs and BAL (median).

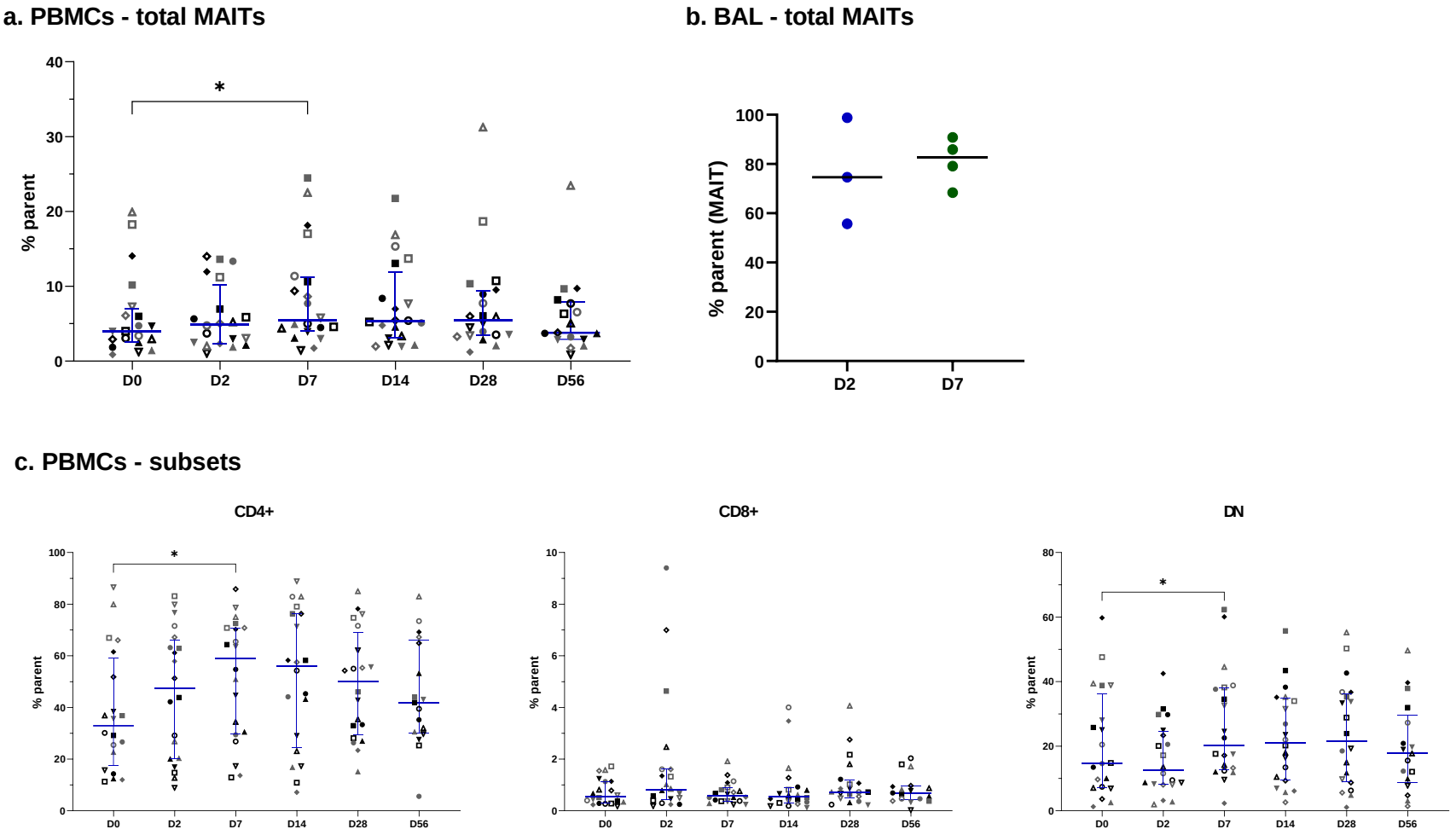


Figure 5.8. Frequency of HLA-DR+ MAIT cells from PBMCs (a and c.) or BAL (b.) using the MAIT/NKT flow cytometry panel. (a+c): Shapes represent each individual, black: D2 bronchoscopy, grey: D7 bronchoscopy. D0 sample taken before BCG aerosol infection. Non-parametric pairwise comparisons compared to baseline only using Wilcoxon corrected with Holm-Bonferroni, following normality testing. Blue line median with IQR. (a) D7 p=0.015; (c) CD4+ D7 p= 0.049; DN D7 p=0.020.

HLA-DR+ total MAIT cells increased in the blood 7 days following infection (Figure 5.8a). This increase was due to a rise in both CD4+ and DN HLA-DR+ MAITs (Figure 5.8c). There was no clear trend in the BAL (Figure 5.8b), however the proportion of HLA-DR+ total MAITs in the BAL was higher at both time points than at any timepoint in the periphery. Further MAIT subsets were not explored in this thesis but will be a part of future planned work.

5.3.4.2 Invariant Natural Killer T cells (iNKTs)

There was no significant difference over time in total iNKTs (Figure 5.9a). This in contrast to the BAL data where there was a trend for an increase at D7 versus D2.

There was a lower proportion of CD8+ iNKTs than either CD4+ or DN subsets in blood at baseline (Figure 5.9c) though no difference in any subset following BCG infection. In the BAL, numbers are small but there appeared to be an enrichment for CD4+ iNKTs at D7 compared with D2 with a corresponding reduction in DN iNKTs. Statistics were not performed due to low numbers.

A median of 82.1% iNKTs were positive for CD161 at baseline (IQR 68.38-85.30), with no significant difference seen at any timepoint (data not shown). In BAL, CD161+ iNKTs were 84.6% at D2 (IQR 75.8-91) and 88% at D7 (IQR 78.0-95.0).

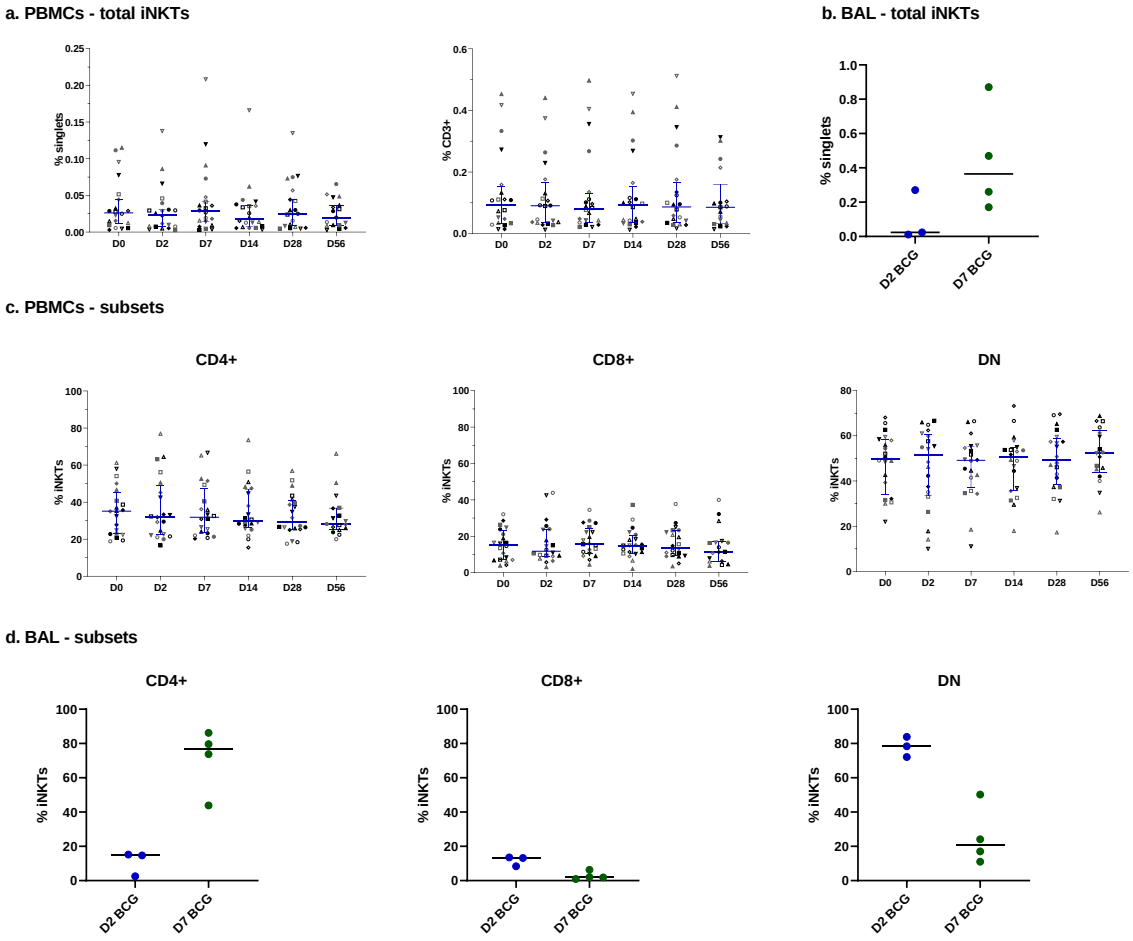


Figure 5.9. Frequency of iNKT cells from PBMCs (a+c) or BAL (b+d) using the MAIT/NKT flow cytometry panel, defined as Vα24+ Vβ11+ CD56+ CD3+. "Singlets": live, CD19-CD14- cells. (a+c) Shapes represent each individual, black: Day 2 bronchoscopy, grey: Day 7 bronchoscopy. D0: baseline sample taken before BCG aerosol infection. Blue/black line median with IQR.

5.3.4.3 CD161+ T cells

Circulating CD161+ MAIT cells made up around 50% of total CD161+ T cells and their proportion did not change following BCG infection (Figure 5.10a). In the BAL, there was no clear trend with such small numbers (Figure 5.10b). Total circulating CD161+ T cells fell at D2, though this effect was lost with correction (Figure 5.10c). In the BAL, there was a trend for total CD161+ T cells to increase at D7, but this trend was lost when comparing to total T cells, likely because all T cells increased at this time point (Figure 5.10d).

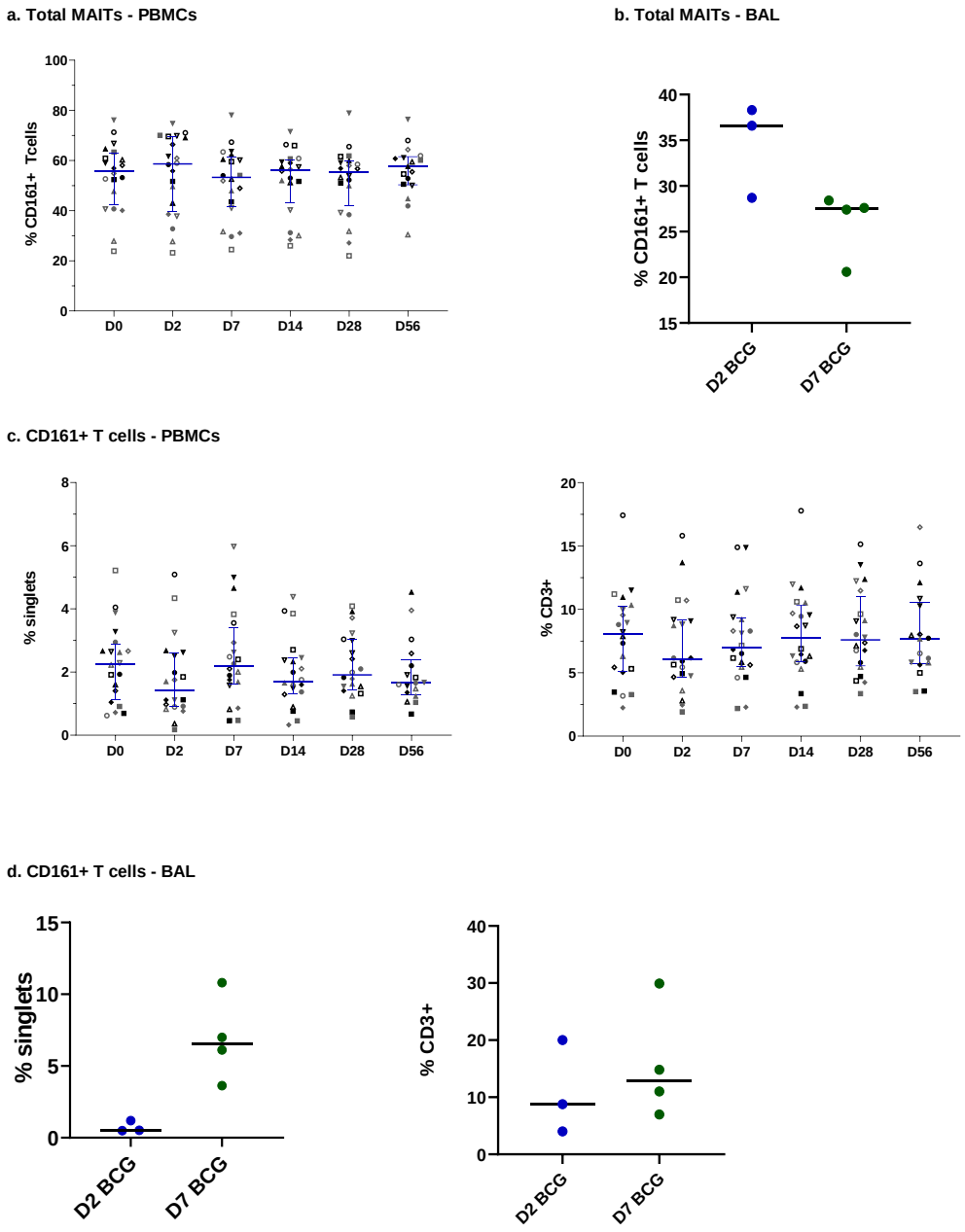


Figure 5.10. CD161+ T cells using the MAIT/NKT flow cytometry panel. (a+b) Frequency of MAIT cells out of CD161+ T cells (live, CD19-CD14- CD3+ CD161+ cells) in PBMCS (a) and BAL (b). (c+d) Frequency of total CD161+ T cells out of singlets (live, CD19-CD14-) or out of T cells in PBMCs (c) or BAL (d). Blue/black line median with IQR.

Circulating CD4+ CD161+ T cells decreased at D2 then increased from D7 through to D28 compared to baseline and the CD8+ CD161+ T cells mirrored this effect. However, after correction only an increase at D2 in CD8+ CD161+ T cells remained significant (Figure 5.11a). There was a similar pattern in the BAL at the same time points (Figure 5.11b). There was no difference detected in circulating DN CD161+ T cells.

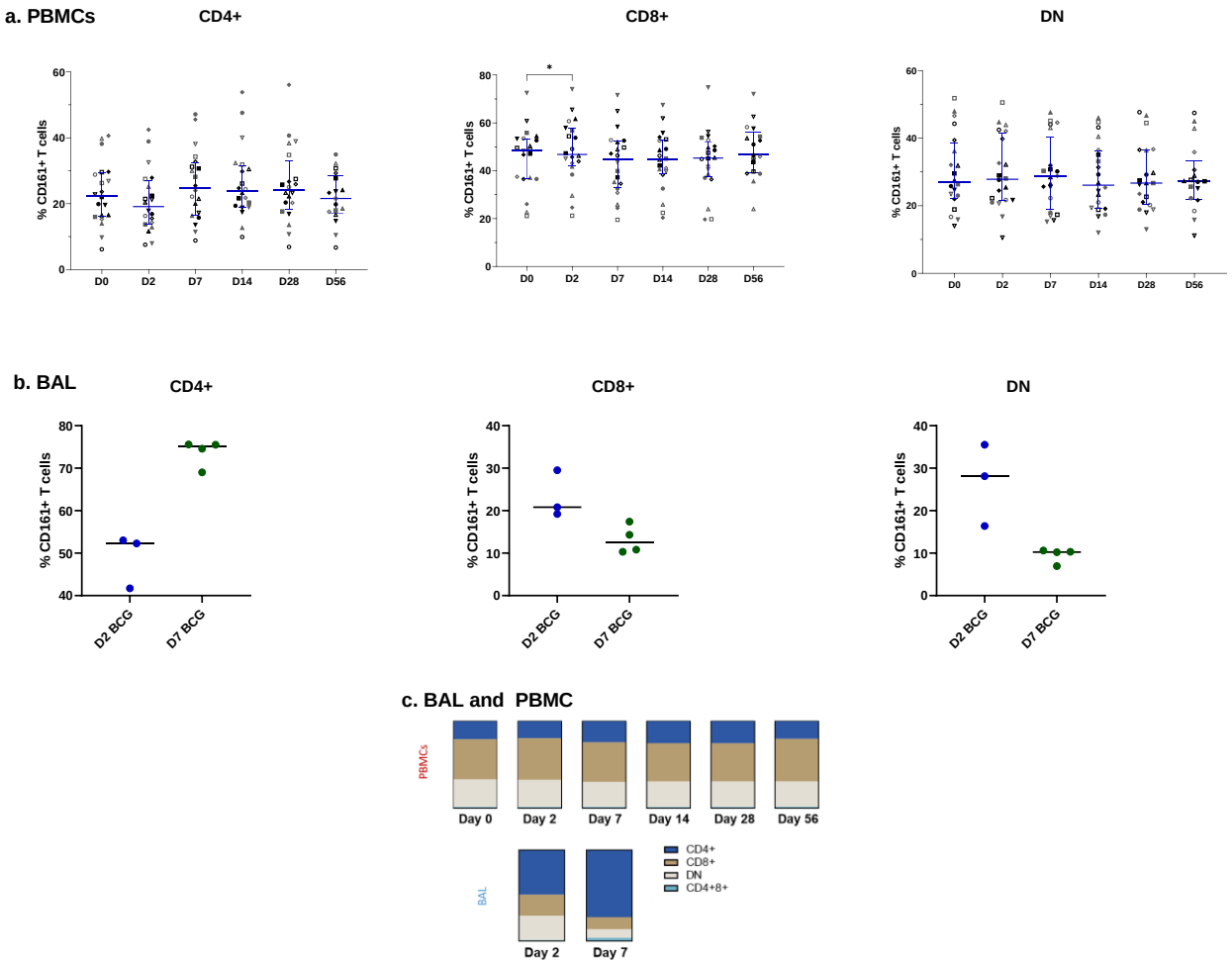


Figure 5.11. Frequency of total CD161+ T cells in PBMCS (a) or BAL (b), or both (c) using the MAIT/NKT flow cytometry panel. All cells are live singlets, CD14- CD19- CD3+ CD161+. (a) Shapes represent each individual, black: D2 bronchoscopy, grey: D7 bronchoscopy. D0: sample taken before BCG aerosol infection. Pairwise comparisons using a mixed model corrected with Dunnett's. Blue/black line median with IQR. CD8+ D2 p= 0.025. (c) Parts of whole schema of same data from PBMCs and BAL (median).

While I was unable to look for antigen specific MAIT cells due to the limitations in panel design, the unstimulated CD161+ CD26+ CD4+ T cell population increased at D28 compared to baseline, though significance was lost on correction (Figure 5.12). There was however a significant increase from D14 onwards when compared with D2, which remained after correction. The majority of this cell population was double negative for TRAV1-2/Vα7.2 and TEM MAIT (Figure 5.12b).

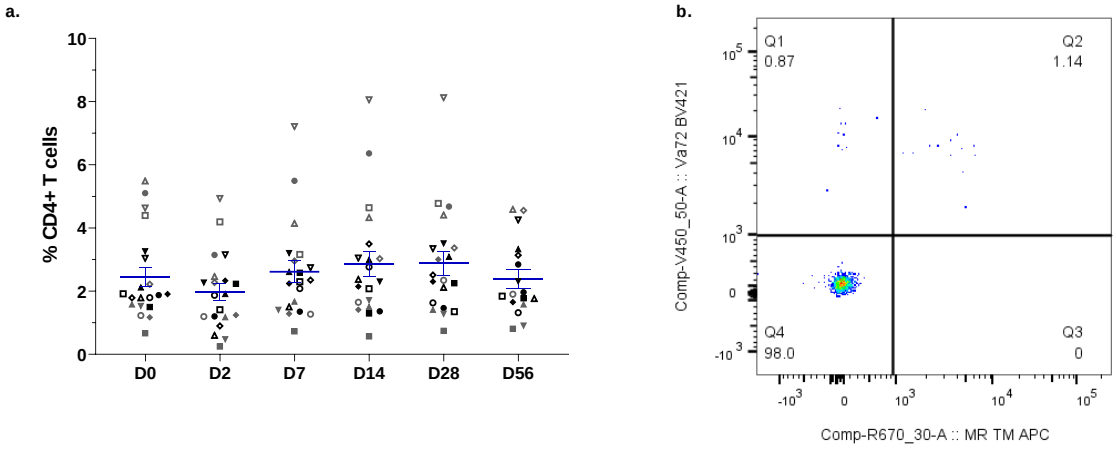


Figure 5.12. CD161+ CD26+ CD4+ T cells. (a) D2-D14 p=0.0032; D2-D28 p=0.0004, non-parametric, all pairwise comparisons, using Wilcoxon corrected with Holm-Bonferroni, following normality testing. Blue line median with IQR. (b) Representative plot of CD161+ CD26+ CD4+ T cell. Negative for 5-OP-U MR1 tetramer and Vα7.2, numbers represent % parent.

There was no significant pattern in the CD8+ T cell population over time (data not shown).

5.3.4.4 γδ T cells

WB γδ T cells were analysed using the Homing panel.

Due to constraints with sample processing on bronchoscopy days, only volunteers having the bronchoscopy also had WBICS at D2 or D7. Hence there were 20 samples D0, D14, D28, D56; but only 10 on D2 and D7. Moreover, due to time constraints and to focus my thesis on early timepoints, I only analysed homing panel samples to D14. Pairwise comparisons for both homing and polycytokine ICS panels are done on 20 samples between D0 and D14, D28, D56, on ten samples between D0 and either D2 or D7, and a Mann Whitney between the 10 unpaired samples D2 and D7. I also ran an unpaired ANOVA or Kruskal Wallace test (depending on normal distribution).

As with the BAL cells, γδ2 stained many more cells than γδ pan and hence total γδ2+ cells (+/-γδ pan) are shown here, presuming weak binding of the γδ pan marker to these cells.[285] There was no difference from baseline in either γδ1+γδ3 (Figure 5.13a) or γδ2 T cells (Figure 5.13b). There was no difference in CD4/CD8 subsets (data not shown). There was also no difference in antigen-specific IFN-γ+ total γδ T cells (Figure 5.13c).

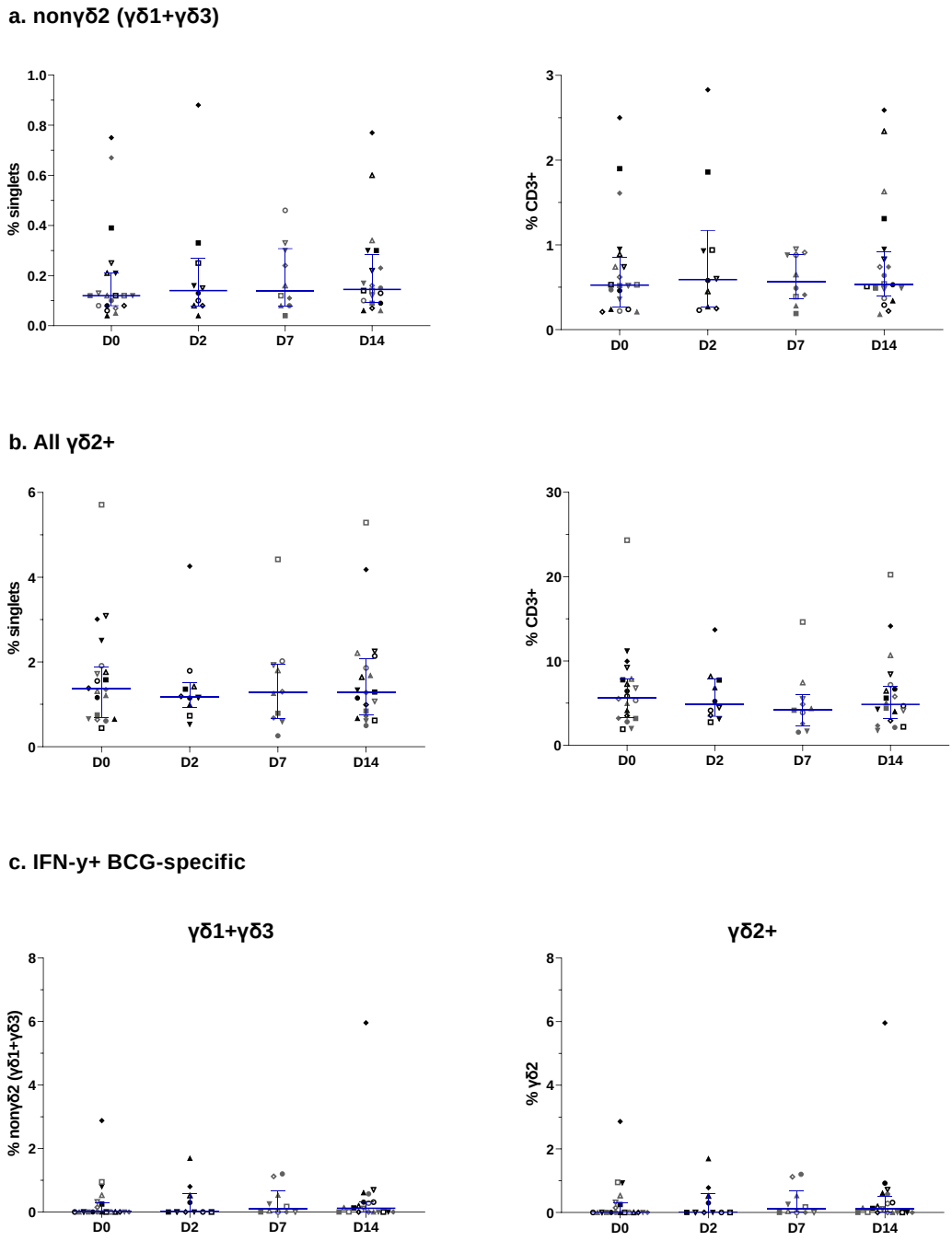


Figure 5.13. Frequency of γδ T cells by singlets or CD3+ defined using unstimulated whole blood cells; IFN-γ+ γδ T cells after overnight stimulation with BCG, calculated as BCG minus unstimulated samples. Samples stained using the "Homing" flow cytometry panel, blue/black line median, "leucocytes": live, singlets, CD19-CD14-. "non-γδ2 (γδ1+γδ3) T cells" defined as live singlets, CD19- CD14- CD3+ γδpan+γδ2-, γδ2 T cells defined as live singlets, CD19- CD14- CD3+ γδ2+, regardless of γδ pan staining (presumed weak binding of γδ pan). Shapes represent each individual; black: D2 bronchoscopy, or grey: D7 bronchoscopy. D0: sample taken before BCG aerosol infection. (b,d) Green dots: cells stained and fixed on same day as BAL collection, black dots: Incubated overnight before staining and fixing. All samples unstimulated.

5.3.5 T cells

Total circulating T cells fell at D2 and there was a trend for an increase at D7 but this did not reach statistical significance (Figure 5.14). The significance at D2 was only present using the Innate panel with 20 volunteers at each timepoint, and was lost when looking at the same samples by the Polycytokine panel (with only 10 volunteers at D2 and D7; Figure 5.14a+b).

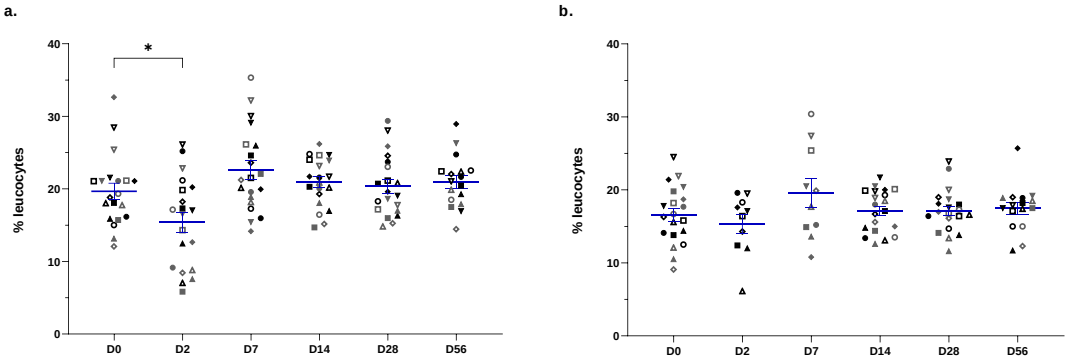


Figure 5.14. T cells in whole blood using (a) the innate or (b) the polycytokine flow cytometry panel. T Cells defined as singlets, CD19-, CD3+, CD14-. "Leucocytes": Live, singlets, CD19-. Shapes represent each individual; black: D2 bronchoscopy, or grey: D7 bronchoscopy. D0 sample taken before BCG aerosol infection. Pairwise comparisons using a mixed model corrected with Dunnett's, following normality testing. Blue line mean with SEM. %leucocytes D2 p=0.028

Using the polycytokine panel, there was no difference in proportion of T cell subtypes (Figure 5.15.a-c). There was also no difference in HLA-DR+ T cells (Figure 5.15d).

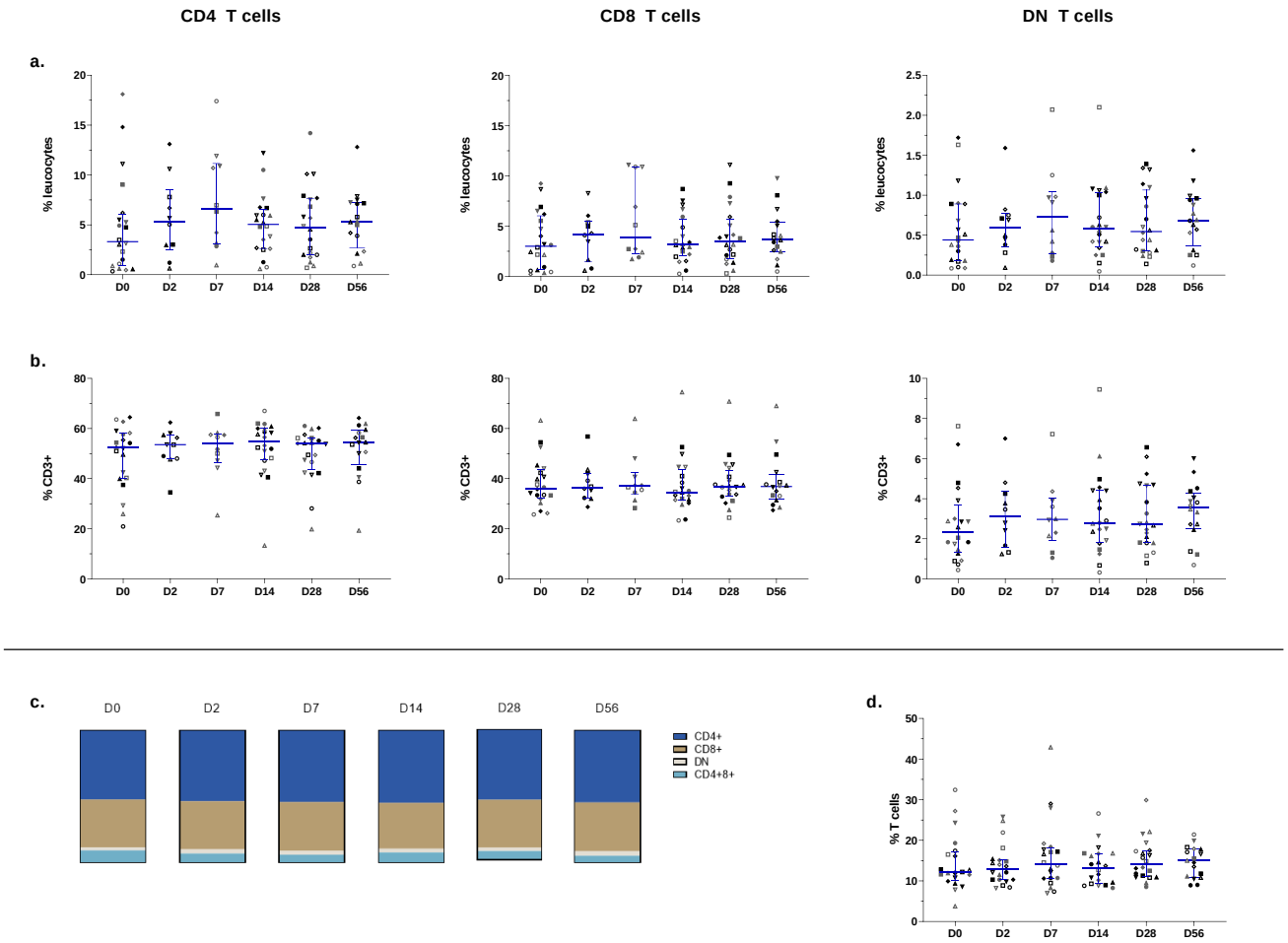


Figure 5.15 (a-c) CD4+, CD8+ or DN (Double negative) T cells defined on unstimulated whole blood cells stained using the polycytokine flow cytometry panel. Frequency by (a) leucocytes or (b) total T cells (defined as singlets, CD19- CD14- CD3+). "Leucocytes": Live, singlets, CD19-. (c) Median frequency of total T cells shown as parts of whole. d. HLA-DR+ T cells defined by the "innate" panel. Shapes represent each individual; black: D2 bronchoscopy, or grey: D7 bronchoscopy. D0 sample taken before BCG aerosol. Blue/black line median with IQR. Double positive T cells not shown but no significance at any timepoint.

Both monocyte and neutrophil: T cell ratios (ML or NL ratio) increased significantly at D2 in the blood (Figure 5.16a.b). In the BAL, though neutrophils increased at D7, as T cells increased further, there was a fall in the NL ratio at D7 compared with D2 (Figure 5.16d). It is unclear if this represents an increase at D2 or a fall at D7 compared to baseline. As I was unable to definitely identify AMs or monocytes using flow cytometry in the BAL, I have shown the AM:T cell ratio as calculated from 10x (Figure 5.16c) which shows a fall at both timepoints compared with saline controls. This appears to be a different pattern to the systemic response in the blood, although a different cell type is being used, and the two techniques for identifying cells may not be comparable.

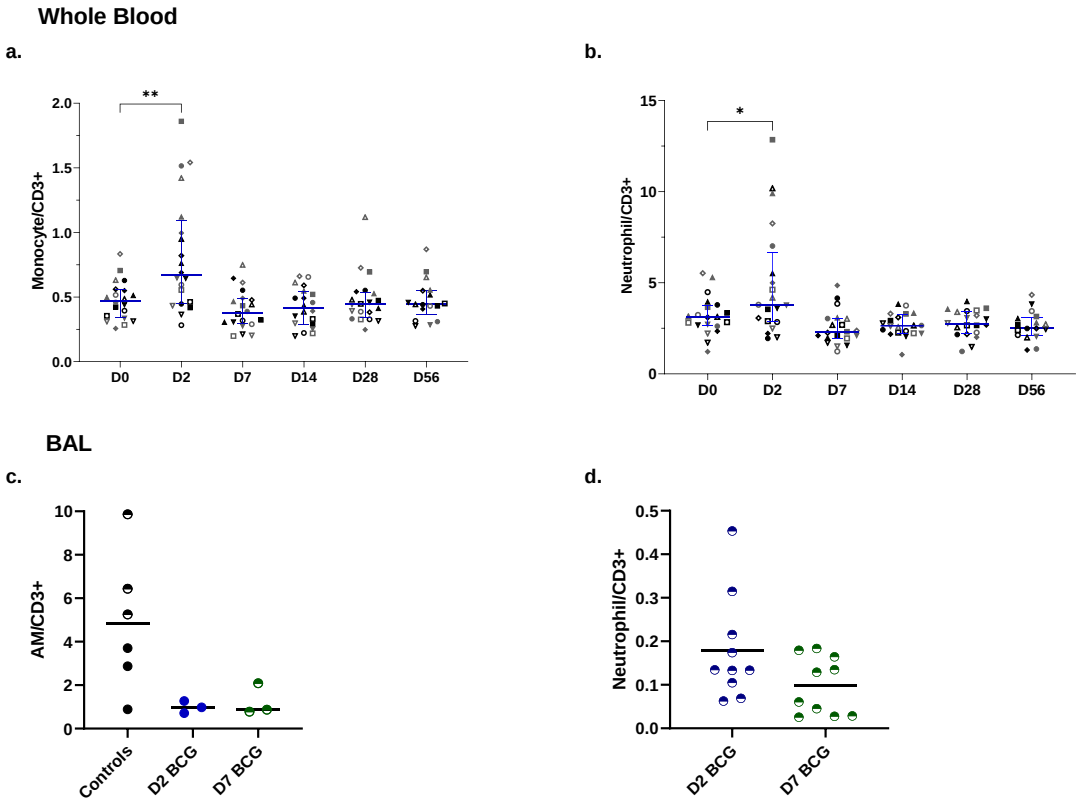


Figure 5.16. (a) Monocyte:T cell ratio, (c) AM:T cell ratio or (b+d) Neutrophil:T cell ratio from whole blood (a+b) or BAL (c+d). Defined by (a,b,d) the "innate" flow cytometry panel" or (c) 10x. (a+b) Shapes represent each individual; black: D2 bronchoscopy, or grey: D7 bronchoscopy. D0 sample taken before BCG aerosol infection. Pairwise comparisons using a mixed model corrected with Dunnett's, following normality testing. Blue/black line median with IQR. (a) ML D2 p=0.002; (b) NL D2 p= 0.038. (c+d) Closed circles D2 BAL, Open circles D7.

5.3.6 Antigen specific responses

There was a significant increase in PPD-specific IFN-y production of PBMCs as detected by ELISpot compared to baseline and compared to the saline control subjects (Figure 5.17). Comparison with saline was done here due to the low inter-individual variability seen in ELISpot. The increase was most significant at D7 but a difference was still detected at D56 following infection (significance was lost at D28 and D56 after correction). There was a trend for a positive correlation between baseline PPD responses and D7 peak response but this did not reach significance (Figure 5.17c).

ELISpot responses from TB041 (aerosol BCG Bulgaria strain) followed a similar pattern to that seen after BCG Danish aerosol infection in TB043 (Figure 5.18). There was no difference between the median area under the curve for TB041 and TB043, nor between any individual timepoint (Figure 5.18c shows no difference on Mann Whitney at D7 peak). In TB041, ELISpot responses were higher and the peak was earlier following BCG Bulgaria aerosol infection than that following intradermal infection (p=0.045 D7 Mann-Whitney), however significance was lost on correction for multiple comparisons (Figure 5.18d).

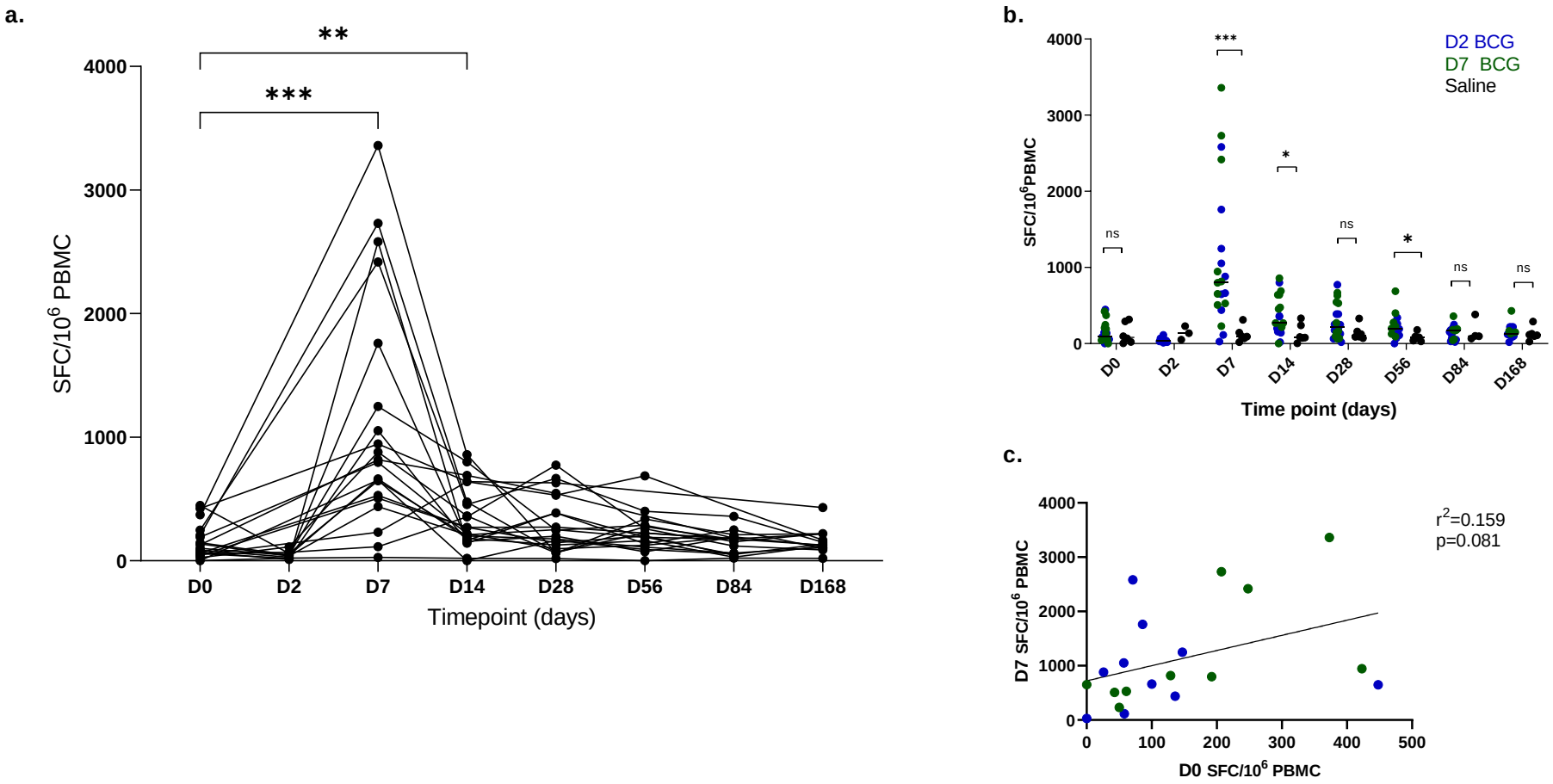


Figure 5.17. *Ex-vivo* IFN-γ ELISpot PBMC responses to overnight stimulation with PPD. a. BCG infected volunteers Group 1 and 2 combined. b. BCG versus saline. c. Simple linear regression D0 v D7 ELISpot responses. (a). Pairwise comparisons comparing baseline only using Wilcoxon corrected with Holm-Bonferroni following normality testing. Note only 10 volunteers at D2 (Group 1 only). D7 p=0.0007, D14 p=0.0029. (b). Mann-Whitney comparisons (not done at D2 as only 3 volunteers in saline group). Line medians. D7 p=0.0006, D14 p=0.0443, D56 p=0.0102. ns: "not significant". Each dot represents an individual.

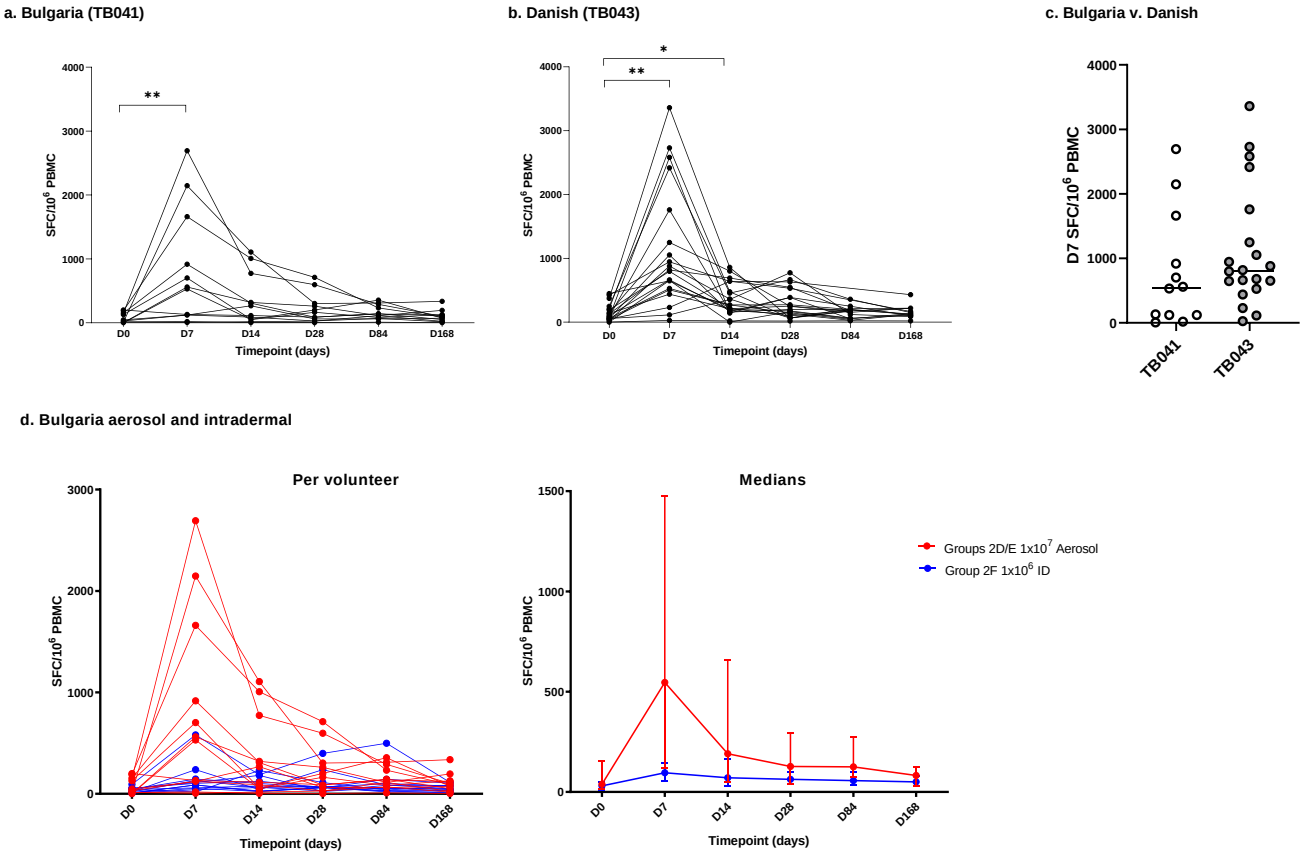


Figure 5.18. BCG Bulgaria (TB041) and BCG Danish (TB043) *ex-vivo* IFN-γ ELISpot PBMC responses to overnight stimulation with PPD. a. BCG Bulgaria aerosol infected volunteers Group 2D/E combined ($1x10^7$ cfu). D7 p= 0.0016 pairwise comparisons comparing baseline only using Wilcoxon corrected with Holm-Bonferroni following normality testing b. BCG Danish aerosol infected volunteers Group 1&2 combined ($1x10^7$ cfu). Data same as Figure 5.17a with D2 and D56 removed to enable comparison with BCG Bulgaria responses c. D7 ELISpot responses Bulgaria versus Danish. Line represents median. No significant difference on Mann-Whitney. (d). TB041 BCG Bulgaria ELISpot responses following aerosol (Groups 2D/E $1x10^7$cfu loaded dose) or intradermal infection (Group 2F $1x10^6$cfu) by individual or median.

All further data is from TB043 only. To investigate the source of the IFN-y, and to describe other cytokine secretion, I stimulated cells overnight with BCG before staining for flow cytometry. CD4+ T cells significantly increased BCG-specific IFN-y production at D7 (Figure 5.19a). IFN-y+ CD8+ T cells significantly increased at D28, though significance was lost following correction with multiple comparisons. Double negative T cells were unremarkable (Figure 5.19b+c).

CD4+ T cells also produced significant levels of TNFα from D7 (though after correction significance only remained at D14) as well as IL-2 at D56 (Figure 5.19a).

There was a significant increase in antigen specific TNFα+ Monocytes at most timepoints, peaking at D7 (Figure 5.20a). IL-17+monocytes also increased at D7.

As there was no CD56+ marker on the Polycytokine panel, I used the Homing panel to delineate any antigen-specific IFN-y+ effects following BCG infection on NK and NKT-like cells (CD3+ CD56+; Figure 5.20b-c).There was a significant increase in BCG-specific IFN-y+ NKT cells at D14. There was no difference in CD3-CD56+ NK cells.

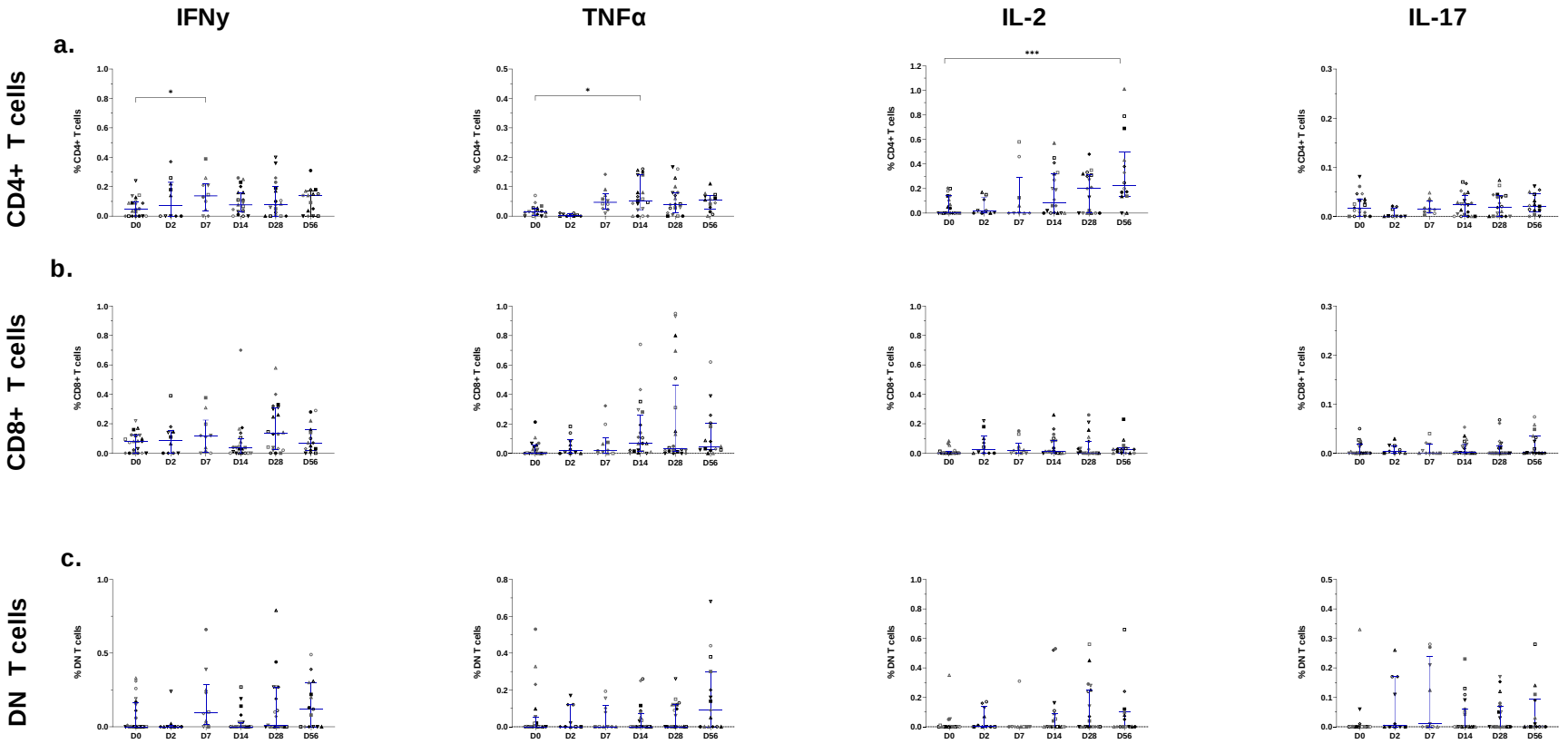


Figure 5.19. Cytokine+ CD4+, CD8+ or DN (Double negative) T cells defined using responses from BCG stimulated whole blood cells minus unstimulated response, using the polycytokine flow cytometry panel. (a) IFN-y+ CD4 D7 p=0.031; TNF-a+ CD4+: D14 p= 0.013; IL-2+ CD4+: D56 p=0.0005. Shapes represent each individual; black: D2 bronchoscopy, or grey: D7 bronchoscopy. D0 sample taken before BCG aerosol infection. Blue line median with IQR. Pairwise comparisons against baseline only, using Wilcoxon corrected with Holm-Bonferroni, following normality testing. Outliers excluded using Grubb's.

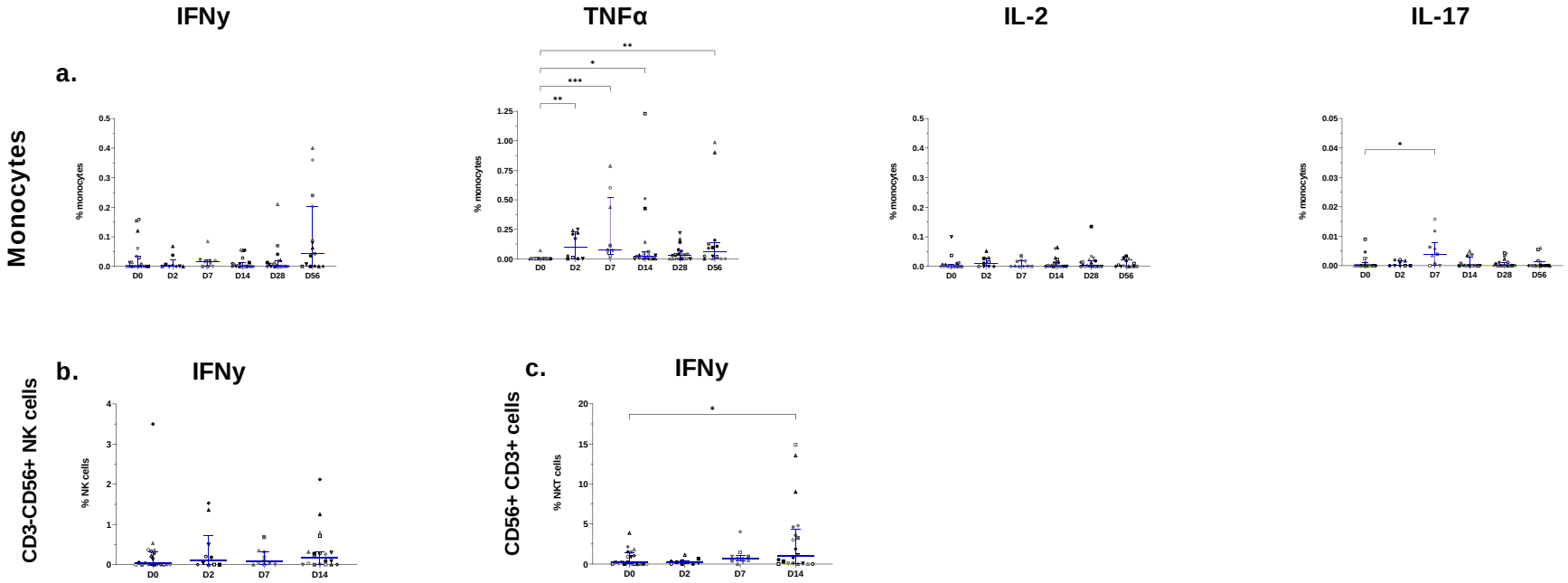


Figure 5.20. Cytokine+ (a), monocytes, (b) NK cells or (c) CD56+CD3+ NKT-like cells, defined using responses from BCG stimulated whole blood cells minus unstimulated response; using the polycytokine flow cytometry panel (a. monocytes) or the homing panel (b-c NK cells and CD3+CD56+ "NKT cells"). (a) TNFα+ monocytes: D2 p= 0.009; D7 p= 0.0005; D14 p=0.039; D56 p=0.008. IL-17+ monocytes: D7 p=0.035. (c) IFN-γ+ "NKT" cells: D14 p=0.011. Shapes represent each individual; black: D2 bronchoscopy, or grey: D7 bronchoscopy. D0 sample taken before BCG aerosol infection. Blue line median with IQR. Pairwise comparisons against baseline only, using Wilcoxon corrected with Holm-Bonferroni, following normality testing. Outliers excluded using Grubb's.

On polyfunctional cell cytokine analysis, CD4+ T cells had a high baseline response which confounded some analyses (significant fall D28 IFN-y+TNFα+IL-2+IL-17+ CD4+ T cells after correction; Figure 5.21a). However, there was a significant increase in antigen-specific IL-2+ CD4+ T cells in combination with or without IFN-y or TNF-a from D14 and/or D56. TNFα and IFN-y alone were also significant from D7 or D14 respectively but lost significance after correction (not shown).

There was no significance after correction in CD8+ T cells (Figure 5.21b) or DN T cells.

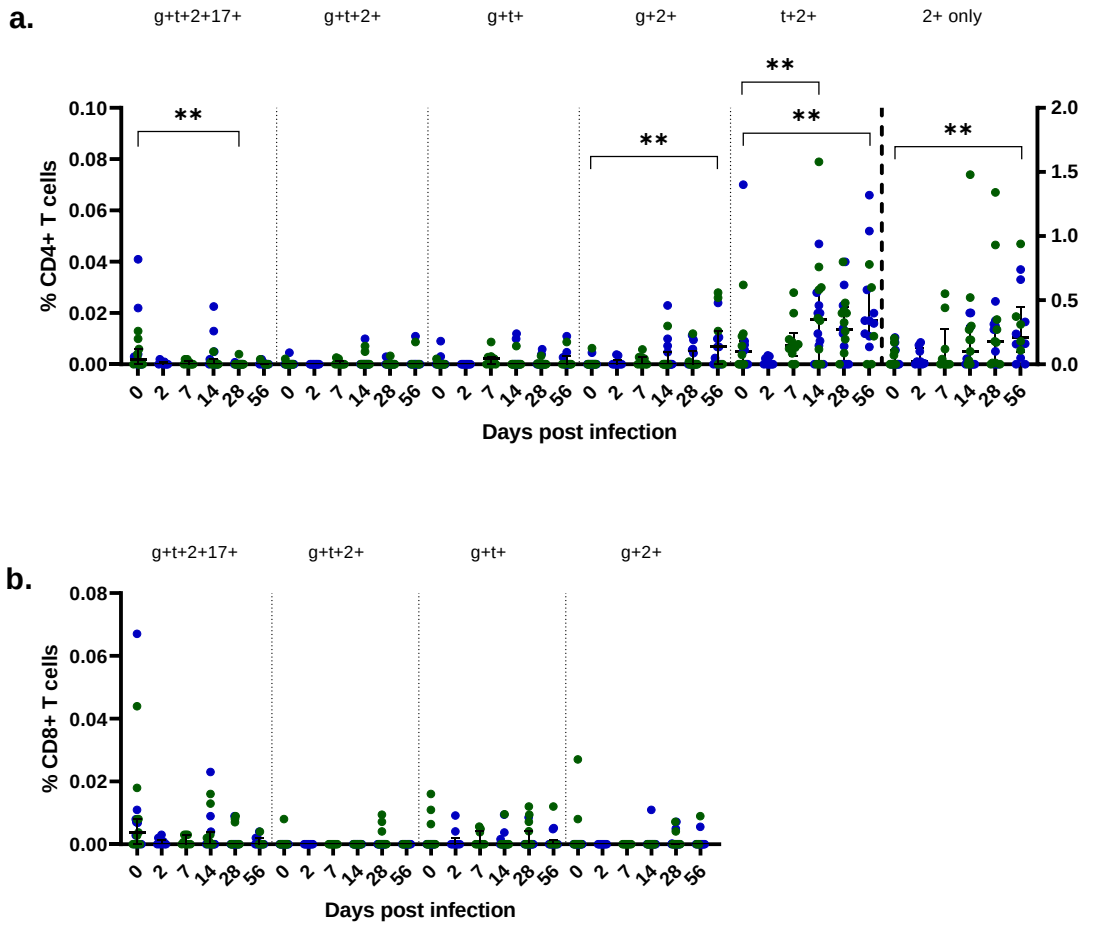


Figure 5.21. Antigen-specific polycytokine producing CD4+ T cells (a) or CD8+ T cells (b.) "g": IFN-y, "t": TNFα, "2": IL-2, "17": IL-17. CD4+ g+t+2+17+ D28 p= 0.0002; g+2+ D56 p=0.0059; t+2+ D14 p=0.008, D56 p=0.004; 2+ D56 p=0.002 by pairwise comparisons against baseline only using Wilcoxon corrected with Holm-Bonferroni, following normality testing. Outliers excluded using Grubb's analysis. blue dots: D2 volunteers, green dots: D7 volunteers. Black lines median and IQR.

Non-specific CD4+ and CD8+ T cells expressing the homing markers CD29+CD49+ or CD103+ reduced at D2 in the blood (Figure 5.22a+b), however significance was lost in the CD4+ T cells on correction. CXCR3+ CD8+ T cells increased at D2. The majority of non-specific CD4+ T cells were both PD-1- and KLRG1- (median 88.10-89.9% total CD4+ T cells across timepoints), with no trend in any KLRG1 PD1 combinations (Figure 5.22c). There was no pattern in other markers investigated and no significant differences in NK cell subsets (data not shown).

I then looked at antigen specific effector cells (defined as IFN-y+ following overnight incubation with BCG minus IFN-y+ unstimulated cells). PD1+, CD29+CD49+ and PD1+KLRG1-BCG-specific IFN-y+ CD4+ T cells increased in the blood at D7, though significance was lost on correction in all but PD1+KLRG1- (Figure 5.23). In all other cell types, cell numbers were too low for any meaningful analysis (less than 50 cells per marker).

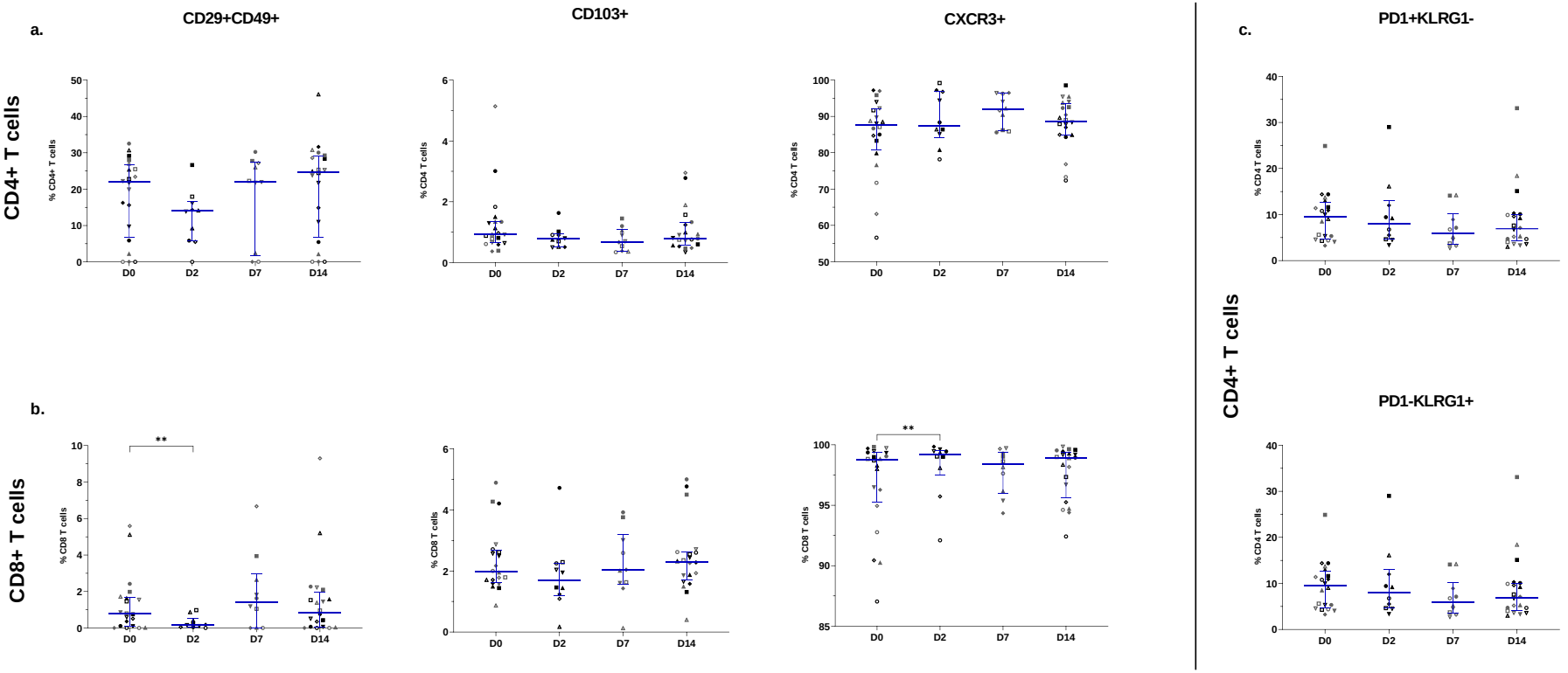


Figure 5.22. Homing markers in unstimulated (a+c) CD4+ T cells or (b) CD8+ T cells; using the "homing" flow cytometry panel in whole blood. Shapes represent each individual; black: D2 bronchoscopy, or grey: D7 bronchoscopy. D0 sample taken before BCG aerosol infection. Blue line median. (b) CD8+ CD29+CD49+ p=0.008; CD8+ CXCR3+ p= 0.004 on pairwise comparisons against baseline only using Wilcoxon corrected with Holm-Bonferroni, following normality testing. Note different axes between (a) and (b) CD29+CD49+.

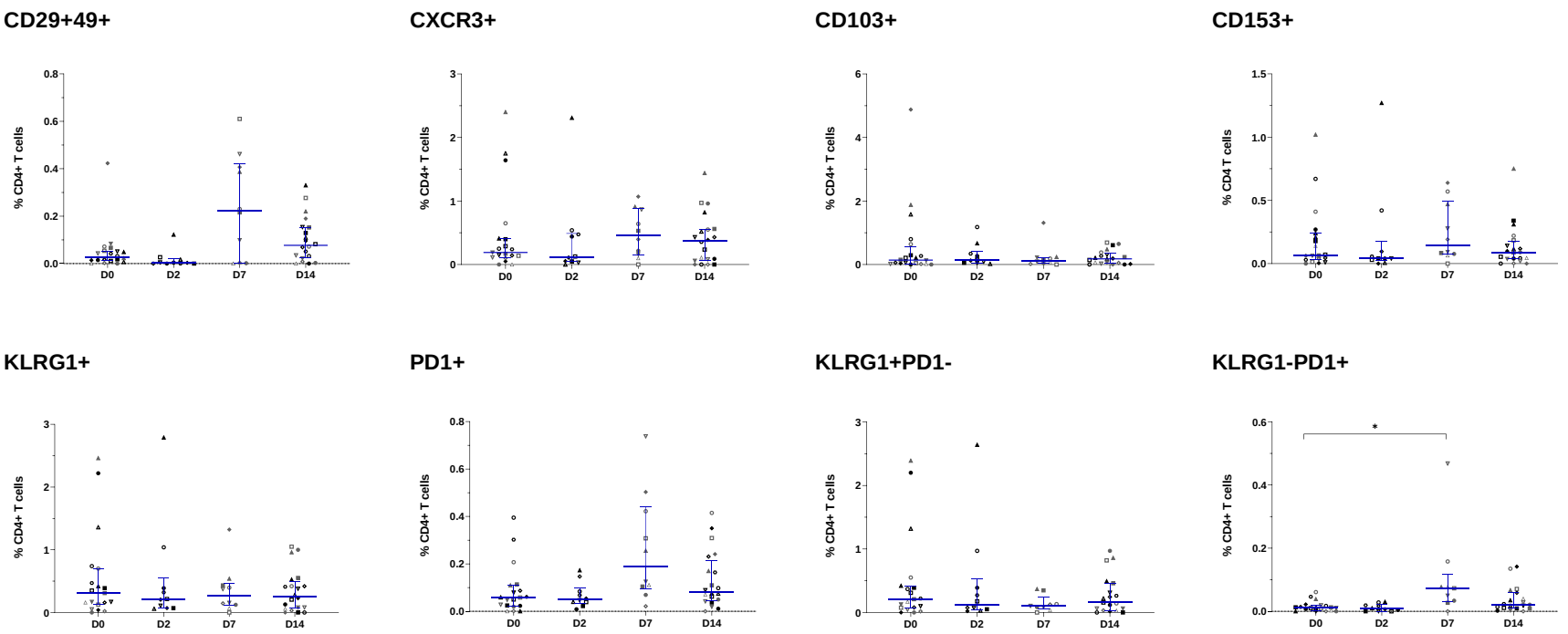


Figure 5.23. Homing markers in BCG-specific IFN-γ+ CD4+ T cells using the homing flow cytometry panel in whole blood. Shapes represent each individual; black: D2 bronchoscopy, or grey: D7 bronchoscopy. D0 sample taken before BCG aerosol infection. Blue line median. KLRG1-PD1+ D7 p= 0.012 on pairwise comparisons against baseline only using Wilcoxon corrected with Holm-Bonferroni, following normality testing.

5.4 Discussion

5.4.1 Granulocytes

Neutrophils, the most prevalent white cell, make up 50-70% of all circulating leucocytes.[194,196] In my dataset, volunteers had a mean baseline of 58% circulating neutrophils, which is consistent with the literature.[194] I saw a reduction in circulating neutrophils after D7 post-BCG but no significant difference in the proportion of eosinophils. This was in contrast to the BAL where both neutrophils and eosinophils increased at D7 compared with D2. However, despite increasing, BAL eosinophils remained within the normal range, and this may be why I did not see a corresponding significant change in blood eosinophils.

As neutrophils only live for 1-2 days, the long-term trend in proportion seen here might reflect ongoing chemokine production in the lung, resulting in neutrophils leaving the circulation and trafficking to the lung; or there may be reduced bone marrow production.[654,655]

In healthy TB contacts, circulating neutrophils fell from 2-6 weeks compared with controls, a similar pattern to that seen here.[211,89] Furthermore, neutrophilia in the blood is associated with poorly controlled active TB disease, which resolves following TB treatment.[656,657] This might suggest that a robust immune response to a mycobacterial aerosol infection is associated with a fall in circulating neutrophils.

In my volunteers the blood NL ratio transiently increased at D2. A persistently high NL ratio (neutrophils/T cells or neutrophils/total lymphocytes) in the blood has been associated with increased risk of progression to TB disease and poor cure rates.[658,659]

The relationship between my findings and an *ex-vivo* functional assay of mycobacterial control will be explored in Chapter 6.

5.4.2 MPs

Systemically there was a trend for MPs to increase from D2, reaching significance at D28, whereas in the BAL fluid, MPs fell at D7 compared with D2. In the BAL, MPs made up a much larger proportion of CD3- cells than in the blood. This difference is likely due to the much higher frequency of neutrophils in blood. The cell population detected here likely includes monocytes, macrophages, DCs, but may also include inactivated neutrophils (downregulating CD66b).[199,573]

5.4.2.1 Monocytes

The percent of total baseline CD14+ CD3- cells (8.23%), which I have defined as total monocytes, is at the high end of the normal range of 1-10% leukocytes,[660,661] suggesting that the monocyte population is similar to that identified in other studies. CD14+ cells increased in the blood at D2 to just over 10% leucocytes, defined as a monocytosis, which resolved by D7.[661] The source of this increase may be high levels of IFN-y inducing myelocytosis in the bone marrow,[172] or it could be caused by the movement of other cells, such as lymphocytes, out of the blood, reflected in the increased ML ratio at this timepoint.

Monocytosis and high ML ratio in the blood is seen in poorly controlled active TB patients and resolves on treatment, while a high ML ratio is also associated with increased risk of TB disease in adults, adolescents and children.[168-171,662] It is unclear if the transient increase seen in my data is comparable to the sustained levels reported in these studies, in terms of association with poor mycobacterial control. Rather the transient rise may just reflect an influx in monocytes from the bone marrow prior to recirculation to the tissues.[663] Further investigation detecting macrophage colony-stimulating factor (M-CSF) and granulocyte-

macrophage colony-stimulating factor (GM-CSF) as well as homing markers may help define the source of these fluctuating cell populations.[663,664]

Monocyte effector subtype may be important than total monocyte proportion.[175,176] In my blood samples, classical monocytes fell and intermediate monocytes rose at D7 post-BCG, though this lost significance on correction. There was no difference seen in the non-classical monocyte population.

Increased CD16+ monocytes (intermediate and non-classical) is associated with poor control. In active TB patients, there are more intermediate and nonclassical monocytes than classical monocytes and this pattern is reversed with TB treatment.[171,665] The reverse pattern was seen in Verrall's healthy "early clearers" study, with a decline in total monocytes for 14 weeks post-exposure in persistently IGRA- contacts, driven by a fall in intermediate and non-classical monocytes.[89] Classical monocytes are better able to migrate to infection sites.[160,163] Reduced levels in TB patients might indicate a defective ability to mount a clearing local immune response.[666,667] Further, *M.tb* manipulates CD16+ monocytes to differentiate into an altered macrophage less capable of controlling *M.tb,* as CD16+ monocytes derived from TB patients have less capacity for antigen presentation and reduced ability to differentiate into DCs than classical monocytes.[668,665]

It is unclear what the trend of falling classical monocytes in my samples might imply. Again, given the transient fall, the explanation may be that the highly migratory effector classical monocytes are migrating to the lung.[666,667] Rather than seeing a fall in non-classical monocytes post *M.tb* exposure, a large cohort study by Joosten et al. of recently exposed healthy contacts saw an increase in CD14+dim monocytes, compared with controls and ID-BCG vaccinated adults (and LTBI and active TB patients). This increase was sustained at 24 months in one of the cohorts and was positively associated with control of mycobacterial growth using the MGIA.[395]

The reason for the difference in findings between Verrall's and Joosten's healthy contacts studies could be due to definition (all CD14+ dim versus CD14+dimCD16+) or median time since exposure between cohorts. Studies vary widely in definition of monocyte subsets, and as shown in my dataset, quality of flow cytometry panel, especially due to the issue of autofluorescence, can impact gating and results, making comparison across studies problematic.[669]

5.4.2.1.1 Cytokine production

There was a significant increase in the production of TNFα by antigen-specific monocytes from D2. The effect was still detectable 2 months post-BCG. More TNFα is produced by peripheral monocytes derived from TB patients than healthy controls.[670] However production is depressed in poor TB controllers, suggesting a robust monocyte-derived TNFα secretion is associated with better control. TNFα increases maturation of phagosomes containing BCG, promoting intracellular killing of the organism in *in-vitro* experiments.[325]

Given I detect increased TNFα production beyond the monocyte life cycle suggests either training, or response to memory adaptive cells.[162] In the above *in-vitro* experiment, even after pre-stimulating monocytes with T cell-derived IFN-y, the difference between healthy controls and TB patient-derived monocytes in terms of BCG-specific TNFα production remained.[325] In more recent studies, circulating monocytes from ID-BCG vaccinated individuals produced more TNFα and IL-1β on *ex-vivo* stimulation with *Mycobacteria* or unrelated pathogens than naïve cells.[299] This was seen at 2 weeks post-vaccination and was still present after a year on stimulation with LPS.[671] The authors determined this was due to trained immunity. Further work is required to establish if the monocytes in my study are exhibiting trained immunity.

I also saw an increase in IL-17 producing monocytes at D7 post-BCG. One study looking at early clearers in the Gambia demonstrated an increase in IL-17 with no associated increase in IL-17+ CD4+ T cells, MAIT or γδ T cells, which might suggest monocytes were the source of this cytokine.[262]

5.4.2.2 DCs

The proportion of circulating DCs in healthy adults varies depending on markers used, and ranges from 1-8% of PBMCs, though the generally accepted frequency is closer to 1%.[672-674] I detected a mean of 4.23% in my dataset. As with the BAL DCs, the population I have defined as circulating DCs likely includes a broader range of cells including CD16- monocytes.[182,575] There was no significant difference in DCs as a percentage of total cells across timepoints post-BCG. However, DCs fell at D14 out of total MPs, as did HLA-DR+ MPs. (DCs were CD16- CD11c+ HLA-DR+ MPs). This may indicate that DCs are tracking to the lungs or to lymph nodes at D14, but further investigation is required to confirm this. Alternatively, another MP population, such as immature DCs, are expanding, driving this change in relative proportions of DCs and HLA-DR+ MPs. Immature DCs are characterised by low HLA-DR expression and display enhanced phagocytic potential in the periphery.[675] *M.tb* reduces maturation and function of DCs in TB patients.[174]

5.4.3 NK cells (CD3-CD56+)

NK cells make up 5–20% of all circulating lymphocytes in humans.[676] NK cells from my samples made up 3.73% of circulating leucocytes at baseline and there was a trend to increase at D7 post-BCG. This mirrors what is occurring in the BAL at the same timepoint. Circulating NK cells were reduced in active TB compared with LTBI patients, while treatment restored circulating NK cells,[240] suggesting systemic NK cells may be associated with TB

control. Further, ID BCG revaccination, which has been associated with protection in some populations, boosts BCG-specific NK cells in the periphery.[300]

CD16+ NK cells seem to be driving the expansion of NK cells in my samples, with a mirroring fall in HLA-DR+ NK cells. While I haven't been able to fully define the NK cells here, $CD56^{dim}CD16^{+}$ NK cells display a cytotoxic phenotype, and have been shown, *in-vitro*, to lyse or induce apoptosis of *M.tb*-infected MPs via perforin and granzyme within 24 hours of co-culture, independent of IFN-y.[230,187,228,229,677] In South African adolescents, latency (a putative marker of control) is correlated with cytotoxicity mediated by CD16+ NK cells.[240] Further, *M.tb* exposed but QFT negative Kenyan adults had more cytotoxic NK cells than QFT positive adults.[244]

All activated NK cells express MHC-II and are capable, in turn, of activating CD4+ T cells.[678,679] The significance of the early fall in HLA-DR+ NK cells with subsequent increase from D28 is unclear, though may indicate a late ongoing driver of NK cell inflammation. Alternatively, investigation into NK cell training would be warranted.[94]

Blood-derived NK cells produce IFN-y on *in-vitro M.tb* co-culture and IFN-y+ NK cells are associated with control in TB patient cohorts.[241-243] Yet, there was no change in the antigen-specific circulating IFN-y+ NK cells in my data set. This may be because the majority of circulating NK cells in my samples were $CD16^{+}$, and $CD56^{bright}CD16^{-}$ cells are the main source of cytokines.[228,229] This compares to the BAL samples where only around 20% of the NK cells were CD16+ and where there was a trend for an increase in BCG-specific IFN-y+ production by NK cells at D7. As I only investigated IFN-y production up to 2 weeks post-infection in NK cells, future work will involve examining later time points, especially given activated NK cells are increasing in the blood from 1 month.

Further, Kleinnijenhuis et al. recently demonstrated no increase in *ex-vivo M.tb* or BCG-specific IFN-y production from peripheral NK cells after ID BCG vaccination.[94] Instead, these

NK cells produced IL-6, IL-1β and TNFα, which are associated with trained immunity. Due to the inherent limitations of flow panel design I was unable to investigate these in my samples, and it will form part of future work on cryopreserved cells.

5.4.4 Donor Unrestricted T cells (DURTs)

5.4.4.1 MAITs

I saw no change in total circulating MAIT cell frequency post-BCG. In NHPs, there was also no difference in MAIT frequencies following aerosol BCG infection.[91,411] Active TB patients have a lower frequency of MAIT cells in the blood than healthy subjects, and MAIT frequency is inversely correlated with disease severity.[257,258] LTBI patients have been reported with higher frequencies of MAITs in the peripheral circulation than healthy controls.[680]

The depletion of MAITs in the periphery in active TB patients is thought to be due to recruitment to the infection site.[105,681] However, not all studies report increased MAITs in the lungs of TB patients, with differences perhaps due to variability in lung tissue samples (biopsies, BAL, pleura) and disease states examined.[682] Here I show no change in circulating MAITs but a trend for increase in MAITs in the BAL at D7. Why there is no change in circulating MAITs in my study may be because a less virulent strain of mycobacterium was used. Alternatively, as data from these TB studies comes from established infections, it could be that early in infection, local expansion of MAITs occurs prior to any corresponding changes in the periphery.

The circulating MAIT cell population in my study was dominated by the CD8+ subset across all time points, which is consistent with the literature.[249] Post-BCG infection I saw an increase in PBMC-derived DN MAITs at D7 compared with D2. By contrast, the BAL was enriched with DN

MAITs at D2, with a shift to CD4+ and CD8+ MAITs at D7. As discussed in Chapter 4, circulating CD8+ MAITs have higher effector function than CD4+ MAITs, while DN MAITs are the terminally differentiated mature subset.[625]

Circulating MAITs from active TB patients are poor producers of IFN-y and have blunted cytotoxicity *ex-vivo*.[258] Functional impairment corresponds with an increase in the activation marker HLA-DR in these patients.[680,683] While I wasn't able to look at PBMC-derived MAIT cytokine or cytotoxicity effector function, I did see an increase in HLA-DR+ MAITs at D7 in the blood, particularly the CD4+ and DN MAIT subsets. Whether this corresponds to an increase or decrease in effector function warrants further investigation.

5.4.4.2 iNKTs

While there was a trend for iNKTs to increase in the BAL at D7 compared with D2 post-BCG, as with MAIT cells, there was no difference seen in PBMC-derived iNKTs. In a study of early clearers in Haitians, there was no difference in peripheral blood iNKT subsets compared with healthy controls.[263] Moreover, there was no change seen in the frequency of iNKTs in the blood 4 weeks post aerosol or IV BCG in NHPs, though the frequency of T_{EM} and T_{RM} circulating iNKTs did increase.[91,411] By contrast, active TB patients have low levels of circulating iNKTs compared with both healthy controls and *M.tb* exposed people.[170,277-279]

CD8+ iNKTs were the least frequent subset seen in the blood in my dataset, which is consistent with the literature.[271,680,684] While there was a trend for DN iNKTs to fall at D7 in the BAL, with an enrichment of the CD4+ subset, there was no difference in the blood compared with baseline. By contrast, in active TB patients there are lower DN iNKTs and more CD4+ iNKTs in the blood compared with controls.[271] In *in-vitro* studies, CD8+ and DN iNKT cells have a Th1-like polarisation and a strong cytolytic effector function and have been correlated with protection against *M.tb*-challenge in NHPs.[276,684] CD4+ iNKT cells have a more

immunoregulatory role, though they can produce both Th1 and Th2-type cytokines.[271] In my samples, there was an increase in antigen-specific IFN-y+ NKT-like cells, at D14 in the blood and at D7 in the BAL, though, due to my panel design, I was unable to determine IFN-y+ production from iNKTs or their subsets. Given NKTs require no priming and can release large quantities of cytokines following TCR recognition of antigen-loaded CD1d on MPs, it is unclear why the increased IFN-y responses seen here is delayed until 1-2 weeks after infection.[684] Due to inherent limitations in with flow panel marker numbers I was unable to further define these cells by effector function.

5.4.4.3 Other DURTs

Only 50% of the circulating CD161+ T cells described here were MAIT cells. CD161+ T cells are likely a combination of MAITs, iNKTs, γδ T cells and Group 1 CD1 cells (GMM-specific and germline-encoded mycolyl-reactive; GEMs).[271,685] I saw an increase of CD161+ T cells at D2 compared with D7 in the BAL but no difference in the blood. Following IV BCG in NHPs, there was an expansion of GMM-specific T cells in both the BAL and blood at 4 weeks which suggests that, in the NHP model, lipid Ag-specific T cells may contribute to an *M.tb* protective immune response.[411]

All γδ T cell results in the blood were unremarkable, possibly due to the technical staining issues described. However, the median baseline frequency of γδ2+ T cells was 5.68% CD3+ cells (IQR 3.3-7.8%) and γδ1+3 T cells were 0.53% CD3+ cells (IQR 0.27-0.85%). This fits with the prevalence described in the literature, where total circulating γδ T cells are 1-5% of lymphocytes, of which γδ2 T cells make up 80-90%.[634] Hence it is unclear why I am not detecting differences following aerosol BCG infection. It may be that power was not sufficient due to low sample size at D2 or D7, or that changes occur after D14 in the periphery.

Alternatively, the changes detected locally and described in Chapter 4 may not translate to detectable responses in the periphery.

I saw an increase in the non-specific CD161+ CD26+ TRAV1-2/Va7.2- CD4+ T cell population. The IFN-y+ subset of this population (which I was unable to investigate due to flow panel design limitations) has been associated with protection in ID BCG vaccinated infants.[686] Furthermore, BCG revaccination in South African adults led to an increase in IFN-y+ antigen-specific CD26+ CD8+ MAITs in the periphery. The association with these cells and control of mycobacterial growth in an *ex-vivo* functional assay will be explored in Chapter 6.

5.4.5 T cells

Following aerosol BCG infection, total circulating T cells fell at D2 and there was a trend for an increase at D7 compared with baseline. At D2, the mean was low normal at 15.4% total leucocytes (normal counts 15-30%).[687] There was no lymphocytopaenia on the clinical full blood count for any volunteer in TB043. Total baseline T cells, as well as CD4+ and CD8+ subsets defined by flow cytometry, were within the normal range for healthy subjects, as were T cells at all other timepoints.[687]

Mirroring the blood, there was an increase in total BAL T cells at D7 compared with D2. It would be interesting to see if the BAL completely mimics the blood, with higher baseline T cells than at D2, but baseline bronchoscopy was not performed. Following aerosol BCG infection in NHPs, a similar biphasic pattern was seen, though this was later than described here, with a fall in BAL and blood at 2 weeks and rise in both compartments at 6 weeks.[422]

It is unclear if the D2 fall in peripheral T cells is due to tracking to the lung, given that by D7 T cells increase in both compartments. A trend for an increase in antigen-specific CD29+CD49+ T cells and a fall at the same time point of the non-specific CD29+CD49+ or CD103+ CD4+ or CD8+ T cells might suggest these cells are homing to the lung, however the picture is unclear

and results may be affected by low cell numbers and large intra-individual variation. After aerosol BCG infection of NHPs, CD29+CD49+ T cells increased in the BAL, but the picture was variable in the blood, as seen here.[426]

CD8+ T cells increased expression of the homing lung marker CXCR3 at D2. CXCR3+ T cells from healthy contacts are associated with control of mycobacterial growth on MGIA.[395] However, baseline levels of CXCR3+ cells were much higher in my samples than reported in the literature (median 98.77% CD8+T cells compared with 25% in the literature),[688] despite using an FMO which gave clear population separation. Hence further panel work-up is required to confirm the results.

An alternative explanation for the D2 T cell drop, other than T cell migration, is proliferation of neutrophils and monocytes changing the proportion of singlets. Other explanations such as T cell death or reduced proliferative capacity,[689] are less likely given the drop occurs so quickly and because there is no indication clinically of BCG-osis or cytokine storm, as discussed in Chapter 3.

In the blood, all three T cell subtypes seemed to contribute to the change in total T cells as there was no difference in proportion of CD4+, CD8+ or DN T cells. This contrasts with the mucosa where there was an enrichment of CD4+ T cells and fall in proportion of CD8+ T cells. Given the importance of CD4+ T cells in the control of TB,[361,412,690] the difference seen here between the mucosal and systemic early immune response is interesting. Low peripheral CD4+ T cells, with or without a corresponding rise in CD8+ T cells has been described in several studies of patients with pulmonary TB, with normalisation on treatment.[691] It is unclear why we are not seeing a change in subtypes in the periphery, especially given the changes I described at the mucosa. It may be that in people with robust T cell peripheral proliferative capacity and thymic function, an influx of CD4+ T cells into the mucosa may not be detectable systemically due to an ability to readily replenish these cells.[692-694] Given the

short time period, it is unlikely the increase in the mucosa is due to local CD4+ T cell expansion following LN priming, unless the CD4+ T cells are from a memory population.[693,695] Alternatively, the change in CD4+/CD8+ T cell ratio seen in the periphery in TB disease may be in response to a chronic assault, perhaps with periodic seeding of the bacterium systemically, which is not relevant to the locally contained, presumably rapidly cleared, mycobacterial infection in my study.

5.4.6 Antigen-specific IFN-y+ cellular immune responses

For the first time, here I demonstrate that aerosol BCG infection in *M.tb*-naïve humans results in a robust systemic mycobacteria-specific cellular immune response by D7. This was clearly demonstrated on ELISpot, with a corresponding significant rise in IFN-y+ from antigen-specific circulating CD4+ T cells on flow cytometry.

Antigen-specific IFN-y+ CD4+ T cells also increased in the BAL at D7 (compared with D2). This is a similar pattern to that seen in mice and an NHP aerosol model where antigen-specific IFN-y+ CD4+ responses in the blood reflect lung responses.[419,426]

5.4.6.1 Transient effector response

The antigen-specific response in my samples was transient, returning to baseline levels by 1-3 months. In viral infections, peripheral effector T cells return to baseline, usually via apoptosis, once the infection has been cleared.[694] Memory T cells remain, but at low frequencies. In yellow fever, effector CD8+ T cell response correlated with viral load and returned to baseline levels at around 1 month.[694] In *M.tb,* due to the ability to develop latency, it is unclear whether IGRA persistence or reversion relates to bacterial clearance, or simply reflects variability in secretion of ESAT-6 and CFP-10 by the bacterium.[26,45,696] There is also ongoing

debate whether protection conferred by ID BCG vaccination is due to bacterial persistence or the development of a memory response. In mice, BCG has been demonstrated in the spleen and draining lymph nodes up to 16 months post ID vaccination, and up to 17 months post intranasal vaccination.[391,697,698] BCG can enter a state of dormancy as it has the same *M.tb* DosS/DosR system.[45] Persistence in immune competent humans beyond one month following ID BCG has not been definitively demonstrated, however disseminated BCG disease has been reported in HIV/AIDs patients decades after ID BCG vaccination.[480,699]

Given these volunteers have robust immune responses, it is likely that the return to baseline effector response is due to BCG clearance. This is supported by my findings in Chapter 3 where no BCG was detected in the sputum at the 3 and 6 month time points. Bronchoscopies from 14 days onwards would help pinpoint the mucosal clearance timepoint and how it relates to the immune signature seen here. However, negative BAL culture would not exclude persistence in regional LNs or other areas.

5.4.6.2 Kinetics

Aerosol BCG infection appears to result in a stronger and earlier systemic peak response than after ID BCG infection. Peripheral PPD-specific IFN-γ ELISpot responses in TB041 volunteers appeared from 7 days, peaking at 1-4 weeks post-ID BCG vaccination. The strength and kinetics of this response after ID vaccination is consistent with that seen in previous trials in our group following ID BCG in BCG-naïve volunteers.[484,700] By contrast, there was a sharp peak in D7 systemic ELISpot responses following aerosol BCG in both TB041 and TB043 and there was a trend for higher peak response than following BCG ID, despite a similar or lower dose being delivered mucosally (see discussion of delivered dose in Chapter 3).[483,484] This is a different pattern to that seen in the NHP model where intra-tracheal BCG resulted in lower systemic ELISpot responses than intradermal BCG.[304] Why an aerosol mycobacterial infection

leads to stronger systemic responses than ID delivery in humans warrants further investigation.

The large surface area of the highly vascular mucosa might allow a more efficient delivery of antigen to the systemic circulation[701] than the more contained subcutaneous delivery, thereby explaining the faster, more robust systemic immune response seen in my study. However, given the inherent immunotolerant environment of the mucosa, mucosal infection or vaccination might equally result in a more attenuated systemic response at the same delivered dose than a pathogen delivered systemically.[702,703] Comparison between the kinetics induced by mucosal and systemic vaccine delivery for other pathogens might be helpful here. Mucosal immunisation can induce similar or stronger local responses than systemic immunisations without compromising systemic responses.[701,704] However there is limited evidence for induction of a stronger or faster systemic immune response such as that seen following aerosol BCG in my study. The recombinant cholera toxin B subunit vaccine has been delivered orally, intranasally, rectally, vaginally or intracutaneously.[705,706] Immune responses induced by the cholera toxin B subunit vaccine delivered mucosally depend on the route of delivery. In humans, nasal immunisation induces development of IgA in the nose, lungs and genital tract, oral vaccination to responses in the small intestine, ascending colon, salivary and mammary gland and rectal immunisation induces local responses limited to the distal colon.[707] While the causal mechanisms have yet to be fully described, these differences are likely due to different homing marker imprinting by DCs, as discussed earlier.[708] While the majority of studies compare mucosal immunisation routes only, [705,706] one study compared antibody responses following oral, intranasal, or systemic cholera toxin B subunit immunisation.[709] Oral immunisation produced more IgG and IgA antibody specific cells in the blood at D7 than the intranasal or intracutaneous route at the same timepoint.[709] This might suggest that in some instances mucosal delivery can produce stronger or faster systemic

responses than following systemic delivery. However, the nasal live cold-attenuated influenza vaccine induces higher local IgA antibodies and cell-mediated immunity, especially in children, but less serum antibody than the inactivated trivalent vaccine delivered subcutaneously or intramuscularly.[710,711] The differences seen with the influenza vaccine may be due to the inherent differences in the vaccines, rather than route of administration. Perhaps the most useful study compared the antibody and cellular immune responses following mucosal (intranasal) or systemic (subcutaneous) inoculation with the live attenuated rubella and mumps vaccine, and with naturally acquired inhaled mumps infection. Systemic rubella immunisation resulted in robust systemic responses but blunted effect at the local respiratory site; while intranasal rubella immunisation elicited a poor systemic response. By contrast, natural inhaled mumps infection, elicited comparable antibody and cellular responses systemically and mucosally; as did subcutaneous mumps vaccination.[712] Hence there is minimal evidence that delivery via the mucosa (gut, respiratory system or genital tract) elicits a stronger early systemic response to systemic delivery, but responses seem dependent on the vaccine type and pathogen. Hence, the stronger faster systemic responses seen following aerosol mucosal BCG infection may simply be due to the specific immune responses elicited by this slow growing mycobacterium.

The kinetics following BCG aerosol infection however, also seems faster than the development of an antigen-specific systemic response to aerosol *M.tb* infection in human field and animal studies. In humans, TST conversion, a measure of delayed Type IV hypersensitivity to ID *M.tb* antigen, appears as early as 2 weeks, but with peak conversion between 4-8 weeks.[369,371] There is a similar kinetic for IGRA conversion.[370] Whether IGRA conversion can occur earlier than this, has not, to my knowledge been described.

In mice the antigen-specific IFN-y+ CD4+ T cell response after *M.tb* aerosol infection is not detected systemically until 2- 3 weeks post infection, and in NHPs, not until 3-4 weeks.[54,91,341,418]

It could be that, due to the inherent limitations of field studies, defining such an early initial immune response to a mycobacterial infection in humans has not been possible. This hypothesis is more likely if the response is transient, such as seen in my study. A transient IGRA conversion is one theory for the "early clearer" phenomenon.[75] Alternatively, *M.tb* virulence factors not present in BCG might delay the immune response, as is seen in mouse models.[54]

This cannot be the explanation in controlled aerosol BCG animal models however. Systemic cellular antigen-specific responses can peak in NHPs as late as 8 weeks post aerosol BCG infection and in some models are delayed compared with ID BCG. [426,104,105,423] By contrast, T cell expansion occurs in NHP lungs 2 weeks after protective IV BCG delivery.[91,392,426] Differences in kinetics may be due to inherent biological differences between animals and humans.[18,2233]

Another hypothesis is that the antigen-specific response I have detected here is a rapid secondary response. Boosting baseline historic BCG vaccine responses with a second ID BCG vaccine is well described.[26,484] Moreover, in previously exposed mice, antigen-specific T cells occur earlier, at 1 week post *M.tb* aerosol infection, though in other mouse studies, baseline PPD-specific responses do not change on re-challenge.[143,341]

Some of my volunteers had high baseline PPD responses on ELISpot and BCG responses on WB ICS. Volunteers are not screened for PPD or BCG reactivity before enrolment. Due to species homology, PPD antigens are expressed in BCG and most non-tuberculous mycobacteria.[69,70] To exclude *M.tb* infection prior to enrolment, all volunteers are screened for reactivity to two *M.tb* specific antigens, CFP-10 and ESAT-6 on IGRA.[71] As there is no BCG-

specific antigen that has been identified to date, historic volunteer exposure to BCG is determined on history and evidence of scar. As not all infant BCG immunisations lead to scar formation,[713] it is possible some of these volunteers had been previously vaccinated with BCG. However, this would not explain the early antigen-specific responses seen in the majority of volunteer samples.

High baseline responses to either PPD or BCG could be due to NTM exposure. NTMs are distributed worldwide but are believed to be of higher incidence in countries around the equator.[5,714] If historic NTM exposure were responsible for the higher baseline or immune priming in the majority of samples, this would suggest higher environmental prevalence in the UK than previous described.[715] While incidence of NTM infection is difficult to determine in the UK as colonisation surveys are not routine practice, UK prevalence of NTM disease is rising.[564,565]

It is uncertain whether NTM exposure reduces the immunogenicity of BCG vaccination.[5,647,716] Here I show that there was a trend for positive correlation at peak ELISpot response with baseline readings, which in my samples suggests masking is not occurring. In a systematic review of UK adolescents with low pre-vaccination PPD responses, intradermal vaccination resulted in high IFN-y+ ELISpot responses, peaking at 3 months and detectable at 12 months.[12] In the Malawi cohort with high baseline PPD-specific responses, there was a lower peak post-ID BCG vaccination and no difference at 1 year compared with controls. These findings contrast with the results shown here where we see a peak response early at D7, despite some volunteers having high baseline PPD responses. Hence it is not certain that BCG or NTM priming accounts for the responses seen in my study. Volunteers may be truly mycobacteria-naïve, with strong early responses due to the novel route of BCG delivery.

To further explore this, I plan to repeat volunteer baseline ELISpot using a recently developed NTM peptide pool. Proliferation assays to detect central memory PPD responses at baseline

are also in process. Preliminary results, however, do not suggest any detectable central memory response.

5.4.6.3 Differences between ELISpot and whole blood ICS assays

IFN-y+ WB ICS results did not reflect the magnitude seen on ELISpot. The difference may be because flow cytometry is less sensitive than ELISpot. However, differences between the two methods may also account for these results. ELISpot was performed fresh, while the whole blood for ICS was frozen then thawed before staining. This results in a modest but definitive decreased mean fluorescent intensity.[717] The ELISpot assay has been standardised to ensure a sensitive highly reproducible assay across studies in our group and the WB ICS assay was developed separately based on work in Hanekom's lab.[497] Therefore, in addition to the inherent differences in the techniques, there are differences in stimulation times.
PPD and PBMCs were used in the ELISpot assay, as BCG and WB causes higher background readings (Harris, Tanner, unpub). However, I used WB and BCG in the flow cytometry ICS. While concordance between PBMC and WB ICS has been demonstrated,[718] I decided to use WB to include neutrophils, serum and other components. Additionally, while there is no difference between BCG and PPD stimulation on WB ICS for classical T cell responses,[719] whole cell stimulation, but not peptide, results in a greater contribution from NK cells and DURTs.[720] BCG might more closely mimic *in-vivo* infection, where live phagocytosed BCG leads to different MHC-II loading than free antigen, I used this in the WB ICS. However, BCG may be less antigenic than PPD on overnight stimulation assays, as only external BCG antigens may be available in that short period, where the secreted antigen is present from the start in PPD. Following ID BCG vaccination in mice, different antigens were recognised at different time points: Culture filtrate at D7 followed by cell wall, membrane, and cytosol-derived antigen.[636]

Both assays were used in this work as ELISpots are a highly sensitive assay, and WBICS has the advantage that further definition of the cell populations using cell surface staining is possible.

5.4.6.4 Cellular cytokine responses

I have shown here that BCG-specific CD4+ and CD8+ cytokine responses occur early after an aerosol BCG infection in healthy adults, they are detectable in the blood, and seem to mirror BAL responses, though with a much smaller effect.

From D7, aerosol BCG led to a systemic Th1 CD4+ T cell phenotype (IFN-y, TNFα and IL-2). In addition, I saw a trend for increased IFN-y+ CD8+ T cells at 1 month. Though different in timing and magnitude, this cytokine phenotype is similar to that described after ID BCG vaccination, and in *M.tb* infected people (LTBI and active TB).[484,721,722]

Th1 cytokines are a hallmark of protective *M.tb* immunity defined by Mendelian susceptibility, TB field studies and animal models.[55,307,372,645] However the significance of early systemic Th1 responses for protection is yet to be defined. Aerosol *M.tb* exposure with IGRA conversion is associated with increased frequencies of circulating Th1 CD4+ T cells, while healthy household contacts ("early clearers") have lower Th1 CD4+ T cells than TB cases.[337,723] Moreover, persistently TST-negative healthy contacts had lower antigen-specific IFN-y+ responses than TST-converters from 3 months.[724] In NHPs given intra-tracheal BCG, peripheral cytokine responses did not correlate with subsequent protection against *M.tb* challenge.[341] The significance of the early cytokine production shown in my study is therefore unclear and will be explored in the next chapter.

While there was a trend for increased IFN-y+ CD8+ T cells at 1 month, this did not reach significance and there was no significant change in any other CD8+ T cell cytokines. This is in contrast to the BAL CD8+ T cells, with increased IFN-y+ production at D7 (though a lower magnitude to CD4+ T cells) and upregulation of Th1 and Th17 cytokine gene expression.

Aerosol BCG infection of rhesus macaques, using the same Omron nebuliser as my study, induced Th1 cytokine+ CD8+ T cells in both BAL and blood.[426] However in two other airway BCG NHP models there were no systemic CD8+ cytokine responses after aerosol BCG, while, there was after protective IV BCG.[91,341] As discussed previously, the role of MHC-1-restricted CD8+ T cells in protection is debated.[388,725] Association with protection is discussed in the next chapter.

There was no difference in the IFN-γ+ DN T cells, which may include DURT populations.[249,269-271,281,282] I also saw no difference in IFN-γ+ γδ T cells compared with baseline. I was not able to specifically look at cytokine production of MAITs or iNKTs but there was an increase in IFN-γ+ CD3+CD56+ NKTs at D14 as discussed earlier.

5.4.6.5 IL-2

In my samples I saw an increase in IL-2+ CD4+ T cells from 1-2 months, and double positive IL-2 with IFN-γ or TNFα. IL-2 is associated with the development of central memory T cells.[331,726] Aerosol BCG infection of NHPs leads to an expansion of circulating CD4+ central memory T cells.[426] IL-2+ T cells, double or triple positive for TNFα or IFN-γ+, are associated with protection against *M.tb* in mice.[726] Further, in the recent BCG revaccination study of South African adolescents, where a protective effect against sustained infection was demonstrated, there was an increase in peripheral IFN-γ+ and/or or IL-2+ CD4+ T cells following BCG revaccination.[26] Future work would include confirmation of central memory cell expansion by staining for central memory markers CD45RA and CCR7.[308]

I also saw an increase in IL-2+ monocytes. Given the main known source of IL-2 is T cells, this warrants further investigation.[727]

5.4.6.6 IL-17

I saw no significant IL-17 production in any T cell. Systemic Th17 CD4+ T cells are lower in TB cases than healthy controls and IL-17 polyfunctional CD4+ T cells have been associated with protection in the NHP and mouse model.[336,337] BCG may not a potent inducer of IL-17. BCG lacks ESAT-6 which induces Th17 differentiation in a TLR-2 dependent manner in mice.[14,652] However, protective intratracheal and IV BCG induces Th17 cells in NHPs.[91,341]

5.4.6.7 Polyfunctionality

In my data set, the only significant Th1 CD4+ polycytokine cells were double positive IL-2 with either IFN-γ or TNFα. There was no CD8+ T cell polyfunctionality detected.

The protective efficacy of polyfunctional Th1 CD4+ T cells is debated, with a recent review showing no effect, and healthy household contacts expressing lower levels than TB adults.[330,337,728] It may be that certain cytokine subtypes are the key to protection or risk, with various effector and memory biomarkers, such as PD-1, KRLG1, HLA-DR, effector memory (CD45RA- CD27-) or central memory (CD45RA–CCR7+) reported in the literature.[318,722,723,728-730]

I may not be seeing a polycytokine CD4+ or CD8+ T cell signature because some data was confounded by high background. A cleaner cytokine panel and full volunteer numbers at D2 and D7 might have reduced the background noise, and increased the power.

5.4.6.8 Antigen-specific biomarkers

HLA-DR+ Th1 CD4+ T cells (IFN-γ +/- TNFα) have been associated with poor TB control.[372-373,374,729,731] HLA-DR+ T cells directly present antigen to other T cells and might be indicative of high antigen-burden environments,[722] which may explain the association with recently

acquired or uncontrolled *M.tb* infection. As I didn't have HLA-DR on my Th1 panel I was unable to look at this marker in association with cytokines. However, non-specific HLA-DR+ T cells did not increase in the WB samples, whereas they did in the BAL (D7 compared to D2). This may simply be due to the antigen load in the BAL, compared with the periphery.

I saw an increase at D7 in circulating BCG-specific effector IFN-y+ PD1+KLRG1- CD4+ T cells. I did not detect a change in the BAL, but as discussed this may be due to low sample size. Lung resident antigen-specific PD-1+KLRG1- CD4+ T cells are associated with protection in mice.[375,458] However, systemic PD-1 has also been described as an exhaustion marker in models of patients with active uncontrolled TB, where treatment reduces PD-1 expression on MAIT cells or CD8+ T cells.[331,680,732] The significance of an early increase in antigen-specific PD-1+KLRG1- CD4+ T cells systemically in humans is yet to be explored.

5.5 Conclusions

In this chapter I have shown that an aerosol BCG infection results in the following changes in the systemic circulation:

- A fall in circulating neutrophils, which has been associated with mycobacterial control. There was no change in eosinophils.
- A transient increase in NL and ML ratio at D2. Chronic increase is associated with poor *M.tb* control but the significance of an early transient increase is yet to be explored.
- Monocyte production of antigen-specific TNFα from D2, which is associated with protection. The effect was still detectable at D56, which may indicate the presence of trained immunity.
- An increase in activated NK cells at D28. This may be indicative of a cytotoxic phenotype, which is associated with protection in field studies.
- No change in circulating total iNKTs, γδ or MAITs, which is consistent with reports from some early clearer studies, though findings are variable.
- A fall in total T cells at D2, an increase at D7, then resolution. This may be due to T cell migration into the lung. No difference was seen in CD4+ and CD8+ T cell subsets, which was in marked contrast to the local mucosal responses.
- A significant increase in total antigen-specific IFN-y+ from D7 with corresponding increase in IFN-y+ CD4+ T cells. These responses were earlier than predicted from animal models.

A summary of the novel findings in this chapter are outlined in Chapter 7, Table 7.1.

In the next chapter I will report the findings of an *ex-vivo* mycobacterial growth inhibition assay and explore correlations with the safety and immunogenicity data discussed in the preceding chapters.

Chapter 6.

Correlates of protection

6 Correlates of Protection

6.1 Introduction

The MGIA is an unbiased summative *ex-vivo* functional assay which has been validated in *M.tb* challenge mouse and NHP models as a surrogate of protection.[449-452] Growth inhibition has been shown to improve in humans following ID BCG vaccination across three different laboratories in the UK and The Netherlands[395,448,456,733] and the BCG MGIA was able to discriminate active TB, latently infected and uninfected UK adults.[447] In addition, BCG correlates with *M.tb* as the *in-vitro* challenge inoculum in human MGIA assays.[447,734] The BCG MGIA is therefore considered a potentially useful surrogate of protective efficacy. While it remains unclear which immune mechanisms mediate this growth inhibition, purported mechanisms include innate effects, IFN-γ+ CD4+ T cells, polyfunctional T cells, humoral responses, or various transcriptomic or cytokine profiles.[395,447,448,733] Here I investigate the change over time in MGIA control of mycobacterial growth following an aerosol BCG infection and correlate any changes to the clinical and immune endpoints described in previous chapters.

6.2 Statement of Authorship

The MGIA was performed by J.Marshall with the supervision of R. Tanner, and the help of S.Harris, E.Hoogkamer and H.Morrison.

6.3 Results

When comparing post-infection control of mycobacterial growth using the MGIA with each volunteer's pre-infection response, overall there was no difference using this assay (Figure 6.1). There was also no difference between BCG and saline controls (data not shown). However, the BCG inoculum control had a high Time to Positivity (TTP) with a mean of 9 days and 20 hours, whereas we aim for a TTP of 7 days in our inoculum control, representing 500cfu. This might indicate that there were insufficient numbers of mycobacteria in this assay, due to use of an old BCG stock in which titre had declined.

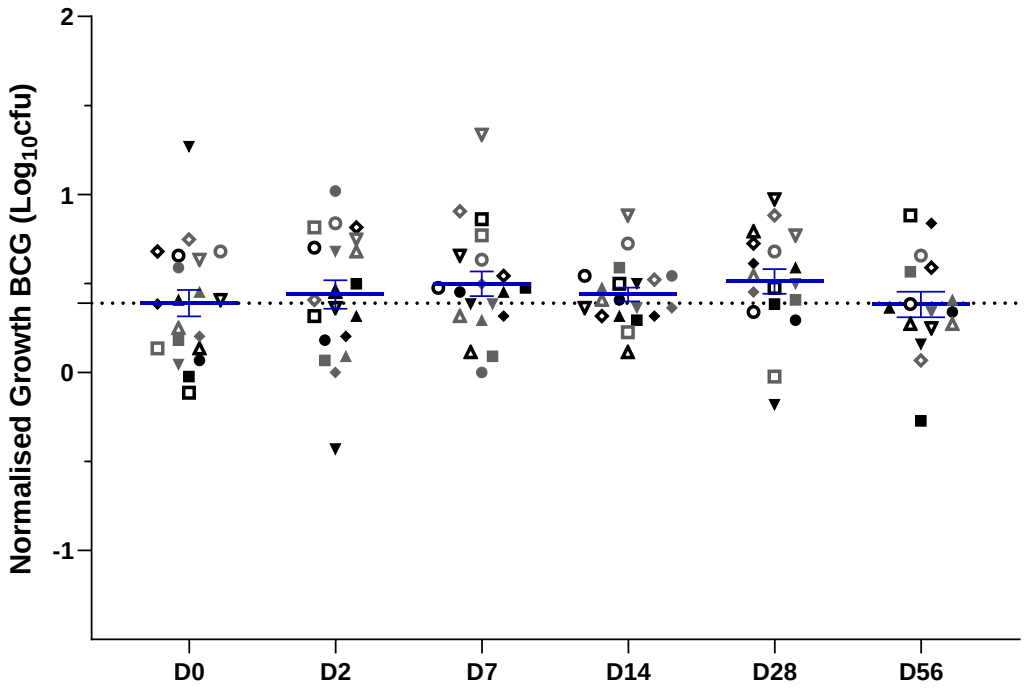


Figure 6.1. Mycobacterial Growth Inhibition Assay of aerosol BCG infected volunteers, using PBMC and autologous serum. Calculated as Log_{10}cfu timepoint minus Log_{10}cfu inoculum control. Blue bars indicate mean with SEM and dotted line represents baseline mean (inoculum control). Shapes show each individual; black D2 bronchoscopy, grey D7 bronchoscopy. D0 sample taken before BCG aerosol infection.

I repeated the experiment over two separate days (due to logistical feasibility) with new BCG stock and this time the inoculum control was as expected, with a mean TTP of 7 days, 0 hours.

However, in this second run, there was a clear batch effect, despite Group 1 and 2 volunteers being evenly spread over the two separate experiments (Figure 6.2a). Neither batch was significantly different over time when analysed separately. I normalised the data by plotting TTP fold change (Figure 6.2b). With the data combined, there was a significant improvement in BCG growth control at D14 compared with D7 (paired t-test p=0.027) but the effect was lost when correcting for multiple comparisons.

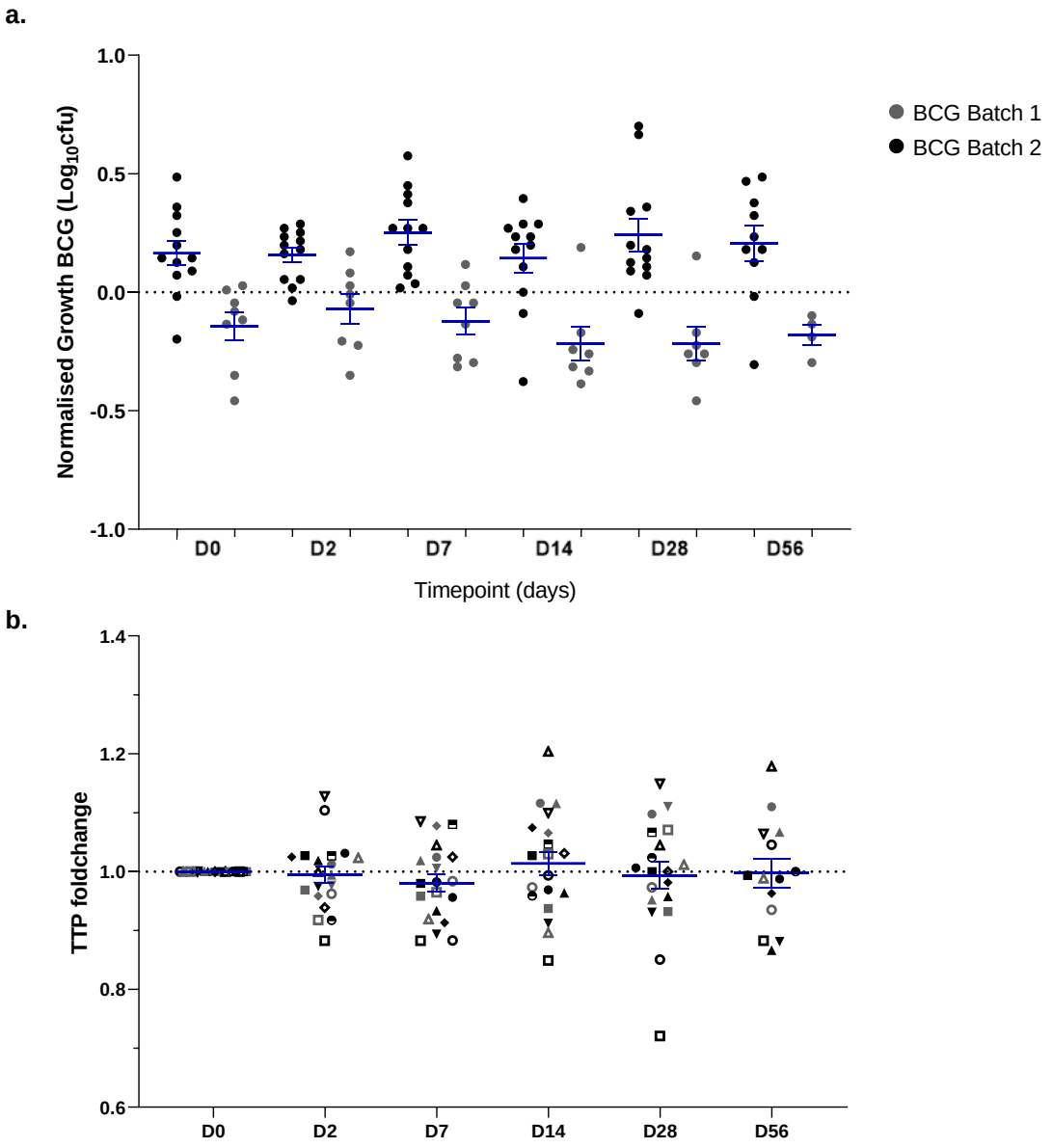


Figure 6.2. Mycobacterial Growth Inhibition Assay of aerosol BCG infected volunteers, using PBMC and autologous plasma. (a) Calculated as Log_{10}cfu timepoint minus Log_{10}cfu inoculum control. Dotted line represents baseline mean (inoculum control). (b) TTP fold change calculated as TTP/TTP baseline. A higher TTP represents better mycobacterial growth control. Blue bars indicate mean with SEM, after normality testing. Grey batch 1; black batch 2, shapes represent each individual.

Work is ongoing to re-optimise the inoculum volume for the assay and all future work will process samples in a single run.

I used the TTP data from the repeat experiment to perform a correlations analysis on all significant results from the mucosal and systemic immune responses, as well as BCG loaded dose, BCG recovery from BAL and adverse events (total, respiratory and systemic). These were compared against ELISpot and MGIA TTP at all timepoints. In total there were 96 correlations performed using a two-tailed Spearman's Rho bivariate correlation. Variables with only D2 or D7 values were split by group for analysis. Below are the significant results, with a moderate or strong negative or positive correlation coefficient (r_s=0.600 or above).[735] Sample sizes of three or less were excluded from the analysis.

There was a positive correlation between baseline antigen-specific IFN-γ ELISpot responses and peak mycobacterial growth control at D14 (Figure 6.3a). Antigen-specific IFN-γ ELISpot responses and TTP also correlated at D14 and D56 (Figure 6.3a), and other timepoints (Table 6.1).

TTP results positively correlated over time. Baseline mycobacterial growth control correlated with TTP at D7. D7 and D14 TTP correlated with later timepoints (Figure 6.3b and Table 6.1).

Table 6.1. Spearman's Rho bivariate correlation between significant clinical and immune variables

$r_s \geq -/+ 0.600$, $p<0.05$; * highlights negative correlations; cells % parent unless otherwise stated

Variable 1	Variable 2	Correlation coefficient	Significance (2-tailed)	n
TTP D0	TTP D7	0.662	0.001	20
TTP D0	IFN-γ+ CD4+ T cells (BAL) D7	-0.857 *	0.014	7
TTP D0	IFN-γ+ CD4+CD8+ T cells (BAL) D7	-0.786 *	0.036	7
TTP D0	CD29+CD49+ IFN-γ+ CD4+ T cells (BAL) D7	-0.786 *	0.036	7
TTP D0	CD103+ IFN-γ+ CD4+ T cells (BAL) D7	-0.964 *	<0.0001	7
TTP D2	HLA-DR+ NK cells % CD3- (BAL) D7	-0.685 *	0.029	10
TTP D2	IFN-γ+ CD4+ T cells (BAL) D7	-0.786 *	0.036	7
TTP D2	CD103+ IFN-γ+ CD4+ T cells (BAL) D7	-0.786 *	0.036	7
TTP D2	CXCR3+ IFN-γ+ CD4+ T cells (BAL) D7	-0.811 *	0.027	7
TTP D7	TTP D14	0.787	<0.0001	19
TTP D7	TTP D28	0.743	<0.0001	19
TTP D7	TTP D56	0.770	0.001	14
TTP D7	ELISpot D14	0.640	0.002	20
TTP D7	CD8+ T cells % leucocytes (BAL) D2	0.778	0.023	8
TTP D7	IFN-γ+ CD8+ T cells (BAL) D7	-0.964 *	0.000	7
TTP D14	TTP D28	0.787	<0.0001	19
TTP D14	TTP D56	0.744	0.002	14
TTP D14	ELISpot D0	0.654	0.002	20
TTP D14	ELISpot D14	0.685	0.001	20
TTP D14	MPs % leucocytes (BAL) D2	-0.638 *	0.047	10
TTP D14	CD4+ T cells % leucocytes (BAL) D2	0.738	0.037	8
TTP D14	CD8+ T cells % leucocytes (BAL) D2	0.929	0.001	8
TTP D14	DN T cells % leucocytes (BAL) D2	0.738	0.037	8
TTP D14	CD4+CD8+ T cells % leucocytes (BAL) D2	0.778	0.023	8
TTP D14	CD4/CD8 ratio (BAL) D7	0.826	0.011	8
TTP D28	CD8+ T cells % leucocytes (BAL) D2	0.778	0.023	8
TTP D28	CD4+CD8+ T cells % leucocytes (BAL) D2	0.729	0.04	8
TTP D28	PD1+ IFN-γ+ CD8+ T cells (BAL) D7	-0.841 *	0.036	6
TTP D56	ELISpot D14	0.851	<0.0001	14
TTP D56	ELISpot D56	0.772	0.001	14
TTP D56	ML ratio (WB) D2	0.601	0.023	14
TTP D56	Eosinophils % CD3- (BAL) D2	0.703	0.035	9
TTP D56	T cells % leucocytes (BAL) D7	0.900	0.037	5
TTP D56	CD4+ % leucocytes (BAL) D7	0.900	0.037	5
ELISpot D0	MPs % leucocytes (BAL) D2	-0.709 *	0.022	10
ELISpot D0	HLA-DR+ T cells (BAL) D2	-0.745 *	0.013	10
ELISpot D0	NK cells %CD3- (WB) D7	0.622	0.003	20
ELISpot D0	HLA-DR+ T cells % leucocytes (BAL) D7	0.879	0.001	10
ELISpot D0	HLA-DR+ CD16+ NK cells % CD3- (BAL) D7	0.758	0.011	10
ELISpot D0	CD4/CD8 ratio (BAL) D7	0.767	0.016	9
ELISpot D0	IFN-γ+ T cells (BAL) D7	0.786	0.036	7
ELISpot D0	IFN-γ+ CD4+CD8+T cells (BAL) D7	0.857	0.014	7
ELISpot D0	CD29+CD49+ IFN-γ+ CD4+ T cells (BAL) D7	0.857	0.014	7
ELISpot D2	ELISpot D14	0.842	0.002	10
ELISpot D2	Total Adverse Events	0.675	0.032	10
ELISpot D2	CD4+CD8+ T cells % leucocytes (BAL) D2	0.731	0.04	8
ELISpot D7	Neutrophils % CD3- (BAL) D2	0.669	0.035	10
ELISpot D7	CD16+ NK cells (BAL) D2	0.745	0.013	10
ELISpot D7	HLA-DR+ T cells % leucocytes (BAL) D7	0.673	0.033	10
ELISpot D7	CD4+/CD8+ T cell ratio (BAL) D7	0.883	0.002	9
ELISpot D14	ELISpot D56	0.882	<0.0001	17
ELISpot D14	CD8+ T cells % leucocytes (BAL) D2	0.881	0.004	8
ELISpot D14	CD4+CD8+ T cells % leucocytes (BAL) D2	0.743	0.035	8
ELISpot D14	CD4+ T cells % leucocytes (BAL) D7	0.728	0.026	9

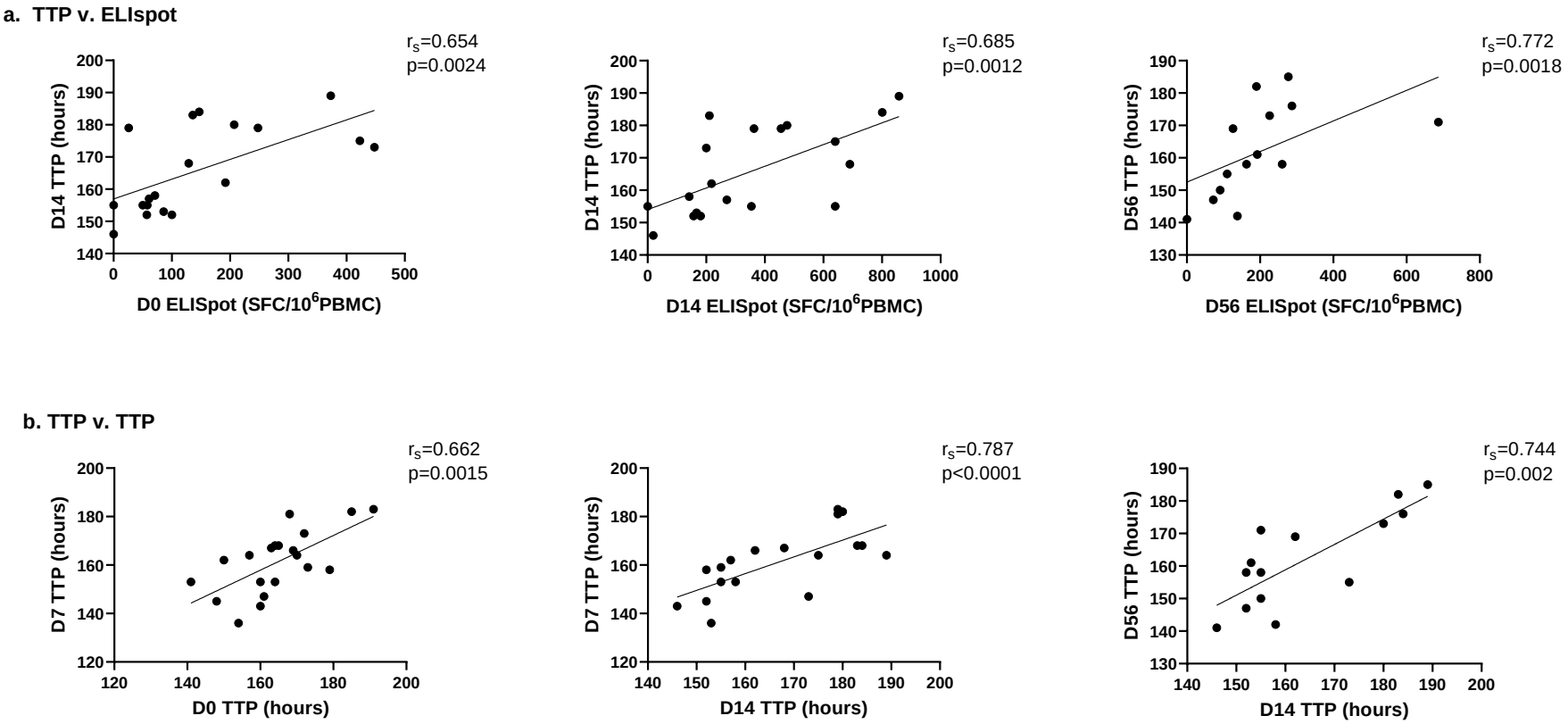


Figure 6.3. Positive correlation between mycobacterial growth control and *ex-vivo* PPD-specific IFN-γ ELISPOT. (a) TTP and ELISpot; **(b)** Selected TTP correlations. Two-tailed Spearman's Rho bivariate correlation. Each dot represents an individual. SFC: spot forming cells. TTP: Time to Positivity on Mycobacterial Growth Inhibition Assay using PBMC, autologous plasma and BCG Pasteur, where higher TTP represents better mycobacterial growth control.

An increase in any T cell subset in the BAL at D2 positively correlated with peak mycobacterial growth control (D14), whereas there was a negative correlation between mononuclear phagocytes (MPs) in the BAL at D2 and subsequent growth control at D14. A higher CD4+ T cell to CD8+ T cell ratio in the BAL at D7 positively correlated with peak D14 growth control.

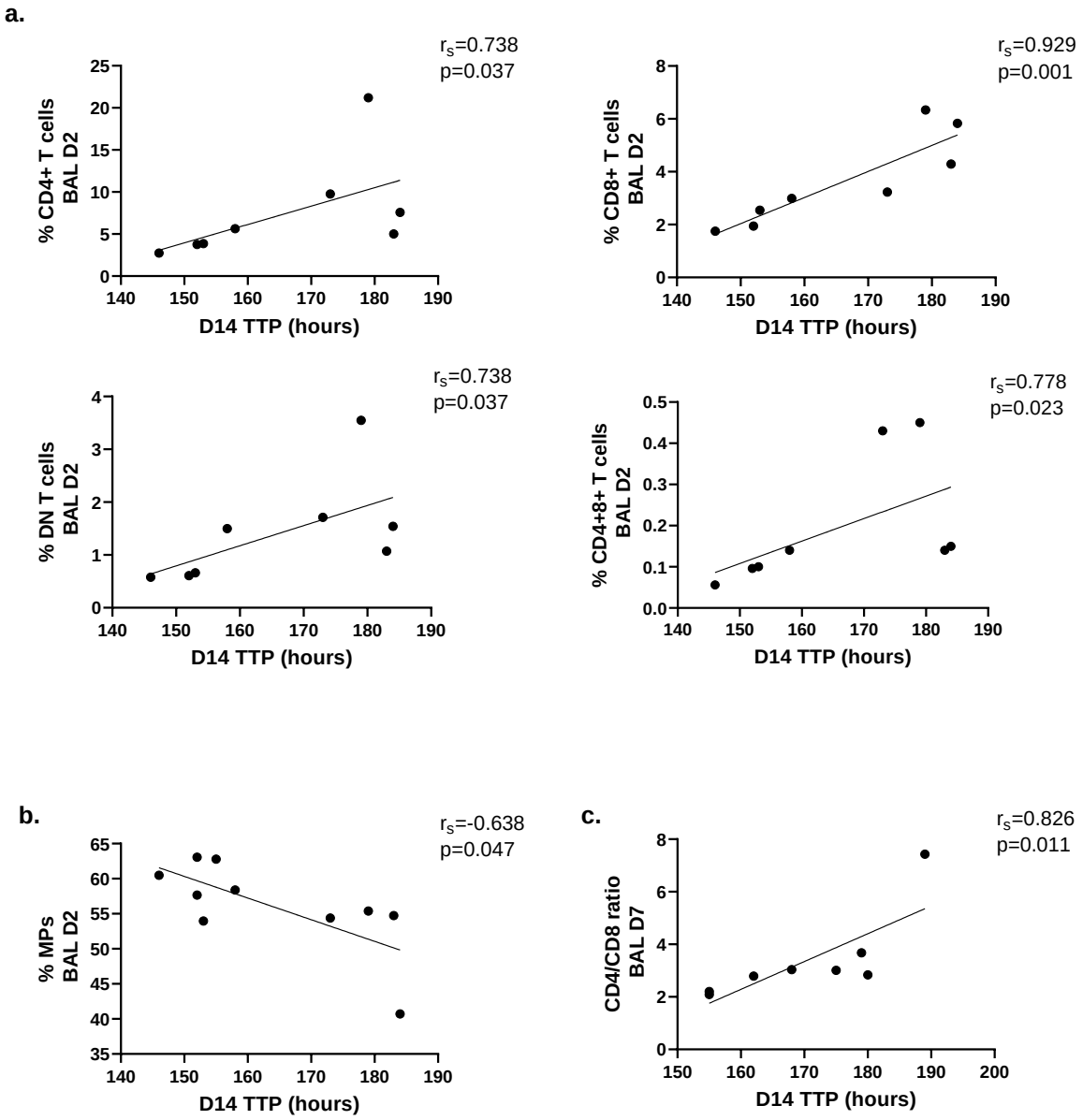


Figure 6.4. Two-tailed Spearman's Rho bivariate correlation between D14 TTP and (a) T cell subsets in bronchoalveolar lavage (BAL) at D2; (b) Mononuclear Phagocytes (MPs) in BAL at D2; or (c) CD4+:CD8+ T cell ratio in BAL at D7. Cells are % total leucocytes as measured on flow cytometry. Each dot represents an individual. TTP: Time to Positivity on Mycobacterial Growth Inhibition Assay using PBMC, autologous plasma and BCG Pasteur, where higher TTP represents better mycobacterial growth control.

There was a negative correlation between D0 or D2 TTP, and activated or lung homing CD4+ T cells or NK cells in the BAL at D7 (Table 6.1). By contrast, baseline antigen specific IFN-y ELISpot responses positively correlated with activated and homing total T cells, CD4+ T cells and NK cells at D7 in the BAL, though not at D2.

In addition, peak ELISpot responses at D7 positively correlated with mucosal D2 neutrophils, CD16+ NK cells and mucosal D7 HLA-DR+ T cells and CD4+/CD8+ ratio (Figure 6.5). There was no association with individual IFN-y producing cells in the BAL or blood, and total IFN-y production as measured by ELISpot at the corresponding timepoint, in this limited correlation analysis.

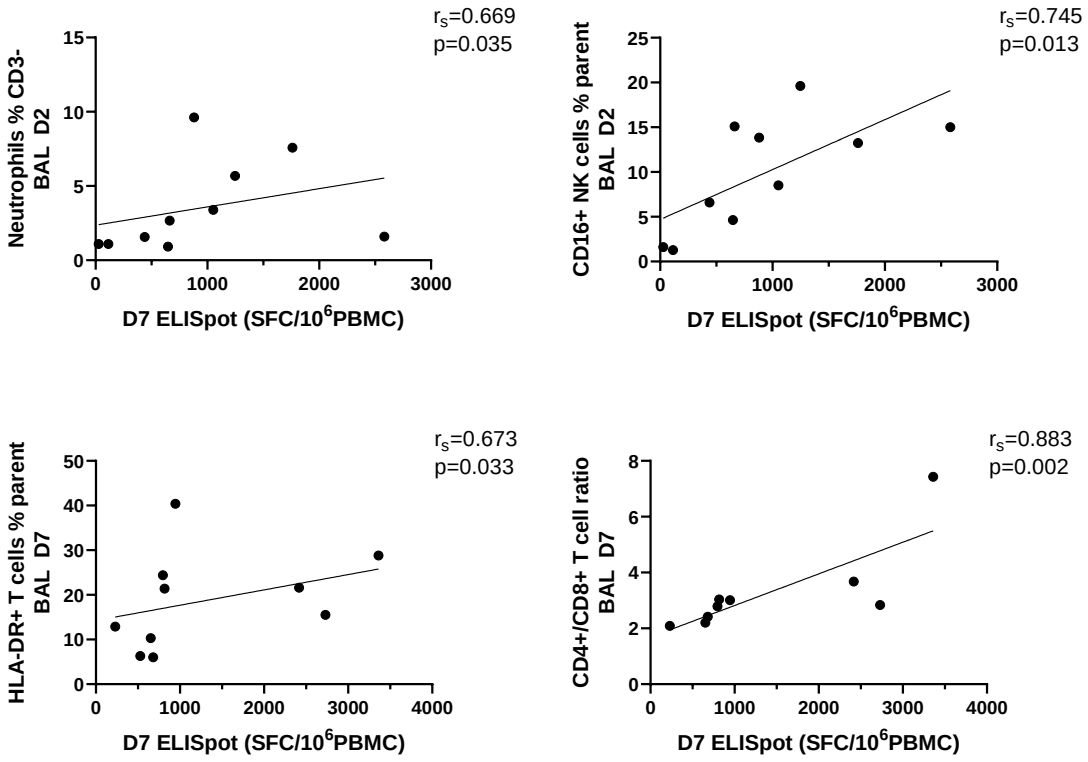


Figure 6.5. Two-tailed Spearman's Rho bivariate correlation between D7 ELISpot and BAL cells at D2 or D7. Cells % defined by flow cytometry. ELISpot: *Ex-vivo* IFN-y ELISpot PBMC responses to overnight stimulation with PPD. Each dot represents an individual. SFC: spot forming cells.

6.4 Discussion

6.4.1 Mycobacterial Growth Inhibition Assay (MGIA)

I did not observe any change in control of mycobacterial growth over time in the initial MGIA. The mycobacterial inoculum was lower than the target inoculum of 500cfu, as evidenced by the long TTP of the inoculum control. An accurate mycobacterial inoculum is critical to sensitivity of the assay, which could explain the results seen here.[452,736]

The repeat MGIA assay had the correct inoculum input, but was run in two batches which showed a significant batch effect. Batch effect is common between different runs of complex functional assays, though the MGIA protocol has recently been optimised to reduce this effect.[455] However, despite using the optimised protocol and the same operators, I saw a batch effect. Normalising based on an inoculum control aids comparability across sites and assay runs.[452,455] However, unusually, the inoculum control TTP was within one hour of each other between batches. I was able to successfully normalise the data using TTP fold change, and saw a significant improvement in mycobacterial growth control after aerosol BCG infection at D14 compared with D7, though there was no significant difference compared with baseline.

It is unclear what caused the batch effect, though current investigation into the condition of the incubators is ongoing. In addition, further work-up of this assay is planned, using positive control samples with known mycobacterial growth control, alongside samples from this study, to confirm the findings here. Therefore, these preliminary results are to be treated with caution and require repeat analysis once a re-validated MGIA had been run, to provide confidence in the conclusions presented here.

Following ID BCG vaccination in humans, the MGIA has previously detected an enhanced ability to control mycobacterial growth, with peak growth control at 56 days.[395,452,455,456,733]

Seeing a protective effect at D14 rather than D56, as seen after BCG ID infection, would fit with the faster kinetics following aerosol BCG infection described in previous chapters. The effect I saw in my study at D14 was transient, though a subset of my volunteers were improving their control at D56. This transient response has been previously described and may indicate the MGIA is mediated by innate mechanisms or that the MGIA response is due to effector, not central memory T cells.[395,448,733] However, the association of MGIA response with many other measures of adaptive immunity suggests that this assay is measuring a sum of the parts.[395,447,448,733]

6.4.2 Correlation between *ex-vivo* MGIA and *in-vivo* immune mechanisms

While there was not a strong overall signal in the change in mycobacterial growth over time, MGIA, the ability to control growth can be measured on an individual level and compared to immune variables in a correlates analysis. This analysis was intentionally limited to significant flow cytometry immune endpoints, with ELISpot and MGIA TTP only, as an initial exploratory analysis. Further work with full analysis of all endpoints, including transcriptomics, is planned and will form a separate body of research that is beyond the scope of this thesis.

Here I showed that local increase in all T cell subsets at D2 and an increase in CD4+ T cells compared with CD8+ T cells at D7, correlated with the peak ability to reduce mycobacterial growth systemically as measured by an *ex-vivo* functional assay. This also correlated with reduced MPs at D2. This would fit with putative mechanisms of protection, where migration away from the parenchyma to the LNs by MPs and an early influx into the local infection site by T cells, particularly by CD4+ T cells promotes local mycobacterial control.[360,362,737] An alternate explanation for the negative correlation between improved systemic control at D14 is that fewer MPs, particularly macrophages, might lead to less infective niches in which the mycobacteria can proliferate and hide from the effector immune response.[53] In either

scenario, the subsequent development of an effective local *in-vivo* clearing immune response may translate into systemic changes in ability to control mycobacterial growth, which are detected on the *ex-vivo* MGIA a week later.

Of note, there were no neutrophils in the PBMC MGIA assay, and hence any influence of neutrophils in *ex-vivo* control would not be detected.

Volunteers who were good mycobacterial controllers at baseline (MGIA D0 TTP), and very early following BCG aerosol infection (TTP D2), had reduced levels of antigen-specific IFN-γ+ T cells, including homing T cells, in the BAL at D7. This might suggest an early clearing response, resulting in reduced antigen and less drive for a local expansion of adaptive immune cells. This hypothesis is supported by the finding that TTP D0 positively correlated with TTP at D7 which correlated with later timepoints, suggesting a good baseline level of mycobacterial control correlates with superior control in this assay following aerosol BCG infection. Whether this early control is due to a rapid innate clearing response is unclear, as there was no correlation between innate immune cells in the BAL or blood and the D0 TTP in my analysis. This may be due to the fact that my analysis included only a limited number of immune variables, or that D2 is too late to detect a rapid innate-mediated clearance.

In addition, there was no correlation between BCG retrieved from the BAL at D2 or D7 and the TTP or any immune correlate. This may be because the analysis was underpowered or because I was unable to establish a quantitative read out for BCG detection. Regardless, because of this, it is difficult to conclude whether the *ex-vivo* MGIA correlates to a clearing response *in-vivo*.

6.4.3 Antigen-specific IFN-γ correlates with protection

Volunteers who had strong ELISpot responses at baseline had increased capacity for mycobacterial growth control on MGIA at D14. ELISpot responses and TTP also correlated at

D14 and D56. This finding supports the epidemiological evidence that antigen-specific IFN-y is required for protection against mycobacteria.[314,738] The association between IFN-y and MGIA has also been reported in mice[736] and human studies.[448,456] In particular, a correlation with PBMC MGIA and PPD-specific IFN-y ELISpot after ID BCG vaccination has been described at 4 weeks in healthy UK adults.[456] In this study, ELISpot responses correlated with MGIA following primary but not secondary ID vaccination. By contrast, in their cohort study of healthy *M.tb* controllers, Joosten et al. did not see an association with IFN-y+ T cells but did see an association with CXCL10+ monocytes in a T cell dependent manner,[395] where IFN-y is an upstream mediator of as IFN-inducible protein 10 (IP-10) and CXCL10.[739]

I did not see a correlation with D14 MGIA and monocytes in the blood, though there was a positive correlation between D2 ML ratio and D56 TTP. In the blood following aerosol BCG infection I saw a transient dip in T cells and corresponding rise in monocytes which resulted in an increased ML ratio at D2 that resolved by D7. The positive correlation with high ML ratio and improved mycobacterial growth control at D56 might suggest a protective role for a high ML ratio in this context. This supports the findings in Joosten's study, but is contradictory to other studies showing an association between high ML and risk of TB progression and poor MGIA growth control.[168,395,447] Potential reasons for these differences, were discussed in Chapter 5.

Baseline antigen specific IFN-y+ ELISpot also positively correlated with activated and homing total T cells, CD4+ T cells and NK cells at D7 in the BAL. Given that baseline ELISpot responses also correlated with improved growth control at D14, these local *in-vivo* responses at D7 may associate with a protective response systemically. Alternatively, they may be an indication of increased antigen in the BAL at D7.[722] Regardless, it is fascinating that baseline immune levels seem to predispose certain volunteers to an enrichment of mycobacterial-specific T cells in the airway by D7. If these volunteers are truly mycobacterial naïve (see discussion in

Chapter 5), this would suggest an inherent predisposition to mycobacterial control, or at least to mounting an early local antigen-specific response, which may be in keeping with the observation of natural *M.tb* controllers in epidemiologic studies. The underlying cause of this priming may be trained immunity from previous pathogen exposure such as cytomegalovirus (CMV),[538,539] or underlying genetic differences e.g. in SNPs such as the leukotriene LTA4H.[351,540]

Peak systemic ELISpot response at D7 positively correlated with mucosal D2 neutrophils and CD16+ NK cells and mucosal D7 HLA-DR+ T cells and CD4+/CD8+ T cell ratio. Neutrophils may aid DC tracking to the LNs and thereby boost antigen-specific responses.[207] In addition, early neutrophilia may be associated with protection.[143] In a whole blood MGIA of healthy *M.tb* contacts, control of BCG was significantly impaired by neutrophil depletion.[211] Whether an increase in mucosal neutrophils and increase in systemic IFN-y+ production correlates with protection is yet to be established. The association between mucosal D2 CD16+ NK cells and antigen-specific IFN-y production in the blood at D7 seen here, is interesting. Activated $CD56^{dim}$CD16+ cytotoxic NK cells lyse *M.tb*-infected cells and hence may be a marker of antigen load.[241-243] Alternatively, NK cells could be a source of the IFN-y on the ELISpot, however this was not supported by my flow cytometry findings in Chapter 5. Similarly, as discussed, HLA-DR+ T cells could be an indicator of increased antigen load, which may be why mucosal HLA-DR+ T cells are correlating with ELISpot in the blood at the same time point. [722] Whether these cells are a main source of IFN-y+ warrants further investigation.

There was no correlation between total cellular derived IFN-y on ELISpot and cell-specific IFN-y production in the BAL or blood at any given timepoint. This is despite IFN-y+ CD4+ T cells increasing in the blood on flow cytometry at the same timepoint as peak ELISpot responses (Chapter 5). The reason for this discrepancy may be the limited number of variables in my

analysis, and for a definitive answer, a full correlates analysis of all variables is required. If after this analysis there is still no correlation with cell-specific IFN-y and ELISpot, it may be that there are other sources of IFN-y that are contributing to the ELISpot that were not detected in my study, such as MAITs and γδ T cells.[249,281,282] Alternatively, the study may be underpowered to detect any correlation, especially as WB ICS was limited to 10 samples at D7.

Interestingly, loaded dose did not correlate with TTP or any immune endpoint that I investigated. This may because the loaded dose range between volunteers was small. However, in a previous study in our group, there was no difference in ELISpot responses following standard dose or one log higher of ID BCG SSI or ID TICE.[483]

There was no direct association seen between mycobacterial growth control and clinical symptoms, BCG retrieved in the BAL, DURTs, single or polycytokine producing cells or other immune endpoints. This may be due to the limited analysis done to date and a more systematic correlation analysis is warranted.

Alternatively, it may be that the MGIA, using PBMC and plasma, is not sufficiently sensitive to detect changes to mycobacterial growth inhibition mediated by peripheral cells, given the infection occurred in the lung mucosa. Many of the peripheral effector immune responses described in Chapter 5, may not correlate with protection. Certainly, variable evidence for systemic immune correlates would support this hypothesis.[73,653] Future work, developing a human BAL cell MGIA is needed, following on from the recent development of a murine lung MGIA.[740] This would also enable any protective effect from surfactant and BALF to be captured in the functional assay.[99]

6.5 Conclusions

- Following aerosol BCG infection there was an improvement in mycobacterial growth control at D14 compared to D7 using the *ex-vivo* functional MGIA.
- This control was earlier to that seen following BCG ID infection and may indicate faster immune mediated protection following infection via the mucosal route.
- MGIA findings reported here need repeating on a re-validated MGIA.
- There was a positive correlation between antigen-specific IFN-y ELISpot response and MGIA growth control.
- Baseline TTP and ELISpot predicted TTP and ELISpot at later timepoints.
- Mucosal T cell subsets positively correlated with peak growth control while MPs negatively correlated.
- These findings support evidence from animal studies and human epidemiological studies that antigen-specific IFN-y is required for protection against mycobacteria. Additionally, the correlation with mucosal responses and growth control highlights the importance of describing local infection site responses.
- ML ratio in the blood at D2 correlated with growth control at 2 months. This contrasts with reports of chronic *M.tb* infection in the literature, where increased ML ratio is associated with poor control.

Ex-vivo MGIA depletion experiments are required to confirm the contribution of these immune variables with growth control, and development of a BAL MGIA may yield important insights between local immune responses and protection.

A summary of the novel findings in this chapter are outlined in Chapter 7, Table 7.1.

Chapter 7.

Conclusions

7 Conclusions

In this body of work, I have developed a human BCG aerosol infectious challenge model and used it to investigate the early immune responses to a mycobacterial infection. I have fulfilled all the aims defined at the beginning of my DPhil.

7.1 Assessing model safety

I have demonstrated that aerosolised BCG (using two strains) can be safely administered to *M.tb* and BCG-naïve UK adult volunteers, up to a loaded dose of $1x10^7$cfu. This challenge model is well tolerated and logistically feasible, confirming my primary hypothesis.

This is the first time BCG has been delivered by aerosol in a controlled study to naïve adults, and the first mycobacterial challenge to be delivered by inhalation, mimicking the natural route of infection. In their PPD and BCG lobar instillation models, Silver and Dhedas' groups reported no significant AEs at D2 and D3.[206,409] I report similar findings here, with a majority of mild AEs and no SUSARs. A subset of volunteers experienced symptoms consistent with systemic and local (pulmonary) immune reactogenicity. All symptoms were transient and self-resolved. However, the BCG Danish strain would be the preferred strain for future challenge studies while it remains the available BCG strain in the UK. This is because BCG Bulgaria inhalation elicited severe symptoms in a small subset of volunteers, while volunteers inhaling BCG Danish reported less severe symptoms at the same dose. Overall, the loaded dose of $1x10^7$cfu elicited strong mucosal and systemic responses while causing few symptoms and hence this dose will likely be used for future studies.

BCG was detected in the airways at D2, D7 and D14 after BCG aerosol inhalation, but was not detected by induced sputum, at 3 months (in TB043). This indicates either a clearing immune response, or dormancy. As volunteers had no long-term symptoms, BCG was likely cleared by

an effective immune response, though long-term dormancy within tissues cannot be excluded. A future body of work is planned to quantify the amount of live and dead BCG in the airways. This would enable correlation with underlying immune processes.

7.2 Using this model to assess the early immune responses in the airway, to better understand protective mechanisms, to further vaccine development.

In this thesis, I have used this challenge model to investigate underlying immune processes. This was an initial exploratory study to develop a pool of potential correlates of protection for further evaluation. Specific immune findings and how they could be targeted for vaccine development are summarised below. This model has allowed me to describe the innate and adaptive local mucosal and systemic immune responses to an aerosol mycobacterial infection. While work is ongoing to correlate these findings with the *ex-vivo* MGIA as a functional read-out of growth control, as the volunteers clinically controlled the BCG infection *in-vivo,* this in an effective model for generating immune response data associated with mycobacterial control. Further work, as described above, may be able to confirm if this model is also a model of mycobacterial clearance. In this study, aerosol BCG infection resulted in adaptive immune responses, and hence the innate immune mechanisms I have described are not a model of "early immune clearance" (a putative innate clearance mechanism by definition independent of the adaptive immune response). Rather the immune responses outlined in this study may represent mechanisms that result in "delayed clearance" (a clearing combined innate and adaptive immune response).

A major limitation of this study is that the immune mechanisms described here may not be directly applicable to an *M.tb* infection due to the lack of some virulence factors (e.g. those encoded at the RD1 locus). While animal models have identified immune evasion strategies employed by *M.tb*, it is uncertain what mechanisms *M.tb* uses to evade recognition and

clearance by the human host. These strategies may be different to animal models, given *M.tb* has co-evolved with humans for millennia. This aerosol BCG model is unable to identify these *M.tb* –specific evasion strategies and hence cannot be used to develop vaccine strategies to overcome such mechanisms. Further, as BCG growth is controlled early and does not usually result in granuloma formation in immunocompetent individuals, this model cannot be used to describe the immune processes which contain *M.tb* within granulomas or which trigger transformation from LTBI to active disease, and hence cannot inform vaccine design aimed at preventing progression to disease. Finally, this model has been developed in a relatively homogenous UK population for logistical and safety reasons. Expansion of the model into a more diverse population would improve the applicability of immune correlates.

In the absence of a virulent *M.tb* human challenge, this aerosol BCG model is a useful tool to understand underlying immune mechanisms and to generate hypotheses for correlates of protection. Validation of immune mechanisms and correlates of protection identified using this BCG aerosol model in *M.tb* animal studies and TB field trials would help identify which mechanisms might also be protective against *M.tb*, which would overcome some of the limitations of this model.

7.2.1 Other uses for this aerosol BCG model

7.2.1.1 Testing candidate TB vaccines

In the absence of a validated attenuated *M.tb* challenge system, this BCG challenge model could be developed to test candidate TB vaccines.[387,741,742] Vaccine efficacy could be tested by demonstrating improved control of BCG growth after BCG aerosol challenge. The current model, where I successfully detected live BCG in the BAL in some volunteers at D2 or D7,

could demonstrate improved control if the candidate TB vaccine led to sterilising immunity in close to 100% of volunteers at D2 or D7. However, such vaccine-induced protection is unlikely. In order to develop a more sensitive model, quantification of BCG in the BAL is required. BCG recovery negatively correlated to BCG delivered dose in the TB041 dose escalation groups (data not shown here) and hence, achieving some degree of BCG quantification in this model is possible.

A limitation of this challenge model for TB candidate vaccine development is that it would not be suitable for the evaluation of vaccines containing RD1 antigens. However, many current candidate vaccines do not contain these antigens. In addition, the clinical protocols and procedures I have created as part of the development of these BCG challenge studies could be easily applied once other mycobacterial challenge agents are available. Given the poor predictive value of pre-clinical models in TB,[30,31] this aerosol BCG model could complement animal studies and allow the rational selection of vaccine candidates for larger scale efficacy trials.

7.2.1.2 BCG aerosol delivery as a novel vaccination route

Aerosol BCG delivery could be developed as an improved route of vaccination. Aerosol vaccine delivery is preferential to ID delivery as it removes the necessity for needles, with the associated risk of needle stick injuries, requirement for health worker training and safe disposal of sharps. Further, aerosol delivery is often preferred by the patient as it is pain free.

In some NHP models, aerosol BCG confers superior protection against and *M.tb* challenge than ID BCG.[743] I have demonstrated that BCG delivered via this route elicits robust antigen-specific mucosal and systemic responses in humans, without the need for an adjuvant,[704]

postulated as required for mucosal vaccine delivery in order to overcome the inherent immunotolerant environment.[702,703] Whether these responses confer similar or superior protective efficacy and durability to ID BCG delivery would require a large efficacy trial.

An aerosol BCG vaccine could also be tested as a boost to an ID BCG vaccination. ID BCG booster vaccines are currently being investigated as protection against sustained infection in adolescents (NCT04152161), as the initial POI trial demonstrated that ID BCG revaccination resulted in 45.4% efficacy against sustained IGRA conversion.[26] Given the robust innate and Th1 adaptive responses elicited from BCG aerosol vaccination in my study, a BCG ID aerosol prime boost may provide robust protection in adolescent and adult populations. However, safety would need to be evaluated first.[704] A previous trial in our group elicited a transient hypersensitivity reaction following MVA85A ID prime aerosol boost vaccination regime, where vaccines were administered one month apart.[387] While this may have been a reaction to the vaccine formulation as opposed to the antigen itself, consideration of the safety of this vaccine combination is warranted. Secondly, excluding the theoretical risk of a Koch reaction when boosting people with pre-existing mycobacterial immunity is required. The Koch phenomenon, development of immunopathology in a person infected with TB due to an exaggerated *M.tb* immune response, was described by Koch in patients with TB disease when using mycobacteria as a therapeutic vaccination. It has also been reported in a therapeutic vaccine mouse model.[741] A small trial assessing responses to aerosol MVA85A in LTBI patients elicited no safety concerns.[742] However, if aerosol BCG were to be delivered worldwide, especially in *M.tb* endemic settings, a phase 1 trial enrolling *M.tb* exposed volunteers would be required. Further, trials assessing safety in a wider population, such as volunteers with respiratory disease such as asthma, would be required before consideration of aerosol BCG as a route of vaccine delivery.

7.3 Immune responses following aerosol BCG infection

This thesis presents the first description in humans of early, D2 and D7, immune processes at the airway parenchymal interface, following an inhaled mycobacterial infection.

Table 7.1 summarises the novel findings in this study, and places these findings in the context of what was previously known in the field. It expands upon Table 1.2 from the Introduction.

Table 7.1. Summary of the main novel findings in the study (red text) in the context of known responses to mycobacterial airway infection in animal models and human TB infection studies (black text)

Cell	Animal studies: mice and other small animal models	Animal studies: non-human primates (NHPs)	Human studies: peripheral blood and genome-wide association	Human studies: lung
Alveolar macrophages (AMs) M1/Classically activated (CD86^{hi} HLA-DR+) or M2/alternatively activated (CD206^{hi} CD14+)	AMs are the first to encounter *M.tb.* and they are the primary infection target of *M.tb* in mouse infection models.[48,103] On mouse *in-vitro* co-culture experiments, AMs recognise *M.tb* PAMPs and ingest the bacteria by phagocytosis. *M.tb* evades killing by inhibiting phagolysosomal fusion and preventing autophagy and reduces antigen presentation.[128,129,137,138] *M.tb* triggers cell necrosis allowing dissemination of bacteria. [129,130,134] Mouse AMs do not upregulate effector function until 10 days after *M.tb* aerosol infection.[54,146] Autophagy of *M.tb* by AMs in *in-vitro* mouse models upregulates MHC-II antigen presentation, stimulating CD4+ T cell responses; also upregulates MHC-I and CD1 antigen presentation inducing CD8+ T cell and CD1-restricted DURTs.[135,136] Mycobacteria-infected mouse AMs aid CD4+ T cell activation and recruitment and are associated	Depletion of total AMs increases animal survival, but depletion of M1 AMs reduces survival and/or limits the granuloma's ability to contain the organism.[150] These M1 AMs are overcome *M.tb* inhibition of lysosome fusion and kill the bacteria through the combined effect of NO, exposure to the acidic environment in the lysosome and autophagy.[121]	CD206 (upregulated in M2 AMs) has is associated with poor TB prognosis.[153] Polymorphisms in the Vitamin D Receptor ApaI Gene reduces risk of TB disease. Activation of the receptor leads to transcription of genes involved in macrophage activation and killing of intracellular bacteria.[152] Human mannose receptor activation by *M.tb* on *in-vitro* co-culture inhibits phagolysosome fusion and reduces antigen presentation.[400] *In-vitro* models have also demonstrated BCG phagocytosis, phagolysosome fusion, MHC-II processing, apoptosis, antigen presentation and Th1 polarisation by human AMs on mycobacterial co-culture.[121,125-127,745]	*M.tb* induces expression of Matrix metalloproteinases (MMPs) by infected macrophages *in-vivo* and are detected next to areas of tissue destruction in patients with active TB.[155,156] MMPs degrade the pulmonary extracellular matrix allowing *M.tb* to disseminate. AMs decrease in BAL at D3 following BCG lobar instillation in mycobacteria-exposed patients.[206] Novel study findings: Less AMs in the BAL post- BCG infection, at both D2 and D7, compared to controls; likely a marker of macrophage infection and subsequent destruction (as seen in mouse models). AMs upregulated genes associated with antigen processing, presentation, MHC-II presentation and lung homing at D2 and D7 post-BCG which is much faster than that seen in mouse models. This study is the first to demonstrate these AM effector responses following aerosol

Cell	Animal studies: mice and other small animal models	Animal studies: non-human primates (NHPs)	Human studies: peripheral blood and genome-wide association	Human studies: lung
	with protection on *M.tb* challenge,[398] and yet AM-depleted mice demonstrate improved recruitment of cytotoxic cells leading to *M.tb* clearance.[145,744]			mycobacterial infection *in-vivo* in humans.
Monocytes	Mouse monocytes are less able to kill intracellular *M.tb,* and hence infection of monocytes by *M.tb* promotes *M.tb* dissemination in mouse models.[131] In mouse and zebra fish *M.tb* infection models, mycobacteria selectively recruit permissive monocytes to the infection site.[131]	ML ratio increased in the periphery of susceptible NHP species by 12 weeks following *M.tb* infection. This was not seen in the more TB disease resistant Chinese cynomolgus macaque.[401]	Increased monocyte lymphocyte (ML) ratio in the peripheral blood is associated with risk of developing TB disease.[168-171] Monocyte primary immunodeficiency results in disseminated BCG infection.[190] Novel findings: CD14+ cells increased in the blood at D2 post-BCG leading to a monocytosis, which resolved by D7. There was an associated transient increased ML ratio at this timepoint. Positive correlation between D2 ML ratio and control of mycobacterial growth on ex-vivo assay at D56.	Novel findings: There was a trend for an influx of non-resident monocyte-derived macrophages into the airway following BCG infection.
Dendritic cells (DCs) The classical DC subtype (cDC) is identified on flow cytometry by CD141/CLEC9 (cDC1); or, CD1c/CCL17 (classical; cDC2); and plasmacytoid DCs (pDC) by CD123. All subtypes are found in the lung, though pDCs are more common in the blood and secondary lymphoid tissue.	Mice depleted of DCs are unable to mount an effective CD4+ T cell response against *M.tb* with subsequent overwhelming *M.tb* infection.[189] The delay in the development of a robust adaptive immune response in mouse models, a key *M.tb* evasion strategy, is likely due to aberrant DC-mediated antigen presentation and delayed	DCs are required to establish an antigen-specific T cell response and control infection on *M.tb* challenge.[192] Delayed DC migration worsens outcome in an NHP model.[192,403]	Human DCs are activated and matured by co-culture with *M.tb* or BCG.[174,187] DC primary immunodeficiency or abrogation results in disseminated BCG infection on intradermal vaccination.[190] Human peripheral DCs co-cultured with *M.tb in-vitro*, phagocytose the bacteria, mature, upregulate HLA-	There is limited information on the phenotype and function of lung DCs in humans as they are in low frequency in BAL fluid, and peripheral blood DCs may not represent what occurs in the lungs, especially as DCs reside more in tissues than the periphery.[746] No change in BAL DC proportion at D3 following BCG lobar instillation

Cell	Animal studies: mice and other small animal models	Animal studies: non-human primates (NHPs)	Human studies: peripheral blood and genome-wide association	Human studies: lung
	migration to the draining LNs.[48,54,148,192] *M.tb in-vitro* co-infection of mouse PBMC-derived DCs, leads to IL-1β-induced Th2 polarisation, and worse bacterial control.[402]		DR expression and induce a Th1 phenotype in CD4+ T cells.[181,183] However, *M.tb* has also been shown to inhibit autophagosome-lysosome fusion in DCs derived from the human circulation, with resultant reduction in IL-12 secretion and poor CD4+ T cell activation on co-culture.[133] pDCs from human PBMCs cultured with BCG show upregulation of maturation markers and granzyme B in response to cDC activation.[404] pDCs in turn promote cDC IL-1β production, which improves growth control.[404] Novel study findings: Circulating DCs (HLA-DR+) fell at D14 out of total MPs.	in mycobacteria-exposed patients.[206] Novel study findings: DCs reduced in the BAL at D7 post-BCG compared to D2. cDCs and pDCs exposed to aerosol BCG infection upregulated Th1 effector gene pathways, and phagocytosis, and HLA-DR upregulation pathways. At D2, cDCs increased expression of CXCL10, CXCL9, IL-1β and IL-6, associated with early clearance and training.[395,404] pDCs also upregulated genes associated with a cytotoxic phenotype, which is consistent with *in-vitro* human peripherally derived pDC phenotype on BCG *in-vitro* co-culture (associated with improved growth control).
Neutrophils	After BCG intradermal or aerosol instillation in mice, neutrophils rapidly track to the site of infection and are a major early source of IFN-γ and chemokines.[204,205,747] Neutrophil depletion in mice leads to prolonged tracking of dendritic cells to the lymph nodes, delayed initiation of the adaptive immune	Higher neutrophil counts within granulomas are associated with greater *M.tb* burden and poor outcome in the NHP model.[405,406] However neutrophil-derived TNFα is associated with protection both early and late in infection and influences adaptive immune responses.[406]	On *in-vitro M.tb* co-culture, human peripherally derived neutrophils kill *M.tb* within one hour and promote apoptosis.[198-200,211,212] Human whole blood depleted of granulocytes, poorly control *M.tb* and BCG, compared with whole blood depleted of CD4+, CD8+ or CD14+ cells. This effect is reversed by adding live granulocytes.[210]	Neutrophils increase in the airway by D3 post BCG lobar instillation in historically *M.tb*-exposed volunteers.[206] Active TB disease is defined by neutrophilia in the BAL and sputum, which reverts on treatment.[218,748]

Cell	Animal studies: mice and other small animal models	Animal studies: non-human primates (NHPs)	Human studies: peripheral blood and genome-wide association	Human studies: lung
	response[207] and increased *M.tb* and/or BCG bacteria load.[208,209] Enhanced neutrophil recruitment decreases BCG bacterial burden.[209] However, lung neutrophilia is a hallmark of *M.tb* infection in susceptible mice.[213-215]		A higher initial neutrophil count is protective against risk of developing LTBI in healthy contacts of TB patients.[211] Healthy contacts then demonstrate a fall in neutrophil count by 2- 6 weeks post putative exposure.[89,211] However, IL-8, a potent neutrophil recruiter, is associated with poor outcome in TB disease[219,220] and high neutrophil count is associated with worse prognosis in patients with established TB disease.[221] Novel study findings: BCG aerosol infection led to a reduction in circulating neutrophils from D7.	Novel study findings: Neutrophils increased in the airway at D7 compared with D2, to the level of a mild neutrophilia. No increase in expression of the neutrophil chemokine CXCL8 gene at either D2 or D7. Neutrophil count increased as DC decreased. Increased neutrophils in the BAL at D2 were associated with stronger antigen-specific systemic IFN-γ responses on ELISpot at D7.
Eosinophils	Mice depleted of eosinophils have a higher *M.tb* bacterial load at 2 months post *M.tb* infection than wildtype mice.[225] *M.tb* infection leads to influx of eosinophils into the airways and lungs of guinea pigs from 11 days.[226] Eosinophils are found in zebra fish *M.marinum* granulomas.[225]	Eosinophils are present in TB granulomas in NHPs.[225]	Patients with active TB disease have depleted peripheral eosinophils compared with LTBI patients, and levels increase within 2 weeks of commencement of TB treatment.[225] Low peripheral eosinophil count correlates with TB disease severity.[407] Novel study findings: No changes in percentage of eosinophils in the blood compared with baseline levels post-aerosol BCG infection.	Eosinophils are present in human TB granulomas. [225] Novel study findings: Eosinophils increase by D7 (compared with D2) in the BAL following BCG aerosol infection . Increased eosinophils in the BAL at D2 were associated with improved growth control on *ex-vivo* MGIA at D56.

Cell	Animal studies: mice and other small animal models	Animal studies: non-human primates (NHPs)	Human studies: peripheral blood and genome-wide association	Human studies: lung
NK cells $CD56^{dim}CD16^{+}$ are more cytotoxic against *M.tb* while $CD56^{bright}CD16^{-}$ cells are robust cytokine producers.[228,229]	IFN-γ+ NK cells appear in the lung 10-15 days following primary infection with *Mycobacteria* spp. and 7 days in mice primed with BCG.[143,239] In a C57BL/6 mice *M.tb* challenge model, depletion of NK cells alone did not increase susceptibility, however depletion of both NK cells and T cells increased susceptibility to TB compared with depletion of T cells alone.[239] Adoptive transfer of NK cells reduced bacterial load.[239]	No change in total NK cell frequency in BAL following aerosol or IV BCG infection in NHPs, (earliest timepoint investigated (2 weeks).[91] Increase in total NK cells and CD16+CD27+NK cell subset in the lungs of LTBI NHPs following *M.tb* aerosol challenge.[408]	Human NK cells recognise *M.tb* infected cells *in-vitro* and induce apoptosis within 24 hours, using perforin and granzyme proteases.[230,231] *In-vitro M.tb* co-culture NK cells secrete Th1 cytokines IFN-γ and TNFα, which activate AM effector function; and NK cells produce IL-22 which controls *M.tb* growth by facilitating phagolysosomal fusion.[232,235] Risk of progression to active TB disease is correlated with preceding low circulating NK cells and dysfunctional peripheral NK cells (low levels of $CD56^{bright}$ NK cells, reduced IFN-γ production), which improves on treatment.[240-243] Healthy persistently IGRA- contacts have more cytotoxic NK cells than IGRA+ contacts.[244] Novel study findings: Trend for increasing circulating NK cells D7 post BCG aerosol infection. Increase in HLA-DR+ CD16+ NK cells at D56. No change in antigen specific IFN-γ+ production by NK cells.	NK cells are increased in the lung of active TB patients[240,245] and NK cells in pleural fluid are the major source of IFN-γ and IL-22 production on *ex-vivo M.tb* stimulation.[235] In human lobar instillation studies, BAL NK cell count do not change after PPD instillation at D2 or D3 in mycobacteria-exposed or naive patients.[206,409,410] However, there is a trend for increased NK cells post-BCG instillation at D3 (mycobacteria-exposed patients).[206] Novel study findings: NK cells increase by D7 (compared with D2) in the BAL following BCG aerosol infection . At D2 and D7, BAL NK cells produced antigen-specific IFN-γ (highest at D7) and displayed a lung homing, Th1/Th17 cytokine and cytotoxic degranulation signature on gene expression. Baseline antigen-specific IFN-γ ELISpot responses positively correlated with D7 BAL NK cells.
DURTs: MAITs	In mice there is a transient accumulation of MAITs in the airway by 10-15 days after *M.tb* or BCG airway infection.[253]	MAITs increase in the airway by 10-15 days after *M.tb* or BCG airway infection.[253,254]	In *in-vitro* experiments, human MAIT cells inhibit the growth of BCG within AMs in an MR1 and Th1 cytokine dependent manner and lyse *M.tb*	MAITs are enriched in the lung in active TB but may have blunted effector function.[257,259] Novel study findings:

Cell	Animal studies: mice and other small animal models	Animal studies: non-human primates (NHPs)	Human studies: peripheral blood and genome-wide association	Human studies: lung
	MAITs reduced mouse survival early in infection, by contributing to delayed CD4+ T cell priming. However, MAITs may reduce bacterial load in chronic disease, in an IL-17 dependent manner.[255]	GranzymeB+IFN-γ+IL-17+TNFα+ MAITs in the airway at 3 weeks post-*M.tb* infection are associated with control to latency.[254] There is no difference in circulating MAIT frequencies following aerosol BCG infection.[91,411]	infected cells via perforin and granzyme.[105] Variable reports about the frequency of MAIT cells in active TB and LTBI patients with some studies finding an inverse correlation with TB risk and others reporting no association.[256-258] Exposed but uninfected household contacts of active TB patients are variably reported as having decreased circulating IL-17+CD8+ MAIT cells or increased Granzyme B and IFN-γ+ effector MAITs compared to contacts who convert.[262,263] Novel study findings: No change in circulating MAITs post-BCG. CD8+ MAITs are the major circulating MAIT cell population. CD8+ MAITs increase at D2 and Double Negative (DN) MAITs fall at D2.	There was a trend for MAITs to increase in the airway at D7 compared to D2. DN MAITs were dominant at D2 but CD4+ MAITs increased at D7. There was an increase in a Th17, Th1 and cytotoxic gene expression signature in BAL MAITs exposed to aerosol BCG.
DURTs: iNKTs	CD4+ and CD4- iNKTs, derived from mouse splenocytes, recognise *M.tb*-infected macrophages and restrict mycobacterial growth, which may be through CD1d dependent GM-CSF production.[272,273] There is no difference in survival against *M.tb* challenge in CD1d-/-	Circulating CD8+ iNKTs correlate with protection against *M.tb* challenge.[276] No change in the frequency of total circulating iNKTs in the blood 4 weeks post aerosol or IV BCG, though however the frequency of T_{EM} and T_{RM} circulating iNKTs increases.[91,411]	iNKTs are decreased in the blood of active TB patients compared to healthy contacts or LTBI patients[170,277] Active TB patients have less Double Negative iNKTs and more CD4+ iNKTs in the blood compared with controls.[271]	iNKT levels are present at low frequencies in the pleura of active TB patients compared with the periphery.[278] Novel study findings: There was a trend for iNKTs to increase in the airway at D7 compared to D2.

Cell	Animal studies: mice and other small animal models	Animal studies: non-human primates (NHPs)	Human studies: peripheral blood and genome-wide association	Human studies: lung
	mice compared with wildtype, however purified iNKT adoptive transfer, or iNKTs activated by the super glycolipid antigen α-galactosylceramide, confers protection.[272,274] iNKT cells are enriched in early granuloma formation in mice.[275]		Early in infection there is no difference seen in peripheral blood iNKT frequency or activation in contacts (IGRA- or IGRA converters) compared with healthy controls.[263] Novel study findings: No change in circulating iNKTs post-BCG. No change in the CD8/CD4 MAIT subsets.	There was a trend for DN iNKTs to fall and CD4+ iNKTs to increase at D7 in the BAL.
DURTs: γδ T cells Vδ2Vγ9 cells are the most common type in human peripheral blood, have an innate-like and T-effector memory (TEM) phenotype, and can recognise phosphoantigen from *M.tb*.[285,286] The non-Vδ2s, Vδ1s and Vδ3s, display a more adaptive phenotype.[287]	Th1 and Th17 γδ T cells track to the lung and draining lymph nodes following aerosol *M.tb* infection.[289] Mice depleted of γδ T cells have an initial high *M.tb* load but control infection better at later timepoints than wildtype mice.[290]	There is an increase of Vδ2Vγ9 T cells in lung and peripheral blood following IV BCG in NHPs at 4 weeks.[291] This is associated with protection to subsequent *M.tb* challenge.[291] *M.tb* exposed NHP Vγ2Vδ2 T cells produce Th1 cytokines, perforin and Granulysin in the lung.[292]	γδ T cells recognise a range of *M.tb* and BCG antigens and the TCR-γ and TCR-δ repertoire expands in patients with LTBI or active TB compared to healthy controls.[288] In infants following BCG intradermal vaccination, NK cells and DN γδ T cells were the highest producers of BCG-specific IFN-γ in blood at 10 weeks.[293] Peripheral cytotoxic γδ T cell response is associated with TB disease risk and progression in healthcare workers compared with healthy contacts; while IL-17+ γδ T cells are increased in active TB patients compared with controls.[294,295] CD4+ γδ T cells expand in latency compared with IGRA- contacts and controls and these cell types have reduced activation on *ex-vivo* stimulation.[263] In this study, there	Novel study findings: γδ2 T cells increased in the airway at D7 compared with D2 in the BAL. There was a trend for increased antigen-specific IFN-γ+ γδ2 at D7 compared with D2 in the BAL. There was an increase in gene expression by γδ2 T cells post-aerosol BCG infection of Type 1 interferons, Th1 polarisation, and cytotoxic pathways but no detectable upregulation of genes associated with Th17 pathways.

Cell	Animal studies: mice and other small animal models	Animal studies: non-human primates (NHPs)	Human studies: peripheral blood and genome-wide association	Human studies: lung
			was no difference in γδ T cells between IGRA- contacts and controls. Novel study findings: No change in circulating γδ T cells post-BCG	
CD4+ T cells and Th1 cytokines	CD4+ T cell deficiency in mice increases *M.tb* burden and mortality.[316,360,749] Adoptive transfer of CD4+ T cells from immunised mice protects naive mice against *M.tb* infection.[413] IFN-γ knock-out mice rapidly succumb to TB disease.[414,415] However antigen-specific IFN-γ production post-BCG vaccination does not correlate with protection against *M.tb* challenge in mice.[416,417] In mice the development of the adaptive Th1 CD4+ T cell response is delayed compared with other pathogens, with influx into the lungs seen 10 days to 3 weeks post infection, during which time bacterial replication continue unabated.[54,91,341,418] Following aerosol *M.tb* challenge, IFN-γ+ CD4+ T cells increase in the BAL at the same time as the blood.[419]	NHPs deficient in CD4+ T cells have worse *M.tb* disease outcome and increased bacterial burden compared with controls.[420] SIV infection in NHPs which causes CD4+ T cell depletion and abrogation is associated with increased risk of TB disease.[421] IFN-γ+ CD4+ T cell response after *M.tb* aerosol infection is not detected systemically in NHPs until 3-4 weeks.[91,341,418] Influx into the airways can be as late as 5-13 weeks post *M.tb* aerosol infection.[422,423] Intra-tracheal BCG results in lower systemic ELISpot responses than intradermal BCG.[424] Following aerosol BCG infection in NHPs, total circulating T cells fall at 2 weeks and rise at 6 weeks.[425] Following aerosol *M.tb* infection, IFN-γ+ CD4+ T cells responses in the BAL mirror the blood (increase at the same timepoint).[426]	HIV co-infection which decreases CD4+ T cell number and function increases risk of progression from LTBI to TB disease and CD4+ T cell count is associated with worse disease outcome.[365,427-429] IFN-γ deficiency leads to disseminated *M.tb* and BCG infection.[430,431] TB vaccine candidates induce potent antigen-specific IFN-γ+ CD4+ T cells, but this doesn't improve protection over BCG alone.[432,433] No association with TB risk and IFN-γ+ CD4+ T cells or any of the other T cell properties in a study of 5,662 BCG-vaccinated infants with a 2 year follow-up.[434] And yet, BCG antigen-specific IFN-γ ELISpot response is associated with reduced risk of TB disease in the first 6-12 months of infancy.[372] Studies of healthy contacts or LTBI patients report conflicting results with *M.tb*-specific Th1 cells increased[435,436] or reduced[437,438]	Total BAL cells contained 19% T cells D2 post-PPD (compared with 10% post-saline), and 40-60% T cells at D3 post-PPD or BCG instillation.[206,409] CD4+ T cells expanded in the airways D2 following PPD bronchoscopic airway instillation in M.tb exposed patients to > 40% BAL cells.[409] Increase in IFN-γ+ CD4+ T cells at D2 and D3 in the airway following PPD or BCG lobar instillation in *M.tb* exposed volunteers. No change following PPD exposure in *M.tb* naïve subjects. BAL from *M.tb* infected patients are enriched for IFN-γ+ antigen specific CD4+ T cells though most of these also produced IL-10, suggesting a negative correlation with protection.[439] However in another study, TB patients switched to a higher IFN-γ producing, Th1 CD4+ T cell signature in the BAL following treatment.[219]

Cell	Animal studies: mice and other small animal models	Animal studies: non-human primates (NHPs)	Human studies: peripheral blood and genome-wide association	Human studies: lung
			compared with active TB disease patients. Systemic TST and IGRA conversion appears as early as 2 weeks, with a peak between 4-8 weeks.[369-371] Novel findings: Total circulating T cells fell in the blood at D2 and then a trend to increase at D7 post BCG aerosol infection. No difference in proportion of circulating CD4+ T cells out of total T cells. Aerosol BCG infection in *M.tb*-naïve humans results in a robust systemic mycobacteria-specific cellular immune response by D7. Demonstrated on ELISpot, with a corresponding significant rise in IFN-y+ from antigen-specific circulating CD4+ T cells on flow cytometry. ELISpot responses are stronger and earlier following aerosol BCG compared with ID BCG. Systemic antigen-specific IFN-y+ responses in the blood at D14 positively correlated with peak mycobacterial growth control on the *ex-vivo* MGIA at the same timepoint.	Novel findings: Influx of CD4 +T cells by D7 compared with D2 in the BAL. CD4+ T cells account for the majority of T cells. Total BAL cells contained 8.76% T cells at D2 with an increase to 37.19% by D7. Increase in antigen-specific IFN-y+ CD4+ T cells in the airway at D7. Upregulation of genes associated with Th1 polarisation and cytotoxicity in CD4+ T cells at D2 and D7 post BCG. These responses increased at the same timepoint in the blood (consistent with mouse and NHP data). IFN-y+ CD4+ T cells in the BAL at D7 correlated with baseline and D2 mycobacterial growth control on the *ex-vivo* MGIA. And also with systemic antigen-specific IFN-y+ responses in the blood at D14.
CD8+ T cells	Following aerosol *M.tb* infection in mice, CD8+ T cells appear from D10 in the lung (the same time as CD4+	CD8+ T cell depletion increases risk of *M.tb* disease in previously	*M.tb* activated human CD8+ T cells on *M.tb* culture are polarised towards a cytotoxic phenotype, but	Antigen-specific CD8+ T cells are detected in the granulomas of LTBI and active TB patients[377]

Cell	Animal studies: mice and other small animal models	Animal studies: non-human primates (NHPs)	Human studies: peripheral blood and genome-wide association	Human studies: lung
	T cells) and directly kill *M.tb* – infected cells through lysis or apoptosis *in-vitro*.[378,379] CD8+ T cell depletion increases susceptibility to *M.tb* infection in mice, however antigen-specific CD8+ T cell responses are not sufficient for protection.[383] Variable evidence from murine models of CD8+ T cell role in *M.tb* protection.[440-442]	infected or BCG vaccinated NHPs.[443] Post aerosol BCG, antigen-specific IFN-y+ CD8+ T cells increase in the BAL, but not until up to 13 weeks post-BCG.[426]	they can also produce Th1 and Th17 cytokines.[381,382] CD8+ T cell deficiency is associated with autoimmunity and not overwhelming mycobacterial infection.[385] Antigen-specific CD8+ T cells are detected in the blood of LTBI and active TB patients[377] Antigen-specific cytokine producing CD8+ cells are insignificant compared with the CD4+ T cell response following viral vector *M.tb* candidate vaccination.[386,387] Novel findings: No difference in the proportion of circulating CD8+ T cells out of total T cells post BCG aerosol infection.	Novel findings: Influx of CD8+T cells by D7 in the BAL. Post-D2 and D7 BCG, BAL CD8+ T cells upregulated genes associated with Th1 polarisation and cytotoxicity pathways. There was a trend for antigen-specific IFN-y+ CD8+ T cells to increase in the BAL at D7 compared with D2. BAL IFN-y+ CD8+ T cells at D2 and the CD4/CD8 ratio at D7 positively correlated with D14 mycobacterial growth control on the *ex-vivo* MGIA. D7 CD4/CD8 ratio also positively correlated with IFN-y+ ELISpot at the same timepoint.

MP phagocytosis of mycobacteria, antigen loading onto MHC-II, and subsequent Th1 CD4+ T cell-help to augment intracellular killing, is understood to be the hallmark of an effective immune response against a mycobacterial infection.[750,751] Impairment of DC-mediated antigen presentation and delayed tracking to draining lymph nodes, with subsequent attenuation of Th1 CD4+ T effector function, is an immune evasion strategy believed to facilitate progression to latency or active disease.[48,54] Here I show that local immune responses, including CD4+ T cell influx and Th1 effector function does not appear to be delayed following aerosol BCG infection, which is in contrast to the kinetics seen in *M.tb* animal infection models (Table 7.1). Antigen presentation, phagosome and apoptotic pathways are upregulated in the airways as early as 2 days after BCG aerosol infection. This is the first time these mechanisms have been demonstrated *in-vivo* in humans. Given these mechanisms are associated with *in-vivo* control of aerosol BCG infection in my model, my results support the idea that development of a vaccine which strongly activates Th1 polarising DCs could induce a protective mucosal T cell response against mycobacterial infection.

Further, other innate immune cells such as NK cells, DURTs and neutrophils increased in the airway and upregulated effector function over this first week, which may play a role in immune defence. These cells demonstrate early intracellular BCG recognition, which has been shown to be important for early clearance. Animal models suggest early recruitment of neutrophils into the lung may be protective against *M.tb* infection,[209,406] while persistent high neutrophilia in the blood is correlated with poor TB control in humans.[219-221] Flow cytometry data in my study suggest that neutrophils did not increase in the airways in my volunteers until after D7, at which time there was a mild neutrophilia. The increase in neutrophils

coincided with a fall in dendritic cells, possibly due to a migration to the LNs for CD4+ T cell activation. In mice infected neutrophils result in robust DC priming which may be what is occurring here.[207] Further, a fall in neutrophils in the blood is associated with resistance to TB infection in human epidemiological studies. I also saw a fall in neutrophils, from D7 in my volunteers. Why low circulating neutrophils is associated with protection needs to be established. It may be reflective of ongoing chemokine production in the lung, resulting in neutrophils leaving the circulation and trafficking to the lung, and hence may be a marker of an early effective local immune response. Alternatively, ongoing neutrophilia may be detrimental to *M.tb* control, due to the associated inflammation and tissue destruction. Further elucidation of protective mechanisms is required. Once causal mechanisms are established, therapeutic targets could be developed to promote early neutrophilia. Conversely, chronic neutrophil production could be blocked by using antibodies to deplete neutrophils or to block their cytotoxic activity.[753]

Both NK cells and DURTs have the potential to produce Th1 cytokines early in infection, without the need for priming by MPs.[2,83,105] Further they have direct cytotoxic potential against mycobacteria-infected MPs.[230,231] Local mucosal NK cells and MAITs were expressing Th1 and Th17 cytokines and a cytotoxic signature within 2-7 days following BCG infection. Significantly, this was not reflected in the blood, highlighting the importance of understanding local immune responses when designing vaccines. Further, baseline antigen-specific IFN-y production predicted NK cell influx in the BAL at D7. This association may be due to inherent genetic differences between participants or due to environmental exposure-induced epigenetic changes.[538,539] If these responses are shown to be protective against *M.tb* in humans, a vaccine which trains NK cells towards a protective cytotoxic or Th1 signature could be developed to confer protection against TB.

The differences described in my study such as faster systemic antigen-specific IFN-γ+ responses than in *M.tb* human infection studies or animal models (see Table 7.1),[369-371] may be because animal models and *in-vitro* human assays are imperfect models for *in-vivo* human responses. In addition, many immune mechanisms described in human natural *M.tb* aerosol infections are based on observations of patterns in the systemic circulation, which can differ from local pulmonary responses. An alternate hypothesis generated by my data is that these differences are because BCG lacks virulence factors such as ESAT-6 postulated to be responsible for many immune evasion strategies.[103,133,485,486] Alternatively, the volunteers in my study may not have been truly mycobacteria-naïve, thereby inducing faster antigen-specific responses. The reason for this difference in kinetics is important to understand and needs further work. Given a fast antigen-specific response seems critical to early mycobacterial clearance or control in animal models,[48,54] understanding the underlying mechanisms which resulted in such rapid responses in my volunteers could be key to developing a protective vaccine.

As discussed, some systemic responses mirrored the airway responses, such as increased T cells at D7, but others exhibited opposing effects, such as neutrophil proportions, which increased in the airway while decreasing in the blood. Further, for some cell types, such as DURTs, there were no changes detectable in the systemic circulation despite these cells increasing in the airway at D7. My thesis therefore highlights the importance of describing immune responses at the site of infection. Biomarkers of infection or protection may be site-specific and not translate to peripheral changes.[91,278,458,459] Vaccines which induce peripheral but not mucosal responses may not be effective for control. Whether route of vaccination impacts this in the human mycobacterial immunity model requires further work. In my study, aerosol BCG infection induced stronger local and systemic antigen-specific IFN-γ than ID

vaccination. Whether this translates to differences in *in-vivo* mycobacterial control needs to be determined.

7.3.1 Novel immune mechanisms, if correlated with growth control, could be used for rational vaccine design to help tackle this pervasive disease.

In the final chapter of my thesis I demonstrated an improvement in mycobacterial growth control at D14 compared with D7 on MGIA, following an aerosol BCG infection. Whether this corresponded with an effective immune response clearing BCG in the airway at this timepoint, remains to be confirmed. MGIA control and peak ELISpot responses were earlier to that seen after ID BCG infection/vaccination and may indicate that a mucosal infection route leads to faster immune mechanisms resulting in BCG clearance.

Ex-vivo MGIA growth control at D14 correlated with systemic antigen-specific IFN-y+ ELISpot responses in the blood at the same timepoint. Volunteers with a high CD4+/CD8+ T cell ratio in the airway at D7, or fewer mucosal MPs at D2 had improved growth control systemically at D14. Further, higher proportions of any T cell type at D2 in the airway (CD4+, CD8+, DN or DP) was correlated with improved control on PBMC MGIA at D14. This was a preliminary correlates analysis and future work exploring correlations between mucosal and systemic responses, and the relationship between high responders, *in-vivo* BCG clearance and *ex-vivo* MGIA growth control is planned. If these immune mechanisms correlate with clearance, vaccines designed to increase early T cell expansion, specifically in the airway, could be developed.

7.4 Future work

As this is the first time early immune responses to an aerosol mycobacterial infection have been described in *M.tb* and BCG-naïve humans, this was an initial exploratory investigation for markers of interest.

A major limitation to this study was the lack of airway saline controls in the flow cytometry work. D2 and D7 post-BCG responses were compared but baseline responses were unknown. Baseline bronchoscopies were not performed on volunteers to avoid disrupting the local immune milieu and for logistical reasons. Further, cell proportions from 10x were unreliable due to the different processing methods employed between D2 and D7. There were also problems with defining the macrophage populations on flow cytometry. In retrospect, to quantify basic cell proportions I should have employed a much simpler method than flow cytometry, by cell counting via cytospin and microscopy. Pooling saline controls from later timepoints in the ongoing TB043 study should provide control data on cell proportions in the airway.

Other work in progress includes analysis of the immunohistochemistry stained endobronchial biopsies and a parallel study in NHPs investigating responses in BAL, lung parenchyma and draining LNs, with the aim of comparing results from this human study across compartments. ATAC-seq analysis of D2 and D7 BAL cells for evidence of training is also ongoing.[754]

This thesis described early cellular immune responses to a mycobacterial aerosol infection, but humoral immune responses need to be defined, given recent evidence for a potential role in protection.[372,396,397] Upregulation of CD16+ NK cells in the BAL at D7 might suggest antibody-dependent cell-mediated cytotoxicity is occurring in the mucosa. Further, the role of the BAL fluid in immune responses warrants investigation.[99-101] Analysis of antibodies,

cytokines and antimicrobial peptides in the BAL fluid is planned. In addition, further work interrogating the DGE and gene pathways of cell subsets is required. The PBMC MGIA findings reported here needs repeating on a re-validated PBMC MGIA, and a BAL MGIA would more closely describe *in-vivo* mycobacterial control.

I have demonstrated baseline ELISpot responses correlate with MGIA control at D14; while baseline systemic *ex-vivo* MGIA control is negatively correlated with mucosal antigen-specific IFN-y+ CD4+ T cells. Further exploration of factors which might affect inherent disposition to mycobacterial control is warranted and could involve investigation of immunological training, or whole genome sequencing analysis for SNPs.[351,538-540] Baseline mycobacterial-specific responses in the volunteers in my study requires further investigation, including previous or ongoing NTM carriage.

Finally, the immune responses described in this exploratory study need to be confirmed in follow up experiments. Sufficiently powered flow cytometry panels targeted at individual cell types, such as dedicated macrophage, NK cell or MAIT cell panels, with ICS staining and effector markers, could confirm gene expression data. *In-vitro* MGIA depletion experiments could confirm the contribution of these immune variables to growth control. *Ex-vivo* functional assays assessing cross talk between primed cells and *M.tb* killing assays, would help define the underlying mechanisms.

7.5 Concluding remarks

I have developed a novel safe and well tolerated aerosol BCG controlled human challenge study. Using this model, I demonstrate that aerosol BCG infection in *M.tb* and BCG-naïve UK adults elicits robust early innate and adaptive immune responses at the mucosa and in the systemic circulation, and BCG airway clearance by 3 months. In addition, I have defined early immune responses which correlate with *ex-vivo* mycobacterial growth control at D14.

There remains much that we do not understand about the interaction between *M.tb* and the host immune response. In a year where any progress gained in ending the TB epidemic has been set back by a decade due to the devastating impact on health systems of the COVID-19 pandemic,[755] this study, which aids understanding of the underlying early clearing immune response, provides an invaluable contribution to the field. As described, this human challenge model could be developed for testing TB vaccine candidates. In addition, this study has created a rich resource of early immune variables from which new hypotheses can be drawn for further investigation. Novel immune mechanisms, if correlated with growth control, could be used for rational vaccine design to help tackle this pervasive disease.

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Appendix A

TB041 Clinical Trial Protocol

CLINICAL TRIAL PROTOCOL

A clinical challenge trial to evaluate controlled human infection with BCG administered by the aerosol inhaled route compared with the intradermal route in healthy, BCG-naïve, UK adult volunteers

Short title: Aerosol BCG challenge trial in healthy UK adults

Trial Reference:	TB041
EudraCT number:	2015-004981-27
REC Reference:	15/SC/0716
IRAS Reference:	192512
Date and Version Number	12 October 2017, V5.0
Chief Investigator:	Professor Helen McShane
Sponsor:	University of Oxford
Local Safety Committee Chair:	Professor Brian Angus
Funding body:	The Gates Foundation and The Wellcome Trust
Author:	Dr Julia Marshall, Dr Michael Riste,

Confidentiality Statement

This document contains confidential information that must not be disclosed to anyone other than the Sponsor, the Investigator Team, and members of the Research Ethics Committee, unless authorised to do so. This information cannot be used for any purpose other than the evaluation or conduct of the clinical investigation without the prior written consent of Professor Helen McShane.

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1 STATEMENT OF COMPLIANCE

Investigator Agreement

"I have read this protocol and agree to abide by all provisions set forth therein. I agree to comply with the International Conference on Harmonisation Tripartite Guideline on Good Clinical Practice."

Professor Helen McShane

Chief Investigator	Investigator Signature	Date

Conflict of Interest

"According to the Declaration of Helsinki, 2008, I have read this protocol, and declare no conflict of interest with any Investigators"

Professor Helen McShane

Chief Investigator	Investigator Signature	Date

2 AMENDMENT HISTORY

Protocol v5.0 Substantial Amendment

Section	From	Changed To
Throughout	Group 2D Group 2E	Dose escalation to 1 x 10^7 cfu BCG Bulgaria in Arm 2 resulting in the addition of an extra group of 3 volunteers (new Group 2D). Group 2E Group 2F
3		Key staff updated
4.1, 4.2, 6.1, 9.1, 12	Arm 2 (30 subjects) The optimal dose of aerosol inhaled BCG Bulgaria (InterVax) will be identified from preliminary results obtained from Groups 2B and 2C (prepared dose 1 x 10^5 or 1x 10^6 cfu) for randomisation groups 2D and 2E Group 2D dose to therefore be either 1 x 10^5 or 1x 10^6 cfu Group 2E dose to be the same as Group 2D	Arm 2 (33 subjects) The optimal dose of aerosol inhaled BCG Bulgaria (InterVax) will be identified from preliminary results obtained from Groups 2C and 2D (prepared dose 1 x 10^6 or 1x 10^7 cfu) for randomisation group 2E Group 2E dose to therefore be either 1 x 10^6 or 1x 10^7 cfu Group 2F prepared dose to be one log lower than Group 2E
4.3	U+Es, LFTs, CRP (except day 2 when CRP only); if raised to be repeated on day 7	Clarification of wording around collection of CRP U+Es, LFTs, CRP (except day 2 when CRP only)
6.2, 9.7, 11.1		Safety review for new Group 2D. To mirror the safety reviews for Groups 2A-2C.
6.2		Addition of rationale for dose escalation to 1x10^7 cfu BCG Bulgaria
6.3, 9.6	Induced sputum procedure Oxford volunteers Groups 2A-2E (ID) at the 4weeks, 3 months, 6 months follow up visits.	Induced sputum procedure Oxford volunteers Groups 2A-2E (not the 2F ID group). Groups 2A-2D and 3 volunteers in 2E will have collection at the day 2, day 7 and 6 months follow up visit; Last 6 volunteers of Group 2E will have collection at the 4weeks, 3 months and 6 months follow up visits
9.5		Explanation that due to the change in schedule of induced sputum collection a small number of volunteers in Group 2E may become unblinded before the day 14 visit.
9.8	Volunteers will be informed that there may be leftover samples of their blood, BAL and sputum.	Volunteers will be informed that there may be leftover samples of their blood, BAL, skin biopsies and sputum.
10.2	• During aerosol inhalation the Investigator will wear appropriate protective clothing (PPE); gown, goggles and face mask.	• During aerosol inhalation all clinical staff will wear appropriate protective clothing (PPE); gown, goggles, overshoes, gloves and FFP3 face mask. The volunteer will

	• The hard surfaces within close range (1-2 metres) of aerosol challenge will be cleaned and disinfected in accordance with local recommendations.	wear a hood in order to contain the aerosol. • The clinic room will then undergo a terminal clean in accordance with local recommendations.
10.3-10.7		Addition of details regarding the agents and devices used as part of the induced sputum procedure; Salbutamol, hypertonic saline and the ultrasonic nebuliser NE-U780
19		2 references added; the European Respiratory Society recommendation for Induced Sputum procedures and the NE-U780 nebuliser manual.

Protocol v4.0 Substantial Amendment

Section	From	Changed To
4.2, 6.3		Addition of footnote to say blood volumes may vary slightly between sites
6.2		Addition of rationale for dose escalation

Protocol v3.0 Substantial Amendment

Section	From	Changed To
3		Key staff updated
Throughout		Addition of Arm 2, using BCG Bulgaria (InterVax)
4, 6.3, 9.6		Addition of Induced Sputum sample
11.1		Clarification that there is no dose escalation and so no safety review for Groups 1C and 1D
16.6		Increase in compensation due to induced sputum sampling necessitating longer visits to clinic

Protocol v2.0 Substantial Amendment

Section	From	Changed To
4.1, 6.3, 7	Intradermal biopsy	Punch biopsy
5		Sii abbreviation removed
6.2	BCG challenge using BCG SSI containing Mycobacterium bovis strain Danish 1331 is preferred as it is licensed in the UK. However BCG SSI can frequently go into global short supply, impacting UK supply. In the event of this occurring, BCG supplied by the SII will be used instead which contains Mycobacterium bovis BCG strain Moscow 361 I and is on the WHO list of prequalified vaccines. The same strain will be used for all volunteers.	BCG SSI containing Mycobacterium bovis strain Danish 1331 will be used in this trial; it is licensed in the UK. The licensed dose of BCG SSI vaccine is 2-8 x 10^5 cfu (0.1ml) intradermally.

	The licensed dose of BCG SSI and BCG SII vaccine is 2-8 x 10^5 cfu (0.1ml) intradermally.	
6.2	The doses which follow will be on the basis of an estimated BCG concentration of 4 x 10^5 cfu.	The doses which follow will be on the basis of an average median BCG concentration of 5 x 10^6 cfu/ml. Further details of safety reviews are given in section 11.1.
6.3	BCG SSI is a licensed vaccine that has been given to over 3 billion people throughout the world since it was first developed in 1921. Full details are given in the SmPC. BCG SII is on the WHO list of pre-qualified vaccines and has a well-defined side effect profile as outlined in the SmPC that is similar to that of BCG SSI. BCG is licensed via the intradermal route. It is not licensed via the aerosol route.	BCG SSI is a licensed vaccine that has been given to over 3 billion people throughout the world since it was first developed in 1921. Full details are given in the SmPC. BCG is licensed for delivery via the intradermal route. It is not licensed for delivery via the aerosol route.
10.1	BCG SSI contains live attenuated Danish strain 1331 Mycobacterium bovis BCG. It is supplied as a powder and solvent for suspension. Each vial contains 2-8 x 10^5 cfu (see SmPC). BCG SII contains live attenuated Mycobacterium bovis BCG Moscow strain 361I. It is supplied as a powder and solvent for suspension. Each vial contains 2-8 x 10^5 cfu (see SmPC). Storage of BCG BCG SSI will be supplied to the CCVTM in Oxford from the Churchill Hospital pharmacy. BCG SII will be shipped to the CCVTM, with shipping at between +2°C and +8°C to ensure cold chain.	BCG SSI contains live attenuated Danish strain 1331 Mycobacterium *bovis* BCG. It is supplied as a powder and solvent for suspension. Each vial contains 2-8 x 10^5 cfu (see SmPC). Storage of BCG BCG SSI will be supplied to the CCVTM in Oxford from the Churchill Hospital pharmacy.
11.1		Dose escalation will not take place in the event of any of the following stopping criteria being met: • **Any SAE deemed to be possibly, probably or definitely related to the challenge agent** • **Solicited respiratory AEs:** If more than one dose of challenge agent (Groups A or B) or more than 25% of doses of challenge agent (Groups C or D) are followed by the same Grade 3 solicited local AE beginning within 2 days after challenge (day of challenge and one subsequent day) and persisting at Grade 3 for >48 hours. • **Solicited systemic AEs:** If more than one dose of challenge agent (Groups A or B) or more than 25% of doses of challenge agent (Groups C or D) are followed by the same Grade 3 solicited

		systemic AE beginning within 2 days after challenge (day of challenge and one subsequent day) and persisting at Grade 3 for >48 hours. • **Unsolicited AEs:** If more than one volunteer (Groups A or B) or more than 25% of volunteers (Groups C or D) develop a Grade 3 unsolicited AE (including the same laboratory AE) that is considered possibly, probably or definitely related to challenge and persists at Grade 3 for >48 hours. 'Solicited respiratory AEs' and 'solicited systemic AEs' are those listed in Table 5 in Section 11.6. 'Unsolicited AEs' are any AE not described herein. If a stopping rule has been met and following an internal safety review it is deemed appropriate to restart dosing, a request to restart dosing with pertinent data must be submitted to the regulatory authority as a request for a substantial amendment. The internal safety review will consider: • The relationship of the AE or SAE to the challenge agent. • The relationship of the AE or SAE to the challenge agent dose, or other possible causes of the event. • If appropriate, additional screening or laboratory testing for other volunteers to identify those who may develop similar symptoms, and alterations to the current Participant Information Sheet (PIS). • New, relevant safety information from ongoing research programs on the various components of the challenge agent. The local ethics committee will also be notified if a holding rule is activated or released. In addition to these pre-defined criteria, the study can be put on hold upon advice of the Local Safety Monitor, Chief Investigator, Study Sponsor, regulatory authority, Ethical Committee(s) or Local Safety Committee, for any single event or combination of multiple

		events which, in their professional opinion, jeopardise the safety of the volunteers or the reliability of the data.
11.6	Outside the diary card periods, respiratory and systemic AEs (listed in section 6.3) **Reporting Procedures for SARs** As BCG is a vaccine with Marketing Authorisation, the mechanism for reporting any SARs to the MHRA is via yellow card.	Outside the diary card periods, respiratory and systemic AEs (listed in Table 5, below) Addition of Table 5 **Reporting Procedures for SARs** These will be reported as per any SAE.

3 KEY TRIAL CONTACTS AND ROLES

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4 SYNOPSIS

4.1 Synopsis

Trial Title	A clinical challenge trial to evaluate controlled human infection with BCG administered by the aerosol inhaled route compared with the intradermal route in healthy UK adult volunteers
Trial Identifier	TB041
Chief Investigator	Professor Helen McShane
Trial Centres	Centre for Clinical Vaccinology and Tropical Medicine (CCVTM), Churchill Hospital, Old Road, Headington, Oxford, OX3 7LE Oxford University Hospital NHS Foundation Trust (OUH) (John Warin Ward, Oxford Haemophilia & Thrombosis Centre, Oxford Centre for Respiratory Medicine), Old Road, Headington, Oxford, OX3 7LE NIHR-Wellcome Trust Clinical Research Facility, Queen Elizabeth Hospital, Edgbaston, Birmingham, B15 2TH
Trial participants	Healthy adult volunteers aged 18-50 years
Planned Sample Size	Arm 1 (30 subjects): Group 1A: 3 volunteers will receive 1 x 10^3 cfu aerosol inhaled BCG SSI, followed by bronchoscopy 14 days later Group 1B: 3 volunteers will receive 1 x 10^4 cfu aerosol inhaled BCG SSI, followed by bronchoscopy 14 days later The next 24 subjects will be randomised to two trial arms: Group 1C: 12 volunteers receiving 1 x 10^5 cfu aerosol inhaled BCG SSI and ID saline placebo, followed by bronchoscopy 14 days later *[NB This group enrolled 4 volunteers before the BCG SSI expired in August 2016. Due to a global shortage of BCG SSI no further volunteers will be enrolled to this Arm]* Group 1D: 12 volunteers receiving aerosol inhaled saline placebo and 1 x 10^5 cfu ID injection of BCG SSI, followed by bronchoscopy and punch biopsy 14 days later *[NB This group enrolled 3 volunteers before the BCG SSI expired in August 2016. Due to a global shortage of BCG SSI no further volunteers will be enrolled to this Arm.]* Arm 2 (33 subjects): Group 2A: 3 volunteers will receive 1 x 10^4 cfu aerosol inhaled BCG Bulgaria (InterVax), followed by bronchoscopy 14 days later Group 2B: 3 volunteers will receive 1 x 10^5 cfu aerosol inhaled BCG Bulgaria (InterVax), followed by bronchoscopy 14 days later Group 2C: 3 volunteers will receive 1 x 10^6 cfu aerosol inhaled BCG Bulgaria (InterVax), followed by bronchoscopy 14 days later Group 2D: 3 volunteers will receive 1 x 10^7 cfu aerosol inhaled BCG Bulgaria (InterVax), followed by bronchoscopy 14 days later The next 21 subjects will be randomised to two trial arms: Group 2E: 9 volunteers will receive the optimal dose of aerosol inhaled BCG Bulgaria (InterVax) identified from preliminary results obtained from Groups 2C and 2D, and ID saline placebo, followed by bronchoscopy 14 days later Group 2F: 12 volunteers will receive aerosol inhaled saline placebo and intradermal BCG Bulgaria (InterVax), at a dose a log lower than 2E then bronchoscopy and punch biopsy
Challenge Schedule	Single challenge at day 0
Follow-up Duration	24 weeks from challenge day
Blood Sampling	See visit schedule
Trial Interventions	Spirometry Challenge by aerosol inhaled route (Groups 1A,1B, 1C, 2A, 2B, 2C, 2D and 2E) or ID route (Group 1D and 2F)

	Saline placebo by alternative route (Groups 1C, 1D, 2E, 2F) Venepuncture Bronchoscopy and BAL Punch biopsy (Group 1D and 2F) Chest x-ray Induced sputum (Groups 2A-2E)	
Trial Duration	Estimated end date is July 2018	
Planned Trial Period	Planned start date is January 2016	
	Objectives	Outcome Measures
Primary	To evaluate the safety of BCG challenge by the aerosol inhaled route in healthy, BCG-naïve UK adult volunteers	Actively and passively collected data on adverse events
Secondary	To evaluate and compare the amount of BCG recovered from BAL in healthy BCG-naïve adults receiving both aerosol inhaled and ID BCG, and punch biopsy in adults receiving ID BCG	Culture (colony counting) and PCR quantification of BCG in BAL and punch biopsy
Tertiary	To identify laboratory markers of the immune response that correlate with the levels of BCG recovered at the challenge site	Established and exploratory markers of innate, cell mediated and humoral immunity in blood and BAL samples
Quaternary	To evaluate and compare the systemic and mucosal cellular and humoral immunogenicity induced by BCG challenge by the aerosol inhaled and ID route, in healthy volunteers	Laboratory markers of cell mediated and humoral immunity, including *ex-vivo* ELISpot in blood and intracellular cytokine staining in blood and BAL samples
Challenge agent	BCG	
Dose(s)	Arm 1: Group 1A: 1 x 10^3 BCG SSI Group 1B: 1 x 10^4 BCG SSI Groups 1C and 1D: 1 x10^5 BCG SSI Arm 2: Group 2A: 1 x 10^4 BCG Bulgaria (InterVax) Group 2B: 1 x 10^5 BCG Bulgaria (InterVax) Group 2C: 1 x 10^6 BCG Bulgaria (InterVax) Group 2D: 1 x 10^7 BCG Bulgaria (InterVax) Group 2E : 1 x 10^6 or 1 x 10^7 BCG Bulgaria (InterVax) Group 2F: One log lower than the Group 2E dose ie either 1 x 10^5 or 1 x 10^6 BCG Bulgaria (InterVax)	
Route of challenge	Aerosol inhalation by nebuliser or ID injection in the deltoid region of the arm	
Allocation Method	Sequential enrolment (Groups 1A, 1B, 2A, 2B, 2C and 2D), variable block randomisation by sequentially numbered sealed envelopes (Groups 1C, 1D, 2E and 2F)	

4.2 Schedule of visits and procedures

Visit number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Timeline (days)*	Screening	0 (Challenge)	2	7	14	28	84	168
Timeline (weeks)*	Screening	0		1	2	4	12	24
Time windows (days)			±1	±2	±21	±7	±14	±21
Inclusion/exclusion criteria	X							
Review contra-indications	X	X			X			
Informed consent	X							
Medical history	X	(X)	(X)	(X)	(X)	(X)	(X)	(X)
Physical examination	X	(X)	(X)	(X)	(X)	(X)	(X)	(X)
Vital signs	X	X	X	X	X	X	X	X
Urinalysis	X							
PFTs	X	X	X	X	X	X		
β-HCG urine test	X	X			X			
BCG Challenge		X						
Biopsy (Groups 1D and 2F)					X			
Bronchoscopy					X			
Chest radiograph	X							
Induced sputum (Groups 2A, 2B, 2C, 2D, 2E)						X	X	X
Local & systemic events/reactions		X	X	X	X	X	X	X
Ediary setup		X						
Ediary final review						X		
Biochemistry (4mL)**/***	X		X	X	X	X		
Haematology (2mL)***	X			X	X	X		
Coagulation (4mL)***	X							
HBV, HCV, HIV (10mL)	X							
HLA typing (4mL)		X						
Exploratory immunology incl ELISpot (10-60mL****)	X	X	X	X	X	X	X	X
Blood vol (mL)	30	64	20	56	66	66	60	60
Cumulative blood vol (mL)***	30	94	114	170	236	302	362	422

X Event scheduled to occur

(X) If considered necessary, emphasising any complaint or change in medications

* Timeline is approximate only, as exact timings (± windows periods) of visits relate to the actual (not intended) date of the previous visit

** U&Es, LFTs, CRP (except day 2 when CRP only)

*** Blood volumes stated for Oxford. The exact volumes of blood taken will depend on the local site – cumulative blood volumes for volunteers outside Oxford may be slightly lower or higher than those of Oxford volunteers, due to the use of different volume vacutainers for biochemistry, haematology and serology samples as per local Trust standard procedures

**** 60ml at all visits except screening (10ml), visit 3 (16ml) and visit 4 (50ml)

Table 1. Schedule of trial procedures

5 ABBREVIATIONS

AE	Adverse Event
AR	Adverse Reaction
BAL	Bronchoalveolar Lavage
BCC	Basal cell carcinoma
BCG	Bacille Calmette-Guérin
β-HCG	Beta - Human Chorionic Gonadotrophin
CCVTM	Centre for Clinical Vaccinology and Tropical Medicine
CFU	Colony-forming unit
CI	Chief Investigator
CIS	Carcinoma *in situ*
CRF	Case Report Form
DNA	Deoxyribonucleic Acid
ELISpot	Enzyme-linked Immunospot
FBC	Full Blood Count
GCP	Good Clinical Practice
GP	General Practitioner (Family Doctor)
HBsAg	Hepatitis B Surface Antigen
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
ICH	International Committee on Harmonisation
ID	Intradermal
IFN-ɣ	Interferon Gamma
IGRA	Interferon-Gamma Release Assay
IM	Intramuscular
LFT	Liver Function Test
LSC	Local Safety Committee
MHRA	Medicines & Healthcare Regulatory Authority
M.tb	*Mycobacterium tuberculosis*
MVA	Modified vaccinia Virus Ankara
NHS	National Health Service
NIHR	National Institute for Health Research
OUH	Oxford University Hospitals
REC	Research Ethics Committee
SAE	Serious Adverse Event
SAR	Serious Adverse Reaction
SmPC	Summary of Product Characteristics
SOP	Standard Operating Procedure
SSI	Statens Serum Institut
SUSAR	Suspected Unexpected Serious Adverse Reaction
TB	Tuberculosis
TMF	Trial Master File
U&Es	Urea & Electrolytes
WTCRF	Wellcome Trust Clinical Research Facility

6 BACKGROUND AND RATIONALE

6.1 Context

Mycobacterium tuberculosis (*M.tb*) is a pathogen with worldwide preponderance which infects humans causing tuberculosis (TB), a transmissible disease resulting in very high mortality and morbidity. It is estimated that a third of the world's population is latently infected with *M.tb*, and these people carry a 10% lifetime risk of developing active life-threatening disease [1]. In 2013, there were 9 million new cases worldwide and 1.5 million people died of TB [2]. Co-infection with human immunodeficiency virus (HIV) greatly increases risk of TB reactivation and death [3, 4]. Diagnosis is challenging and drug treatment can be prolonged, harmful, costly and complex. For these reasons an effective vaccine is a global public health priority.

The Bacille Calmette-Guérin (BCG) vaccine is the only licensed *M.tb* vaccine and it has been administered globally to several billion people over a 90 year period, by the intradermal route [5]. Although it does not protect against pulmonary TB in endemic areas, it is effective in preventing disseminated TB disease including tuberculous meningitis in childhood [4, 6, 7].

Recent advances in TB vaccine development have primarily been in the area of viral-vectored vaccines, given in prime-boost regimes with BCG as the priming vaccine. The most advanced of these vaccine candidates, MVA85A, had promising phase I results in the UK when given by the intradermal route, but subsequently showed significantly lower immunogenicity in phase II efficacy trials in South Africa [8, 9]. The failure of the MVA85A vaccine to improve efficacy in BCG-vaccinated infants and adults highlights our inability to predict which candidate TB vaccines might work in humans. The predictive value of preclinical animal models remains uncertain, and we do not have a validated immunological correlate of protection with which to guide vaccine design and the selection of which candidate vaccines should progress to efficacy trials.

Historically, the main immunological readout for assessing immunogenicity in TB vaccine trials has been measurement of antigen specific interferon-gamma (IFN-γ) release by T cells in one of several immunological assays [10]. There is considerable evidence to support the use of IFN-γ as an immunological readout [11-13], but whilst IFN-γ is essential for protective immunity against *M.tb*, it may not be sufficient. There are other cytokines, such as tissue necrosis factor alpha (TNFα), which are known to be important, as well as other functions of T cells which may be involved [10, 11].

Challenge models

Currently, there is no reliable alternative to large, randomized controlled trials in order to assess vaccine efficacy against TB. These efficacy trials for novel TB vaccines are challenging, time consuming and very costly [14]. The development of a safe controlled human mycobacterial challenge model which would ultimately be validated against field efficacy studies could greatly facilitate TB vaccine development. Such a model could be used both to guide vaccine selection and facilitate identification and validation of potential immunological correlates of protection.

In vaccine development for other pathogens such as malaria and para-typhoid, where animal models and immunological readouts are of uncertain or limited relevance, such challenge studies have been shown to be of great utility. So far it is has not been possible to consider a human challenge approach in TB vaccine development because humans cannot be safely challenged with wild type virulent *M.tb*. Efforts to develop safe, attenuated strains of *M.tb*, with reporter genes to facilitate sensitive detection of changes in bacterial load, are progressing (Walker et al, personal communication). In the meantime, however, progress with establishing basic parameters surrounding a clinical challenge model can be conducted using the existing TB vaccine, Bacille Calmette-Guérin (BCG), a live attenuated strain of *M. bovis*, which is licensed for human use. Using BCG as a model organism allows us to establish the optimal parameters for a

human challenge model, which can then be applied to the challenge reporter strain when it becomes available.

Three studies to date have evaluated BCG as an intradermal challenge agent. In this approach, a skin biopsy is taken two weeks after BCG 'challenge' and the mycobacteria in the biopsy are quantified by culture and PCR [15-17],). This model has demonstrated that viable BCG, detected using culture and PCR, can be recovered from a skin biopsy taken two weeks after BCG challenge. Furthermore we have demonstrated that prior BCG vaccination modulates mycobacterial recovery from subsequent BCG challenge, in a population where BCG has previously been shown to protect against TB [15, 16, 18]. We have also shown that the magnitude of the immune response correlates inversely with mycobacterial growth and have used the challenge model to identify mechanisms responsible for this response [19].

Basis for the aerosol inhaled route

A limitation of the BCG challenge work conducted to date is the intradermal route of delivery. The natural route of infection for *M.tb* is by inhalation of aerosolised infectious droplets containing tubercle bacilli, leading to the establishment of primary infection in the lung. The lung has a distinct mucosal immune system characterized by bronchus associated lymphoid tissue (BALT), which is well adapted to encounter and process antigens such as *M.tb*. Administering BCG challenge via the airway should therefore have the advantage over other routes, of more correctly reflecting the mucosal response to infection with *M.tb,* thus helping us to understand the evolution of early mycobacterial infection in the lung. A successful challenge model by this route will be of great value in testing the efficacy of future vaccines. The inhaled route is a well-established route of drug delivery. Aerosolised droplets of bronchodilating, anti-inflammatory, and antimicrobial drugs are administered by inhalation to millions of patients each day. In the 1960s, BCG was safely delivered by aerosol in a small study in healthy subjects and in two studies in patients with lung cancer [20, 21]. Further clinical studies evaluating the feasibility of an aerosol challenge strategy are needed.

Aerosol delivery devices

Currently the World Health Organisation (WHO) is making a major investment in developing new aerosol devices, providing a portable, low cost method of vaccine delivery [22]. These devices are small, lightweight, portable, and aerosolise through a mesh to provide small and consistent particle size which deliver a vaccine or, for this trial, challenge agent, to the distal airway mucosa more consistently and precisely than conventional jet mechanisms.

One such mesh nebuliser is the MicroAIR NE-U22 (Omron® Healthcare Limited, Japan) which uses ultrasound to push liquid through a fine metal mesh. This generates an aerosol mist with a particle diameter of about 4 μm. It is in current use with licensed drugs such as bronchodilating and antimicrobial agents and achieves good bioavailability. BCG particles are typically around 2-4μm in size and can therefore be aerosolised with minimal damage [23-25]. We have used this device in our previous human aerosol TB vaccine clinical trials, TB026 and TB035, with a good usability and safety profile.

A GLP study assessing the toxicity of BCG administered via the intranasal route has recently completed. Administration of BCG SSI to BABL/c mice by the intranasal route was associated with changes in the lungs and lymph nodes consistent with exposure to the test item and induction of an adaptive immune response. A recent study has demonstrated BCG viability following aerosol delivery to the lungs of rhesus macaques using this nebuliser, with a good safety profile and PPD-specific cellular immune responses in BAL samples [25].

Bronchoscopy and bronchoalveolar sampling

BAL samples enable us to quantify the growth of the challenge agent itself through culture and PCR assessment of BCG CFU counts in the BAL sample. This is of key importance in evaluating this as a viable challenge route for the future assessment of vaccines. BAL samples also provide a relatively easily obtained sample of the lung's immune response to the challenge agent [26]. Protection from TB induced by intra-pulmonary vaccination correlates with cellular immune responses detected in the BAL samples of immunised mice [27]. In macaques receiving aerosolised MVA85A we detected significant levels of antigen-specific cellular immune responses in BAL samples [28], while in TB026, where 12 UK adults received aerosol MVA85A, we detected significant levels of antigen-specific cellular immune responses in these BAL specimens, with higher frequencies in the aerosol group [29]. Both CD4+ and CD8+ antigen specific T cells were detectable in the BAL. In macaques receiving aerosolised BCG, polyfunctional T cells and significant levels of antigen-specific cellular immune responses were detected in BAL specimens [25].

BAL samples are obtained by performing a bronchoscopy, a widely and safely used procedure. The short procedure involves the insertion of a narrow flexible fibre-optic tube into the airway under light intravenous sedation and topical local anaesthesia. Under direct vision, saline is delivered to a section of lung mucosa and then recollected by suction. In clinical trials TB026 and TB035 bronchoscopies have been well tolerated by volunteers.

6.2 Rationale

Hypothesis

We postulate that aerosol inhalation is a practical and feasible route of BCG challenge with an acceptable safety profile in healthy volunteers. We hypothesise that an aerosol BCG challenge will result in the recovery and quantification of BCG through the BAL procedure, and will provide a more physiological model of early mycobacterial infection.

Challenge Agent and dosage

BCG SSI or BCG Bulgaria (InterVax) will be used for this study. From previous studies there is evidence that only 10% of the intradermal dosage is required for mucosal administration (29, 30). With this in mind, and as this trial will be the first GCP clinical trial to give aerosolised BCG to humans, we plan to start at a very low challenge doses with incremental dose increases.

Arm 1 (BCG SSI)

The intradermal licensed dose of BCG SSI is 2-8 x 10^5 cfu (0.1ml)

The doses for the groups in Arm 1 will be on the basis of a median BCG concentration of 5 x 10^6 cfu/ml. Group 1A will receive 1 x 10^3 cfu BCG SSI by aerosol inhalation. The first volunteer in this group will be challenged ahead of other volunteers and no other volunteers will be challenged until at least 7 days has elapsed. The CI will perform a review of the 7 day safety data and decide if it is safe to challenge the remaining volunteers. Further details of safety reviews are given in section 11.1.

There will be a second review of safety data after challenge of all volunteers in Group 1A.

We will then dose escalate to 1 x 10^4 cfu BCG SSI for Group 1B in the same manner, with a safety review 7 days after challenge of the first volunteer. There will be a second review of safety data after challenge of all volunteers in Group 1B.

Once Group 1B is fully enrolled and the second safety review is completed, we will start randomisation to Groups 1C and 1D, with dose escalation to 1 x 10^5 cfu. For safety reasons, the

first volunteer will be assigned to Group 1C (aerosol BCG SSI plus ID saline placebo) and there will be no further challenges until 7 days have elapsed and the safety data reviewed. If the CI decides it is safe to proceed, the remaining volunteers will then be randomised and challenged.

Arm 2 (BCG Bulgaria (InterVax))

The intradermal licensed dose of BCG Bulgaria (InterVax) is 1.5-6.0 x 10^5 cfu (0.1ml).

The doses for the groups in Arm 2 will be on the basis of an average BCG concentration of 4 x 10^6 cfu/ml. Group 2A will receive 1 x 10^4 cfu BCG Bulgaria (InterVax) by aerosol inhalation. The first volunteer in this group will be challenged ahead of other volunteers and no other volunteers will be challenged until at least 7 days have elapsed. The CI will perform a review of the 7 day safety data and decide if it is safe to challenge the remaining volunteers. Further details of safety reviews are given in section 11.1.

There will be a second review of safety data after challenge of all volunteers in Group 2A.

We will then dose escalate to 1 x 10^5 cfu BCG Bulgaria (InterVax) for Group 2B in the same manner, with a safety review 7 days after challenge of the first volunteer. There will be a second review of safety data after challenge of all volunteers in Group 2B.

We will then dose escalate to 1 x 10^6 cfu BCG Bulgaria (InterVax) for Group 2C, with a safety review 7 days after challenge of the first volunteer. There will be a second review of safety data after challenge of all volunteers in Group 2C.

We will then dose escalate to 1 x 10^7 cfu BCG Bulgaria (InterVax) for Group 2D, with a safety review 7 days after challenge of the first volunteer. There will be a second review of safety data after challenge of all volunteers in Group 2D.

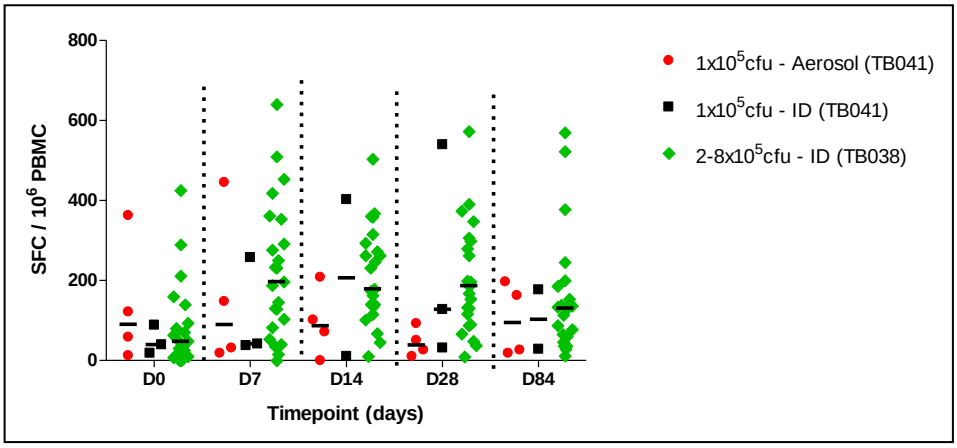
Once Group 2D is fully enrolled and the fourth safety review is completed, we will start randomisation to Groups 2E and 2F. The dose used for challenge of the volunteers in Group 2E will be either 1 x 10^6 cfu or 1 x 10^7 cfu, depending on which dose shows the most promising results from Groups 2C and 2D. The dose used for intradermal challenge in Group 2F will be a log lower than Group 2E.

The rationale for dose escalating to 1 x 10^6 cfu was based on the following:

1. We will carefully dose escalate according to the schedule above and will not dose escalate beyond a dose that is not tolerated. If the 10^5 cfu dose is not well tolerated, we will not dose escalate further.
2. The amount of viable BCG cfu recoverable from a vial of BCG is always lower than the expected amount based on the SMPC. Therefore we anticipate dosing using the SMPC with a dose of 10^6 cfu will result in a delivered dose that is less than 10^6 cfu. The licensed dose of BCG SSI is 2-8 x 10^5 cfu.
3. In a previous BCG study we have administered 3 x the standard dose of BCG by intradermal injection, and this was well tolerated [17]. This was a non-CTIMP challenge study (TB031) where 10 participants received BCG SSI intradermally at a dose range of 5 x 10^5 to 2.4 x 10^6 cfu. As this higher dose of BCG SSI was previously shown to be well tolerated (no SAEs, with local and systemic AEs being mostly mild in nature), we would anticipate a similar safety profile in TB041 where participants will receive 1 x 10^6 cfu of the BCG Bulgaria strain. Furthermore, an additional 10 participants received an intradermal immunization with BCG Tice at a dose range of 5 x 10^5 to 2.4 x 10^6 cfu. This was also well tolerated with local and systemic AEs being mostly mild in nature.

4. To date in this study, administering a dose of 1 x 10^5 cfu has been well tolerated. Furthermore, the immunogenicity data on this dose demonstrates a relatively low immunogenicity compared with both the intradermal route, and with a previous clinical trial with BCG (TB038), Figure 1 below:

Figure 1: PPD ELISpot responses after BCG SSI 10^5 cfu delivered by either aerosol or intradermal route, compared with a prior clinical study with BCG SSI (TB038) where the dose was 100µl.



The rationale for further dose escalating to 1 x 10^7 cfu (for aerosol delivery only) is based on the following:

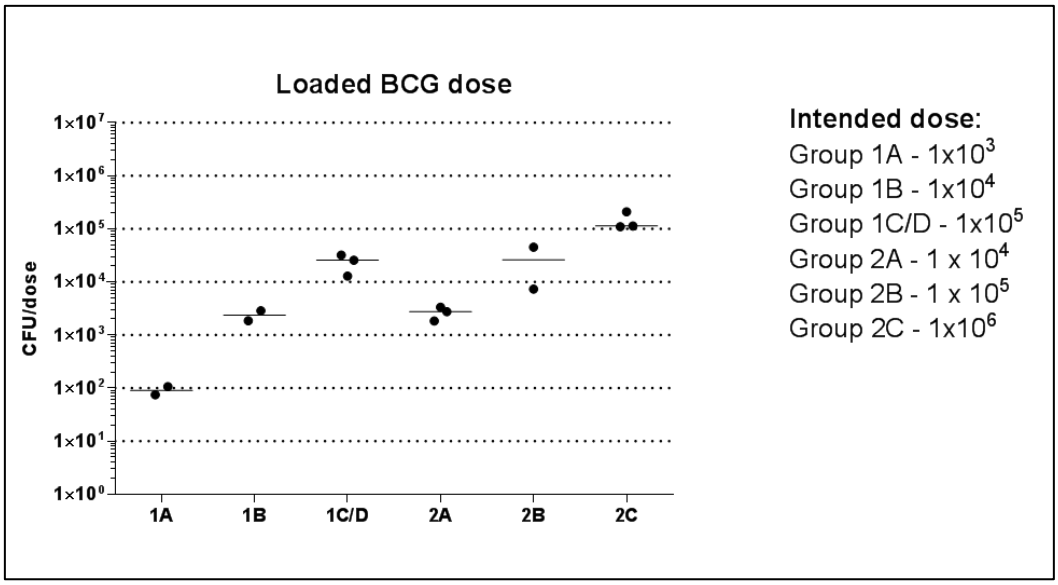
1. The safety data for all volunteers dosed to date has been reviewed and there have been no safety concerns in any volunteer. This includes data from all 3 Group 2C volunteers up to Day 14 who received a dose of 1x 10^6 cfu via aerosol. This dose was well tolerated. The progress to date of volunteers is shown below (green indicates visit completion).

Group	Vol no.	D0 Challenge	D2	D7	D14 Bronch	D28	D84	D168
2A	TBT-0411017	12/04/2017	13/04/2017	19/04/2017	26/04/2017	10/05/2017	05/07/2017	27/09/2017
2A	TBT-0411013	19/04/2017	20/04/2017	26/04/2017	03/05/2017	17/05/2017	10/07/2017	11/10/2017
2A	TBT-0411019	01/06/2017	02/06/2017	07/06/2017	22/06/2017	29/06/2017	06/09/2017	16/11/2017
2B	TBT-0411021	14/06/2017	16/06/2017	21/06/2017	29/06/2017	10/07/2017	12/09/2017	29/11/2017
2B	TBT-0411023	09/08/2017	11/08/2017	15/08/2017	24/08/2017	06/09/2017	01/11/2017	22/01/2017
2B	TBT-0411024	09/08/2017	11/08/2017	15/08/2017	24/08/2017	13/09/2017	01/11/2017	22/01/2017
2C	TBT-0411025	16/08/2017	17/08/2017	23/08/2017	30/08/2017	12/09/2017	07/11/2017	30/01/2018
2C	TBT-0411029	20/09/2017	22/09/2017	28/09/2017	04/10/2017	18/10/2017	13/12/2017	07/03/2017
2C	TBT-0411031	20/09/2017	22/09/2017	27/09/2017	04/10/2017	18/10/2017	13/12/2017	07/03/2017

2. The amount of viable BCG cfu recoverable from a vial of BCG is always lower than the expected amount based on the SmPC. The viable BCG cfu from the vials of BCG Bulgaria

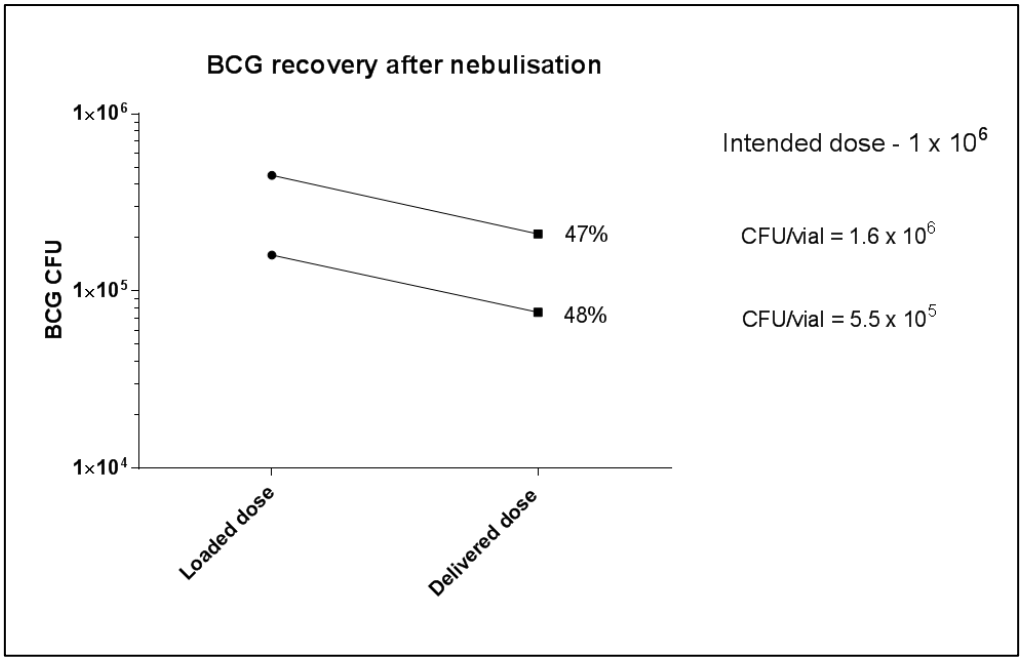
reconstituted in the trial to date is shown below, Figure 2. It is consistently half to 1 log lower than the 1.5-6 x 10^5/ml cfu stated in the SmPC.

Figure 2: BCG dose loaded into nebuliser following BCG reconstitution.



3. We have developed a model to help quantify the actual delivered dose of nebulised BCG through the nebuliser. This involved using a vacuum pump attached to an impinger to collect the aerosol expelled by the Omron nebulizer. The amount of BCG in this collected sample was quantified by plating onto solid agar. This model consistently measured an approximately 50% loss of BCG compared with the amount of BCG loaded into the nebuliser. See Figure 3 below. Of importance, this model does not take into account the further certain significant loss of BCG that occurs in a clinical setting through loss of aerosol into the environment, as the volunteer does not breathe in continuously, as with our vacuum model, and the nebuliser continues to aerosolise the BCG between breaths.

Figure 3: BCG recovery after nebulisation using vacuum model



Our target continues to be to deliver the standard licensed BCG Bulgaria dose of approximately 4 x 10^5 BCG cfu via aerosol into the airway of the volunteer (if safety allows) and to compare this with the same dose delivered intradermally. As outlined above, the amount of viable units in the vials is consistently a half to one log lower, justifying our previous rationale for a dose escalation to 1 x 10^6 BCG cfu.

However, we have now further demonstrated that there is at least another 50% to one log loss of BCG in the process of nebulisation. Hence in order to account for these losses and achieve the actual delivered dose of approximately 4 x 10^5 cfu into the airways of the volunteer, we would need to load the nebuliser cup with 1 x 10^7 cfu ("prepared" dose).

Given the above, we need to ensure that we are delivering a comparable dose of BCG via the two routes (ID or aerosol). Therefore to account for the loss that occurs during nebulisation we plan to load the nebuliser cup (Group 2E) with a dose 1 log higher to the amount loaded into the syringe for intradermal injection (group 2F). The prepared dose of Group 2E will depend on safety data from Groups 2C and 2D, as previously.

6.3 Risks and Benefits

Potential risks

The potential risks to participants in this trial include risks associated with:

1. Venepuncture and intravenous cannulation

Localised bruising and discomfort can occur at the site of venepuncture. Infrequently fainting may occur. The total volume of blood drawn over a six month period will be 422mL (blood volumes for volunteers outside Oxford may be slightly lower or higher than those for Oxford volunteers due to use of different volume vacutainers, following local Trust SOPs), which should not compromise these otherwise healthy volunteers, as they would donate 470mL during a single blood donation for the National Blood transfusion Service over a 3-4 month period. Volunteers will be asked to refrain from blood donation for the duration of their involvement in the trial.

An intravenous cannula is routinely inserted into a peripheral (usually forearm) vein prior to bronchoscopy in order to administer intravenous sedation. This is removed once the procedure is completed. The risks of cannulation are identical to those associated with venepuncture but include an additional small risk of soft tissue infection. This risk will be minimised by an aseptic insertion technique and is easily recognisable and treatable. The short duration of cannulation (a few hours) further minimizes this risk.

2. BCG challenge

BCG SSI is a licensed vaccine that has been given to over 3 billion people throughout the world since it was first developed in 1921. BCG Bulgaria (InterVax) is on the WHO list of pre-qualified vaccines and has a well-defined side effect profile that is similar to that of BCG SSI. Full details are given in the SmPCs. BCG is licensed for delivery via the intradermal route. It is not licensed for delivery via the aerosol route. The potential known adverse events are:

Local reaction from ID administration (Group 1D and 2F)

An inflammatory reaction as manifested by redness and swelling is expected to occur at the site of injection, followed by a local lesion that may ulcerate. This heals over some weeks to months to leave a small flat scar. It is also possible to develop some swelling (<1cm diameter) of axillary lymph nodes.

Uncommonly (less than 1 in 100 people) swelling of axillary lymph nodes to more than 1cm across, or an ulcer that discharges fluid at the injection site may occur.

Rare side effects (less than 1 in 1000 people) include inflammation of lymph nodes leading to abscesses and discharge of fluid from the swellings. Such a reaction would require treatment with antibiotics.

Local reaction from aerosol inhaled challenge (Groups 1A, 1B, 1C, 2A, 2B, 2C, 2D and 2E)

Expected reactions to aerosol challenge include mild throat discomfort and coughing. Transient bronchospasm (causing wheeze) is possible. As this is a novel route for BCG there are no other expected reactions.

Systemic reactions

Systemic reactions to intradermal BCG are rare (less than 1 in 100 people) and include low-grade fever and headache, and allergic reactions. In a previous trial of aerosol BCG around 1/3 of participants experienced fever and malaise. Disseminated complications of BCG, such as bone infections, have also been reported, but are extremely rare and have usually been reported in immunocompromised, not immunocompetent, individuals.

Allergic reactions

Allergic reactions from mild to severe may occur in response to any constituent of a medicinal product's preparation. Anaphylaxis is extremely rare (less than 1 in 1000 people) but can occur in response to any vaccine or medication.

3. Bronchoscopy

Bronchoscopy is a widely and safely used investigative procedure in clinical research studies involving both healthy volunteers and patients with respiratory conditions such as asthma and interstitial lung disease [30, 31]. Clinical guidelines for performing investigative bronchoscopy in research studies are well established [32].

The bronchoscopies will be carried out in a dedicated NHS bronchoscopy suite with an excellent safety record by highly skilled and experienced consultant respiratory physicians. Intravenous sedation and topical local anaesthesia are administered prior to bronchoscopy to reduce discomfort, facilitate the procedure, and remove memory of the event (In 98% of cases, subjects

have no memory of the procedure). Trained, experienced staff and facilities for resuscitation and drugs for reversal of sedation will be available. No transbronchial or endobronchial biopsies will be taken. To further minimise risk, volunteers will be excluded if they have an abnormal chest radiograph, a significant smoking history, a history of atopy or any evidence of lung disease, including asthma (as defined by: a clinical diagnosis of asthma; prescription of asthma medication; airflow obstruction on spirometry; history of nocturnal or exercise-induced wheeze).

The risks of bronchoscopy are discussed at the time of consent and comprise: adverse reaction to sedation or local anaesthetic, sore throat and /or transient hoarse voice, laryngospasm/bronchospasm, hypoxia, post-procedure flu-like symptoms (1-2 days) and risk of death (<1 in 100,000).

- Bronchoalveolar lavage is a routine procedure in investigative bronchoscopy and carries a minimum bleeding risk. The risk of infection or febrile reactions will be minimised by full bronchoscope asepsis and bronchoscope superflush.
- Respiratory depression secondary to sedation is rare. No rescue medication for oversedation has been required for volunteers participating in our aerosol clinical trials (TB026 and TB035) to date.
- Allergic reactions from mild to severe may occur in response to any constituent of the local anaesthetic or sedative agents. Anaphylaxis is extremely rare but can occur.

The Summary of Product Characteristics (SmPC) for the local anaesthetic and sedative agents contain full details of the indications and side effects of these licensed medications.

4. Punch biopsy *(Group 1D and 2F only)*

- There is a small risk that the punch biopsy site may become infected. If this did occur, treatment with antibiotics may be required. The punch biopsy site will heal to form a small scar. Allergic reactions from mild to severe may occur in response to any constituent of the local anaesthetic or sedative agents. Anaphylaxis is extremely rare but can occur.

5. Chest radiograph

A chest radiograph is a painless radiological investigation which exposes volunteers to approximately 0.02 milliSieverts of radiation, equivalent to around 3 days of natural background radiation. The additional risk of cancer due to one chest radiograph is insignificant (1 in 900,000).

6. Spirometry

Vigorous respiratory manoeuvres such as forced expiration through the spirometer can occasionally lead to coughing or light-headedness, but these symptoms are mild and rapidly self-limiting.

7. Induced sputum *(Groups 2A-2E)*

Inhalation of hypertonic saline to induce sputum production can occasionally result in excessive bouts of coughing or sensation of shortness of breath but these symptoms are mild and rapidly self-limiting. Rarely, hypertonic saline can induce bronchoconstriction but this is very unlikely to occur in our cohort of patients who have no history of atopy or asthma. Hypertonic saline-induced bronchospasm is quickly reversed by treatment with an inhaled short-acting B_2 agonist. Volunteers will be pre-treated with nebulised salbutamol and their lung function will be monitored throughout the procedure.

Potential benefits

Volunteers are not expected to benefit directly from participation in this trial. Volunteers will gain some information about their general health as a result of the screening history, examination, blood tests, urine tests, chest radiograph and spirometry. They may also gain health information from the bronchoscopy. Volunteers found to have a previously undiagnosed condition thought to require further medical attention will be referred appropriately to their GP or an NHS specialist service for further investigation and treatment, with their permission.

It is hoped that their contribution will further the development of a safe and successful vaccine for TB and our knowledge about TB infection and protection.

7 OBJECTIVES AND OUTCOME MEASURES

Objectives	**Outcome Measures**	**Timepoint(s) of evaluation of this outcome measure**
Primary Objective To evaluate the safety of BCG challenge by the aerosol inhaled route in healthy volunteers	Actively and passively collected data on adverse events	At each visit and via e-diary for 28 days after challenge
Secondary Objective To evaluate and compare the amount of BCG recovered from BAL in healthy BCG-naïve adults receiving both aerosol inhaled and ID BCG and from punch biopsy in adults receiving ID BCG	Culture and PCR quantification of BCG in BAL and punch biopsy	Day 14
Tertiary Objective To identify laboratory markers of the immune response that correlate with the levels of BCG recovered at the challenge site	Established and exploratory markers of innate, cell mediated and humoral immunity in blood and BAL samples	At each visit
Quaternary Objective To evaluate and compare the systemic and mucosal cellular and humoral immunogenicity induced by BCG challenge by the aerosol inhaled and ID route, in healthy volunteers	Laboratory markers of cell mediated and humoral immunity, including *ex-vivo* ELISpot in blood and intracellular cytokine staining in blood and BAL samples	At each visit

Table 2. Objectives and Outcome Measures

8 PARTICIPANT IDENTIFICATION

8.1 Trial Participants

Healthy, BCG-naïve UK adults have been chosen because of their low baseline level of anti-mycobacterial immunity and therefore reduced ability to suppress BCG growth. This maximizes the chance of detecting BCG at the challenge site.

8.2 Inclusion Criteria

Volunteers must meet all of the following criteria to enter the trial:

- Healthy adult aged 18-50 years
- Resident in or near Oxford (CCVTM or OUH) or Birmingham (NIHR-WTCRF) for the duration of the trial period
- Screening IGRA negative
- Chest radiograph normal
- No relevant findings in medical history or on physical examination
- Allow the Investigators to discuss the individual's medical history with their GP
- Use effective contraception (see below) for the duration of the trial period (females only)
- Refrain from blood donation during the trial
- Give written informed consent
- Allow the Investigator to register volunteer details with a confidential database (The Over-volunteering Protection Service) to prevent concurrent entry into clinical studies/trials
- Able and willing (in the Investigator's opinion) to comply with all the trial requirements

8.3 Exclusion Criteria

Volunteers must meet none of the following criteria to enter the trial:

- Previously resident for more than 12 months concurrently in a tropical climate where significant non-tuberculous mycobacterial exposure is likely
- Participation in another research trial involving receipt of an investigational product in the 30 days preceding enrolment, or planned use during the trial period[a]
- Prior vaccination with BCG or any candidate TB vaccine
- Administration of immunoglobulins and/or any blood products within the three months preceding the planned trial challenge date
- Clinically significant history of skin disorder, allergy, atopy, immunodeficiency (including HIV), cancer (except BCC or CIS), cardiovascular disease, gastrointestinal disease, liver disease, renal disease, endocrine disorder, neurological illness, psychiatric disorder, drug or alcohol abuse
- Concurrent oral or systemic steroid medication or the concurrent use of other immunosuppressive agents
- History of anaphylaxis to vaccination or any allergy likely to be exacerbated by any component of the trial agent, sedative drugs, or any local or general anaesthetic agents
- Pregnancy, lactation or intention to become pregnant during trial period

- Any respiratory disease, including asthma
- Current smoker
- Clinically significant abnormality on screening chest radiograph[b]
- Clinically significant abnormality of spirometry
- Any nasal, pharyngeal, or laryngeal finding which precludes bronchoscopy
- Current use of any medication taken through the nasal or inhaled route including cocaine or other recreational drugs
- Clinical, radiological, or laboratory evidence of current active TB disease
- Past treatment for TB disease
- Any clinically significant abnormality of screening blood or urine tests[b]
- Positive HBsAg, HCV or HIV antibodies[b]
- Any other significant disease, disorder, or finding, which, in the opinion of the Investigator, may either put the volunteer at risk, affect the volunteer's ability to participate in the trial or impair interpretation of the trial data

[a] Volunteers will be excluded from the trial if they are concurrently involved in another study or trial that involves regular blood tests or an investigational medicinal product. In order to check this, volunteers will be asked to provide their National Insurance or Passport number (if they are not entitled to a NI number) and will be registered on a national database of participants in clinical trials (www.tops.org.uk).

[b] Volunteers who are excluded from the trial because they have been discovered during screening procedures to be suffering from a previously undiagnosed condition thought to require further medical attention will be referred appropriately to their GP or an NHS specialist service for further investigation and treatment.

Effective contraception for female volunteers

Female volunteers are required to use an effective form of contraception during the course of the trial. Although no harmful effects on the foetus have been observed from BCG during pregnancy, it is wise to avoid vaccination during pregnancy, particularly during the first trimester.

Acceptable forms of contraception for female volunteers include:

- Established use of oral, injected on implanted hormonal methods of contraception
- Placement of an intrauterine device (IUD) or intrauterine system (IUS)
- Permanent sterilisation or bilateral tubal occlusion
- Barrier methods of contraception (condom; or occlusive cap with spermicide)
- Male sterilisation, if the vasectomised partner is the sole partner for the subject
- True abstinence, when this is in line with the preferred and usual lifestyle of the subject (periodic abstinence and withdrawal are not acceptable methods of contraception)

9 TRIAL DESIGN AND PROCEDURES

This is a randomised controlled clinical challenge trial of BCG administered by the aerosol inhaled route compared to the intradermal route in healthy UK volunteers.

9.1 Trial Numbers and Groups

For Arm 1 30 volunteers will be enrolled; the first three to Group 1A, the next three to Group 1B, then 24 randomly allocated to either group 1C or group 1D (the 1st volunteer will be in Group 1C).

[Arm 1 of this trial enrolled 13 volunteers before the BCG SSI expired in August 2016. Due to a global shortage of the vaccine, there is no way of knowing when more BCG SSI can be obtained. Recruitment for Arm 1 was therefore stopped in August 2016. There were no safety concerns for any of the groups.]

For Arm 2, 33 volunteers will be enrolled; the first three to Group 2A, the next three to Group 2B, the next three to Group 2C and the next three to Group 2D. Then 21 volunteers will randomly be allocated to either Group 2E (9 volunteers) or Group 2F (12 volunteers).

Arm 1		
Group	Sample size	Intervention
Group 1A	3	Aerosol inhaled BCG SSI 1 x 10^3 cfu then bronchoscopy
Group 1B	3	Aerosol inhaled BCG SSI 1 x 10^4 cfu then bronchoscopy
Group 1C	12	Aerosol inhaled BCG SSI 1 x 10^5 cfu and intradermal saline placebo then bronchoscopy
Group 1D	12	Aerosol inhaled saline placebo and intradermal BCG SSI 1 x 10^5 cfu then bronchoscopy and punch biopsy
Arm 2		
Group	Sample size	Intervention
Group 2A	3	Aerosol inhaled BCG Bulgaria (InterVax) 1 x 10^4 cfu then bronchoscopy
Group 2B	3	Aerosol inhaled BCG Bulgaria (InterVax) 1 x 10^5 cfu then bronchoscopy
Group 2C	3	Aerosol inhaled BCG Bulgaria (InterVax) 1 x 10^6 cfu then bronchoscopy
Group 2D	3	Aerosol inhaled BCG Bulgaria (InterVax) 1 x 10^7 cfu then bronchoscopy
Group 2E	9	Aerosol inhaled BCG Bulgaria (InterVax) either 1 x 10^6 or 1 x 10^7 cfu and intradermal saline placebo then bronchoscopy. Dosing will depend on the optimal dose identified from results obtained from Groups 2C and 2D
Group 2F	12	Aerosol inhaled saline placebo and intradermal BCG Bulgaria (InterVax), at a dose a log lower than 2E ie either 1 x 10^5 or 1 x 10^6 cfu, then bronchoscopy and punch biopsy

Table 3. Trial groups

9.2 Recruitment

Volunteers may be recruited by use of an advertisement formally approved by the ethics committee and distributed or posted in the following places:

- In public places (including NHS hospitals and university buildings) with the agreement of the owner or proprietor
- In newspapers or other literature for circulation
- On radio via announcements
- On a website operated by our group or with the agreement of the owner or operator
- By e-mail distribution to a group or list only with the express agreement of the network administrator or with equivalent authorisation
- On stalls or stands at exhibitions or fairs
- Direct mail-out: this will involve obtaining names and addresses of adults via the most recent Electoral Roll. The contact details of individuals who have indicated that they do not wish to receive postal mail-shots would be removed prior to the Investigators being given this information. The company providing this service is registered under the Data Protection Act 1998. Investigators would not be given dates of birth or ages of individuals, but the list supplied would only contain names of those aged between 18-50 years (as per the inclusion criteria).
- Oxford Vaccine Centre databases: we may contact individuals from databases of groups within the CCVTM (including the Oxford Vaccine Centre database) of previous trial participants who have expressed an interest in receiving information about future studies for which they may be eligible.

A copy of intended advertising will be submitted with the initial application for ethical approval and any significant changes to this advertisement will be submitted as an amendment to the ethics committee for approval before use.

Volunteers who express an interest in the trial will be given a Participant Information Sheet and asked to register for the trial through the website. Their details will be passed to the trial team to arrange a pre-screening call and screening appointment.

9.3 Informed Consent

Written informed consent will be obtained at screening by a GCP trained Investigator.

The volunteer must sign and date the latest approved version of the consent form before any trial specific procedures are performed.

The Participant Information Sheet will be made available to the volunteer no less than 24 hours prior to attending for screening. The details enclosed will be discussed with the volunteer, including:

- the aims and nature of the trial
- the agents used
- the schedule of visits and tests to be carried out
- the implications and constraints of the protocol
- compensation for the volunteer
- known side effects and risks of taking part in the trial

The following general principles will be emphasised:

- Participation is entirely voluntary
- The volunteer may withdraw from the trial at any time for any reason
- Withdrawal or refusal to participate involves no penalty or loss of medical benefits

- The volunteer is free to ask questions at any time to allow him or her to understand the purpose of the trial and the procedures involved
- There is no direct benefit from participating
- The volunteer's GP will be contacted to corroborate their medical history and confirm that the volunteer does not meet any of the exclusion criteria
- The volunteer will be registered on the TOPS database (The Over-Volunteering Protection System)
- The volunteer's blood samples taken as part of the trial will be stored indefinitely for further research. The volunteer may elect to have their samples destroyed after the period required to meet Good Clinical Practice and regulatory requirements.

The volunteer will then have time to consider whether or not to participate. If the volunteer decides to participate, they will sign and date two copies of the consent form, one for them to take away and keep, and one for the Investigator which will be retained at the trial site. These forms will also be signed and dated by the Investigator. This will occur before any trial specific procedures are performed.

9.4 Screening and Eligibility Assessment

Once the volunteer has given their consent to undergo screening, a baseline medical history (including concomitant medication) and physical examination will be performed by a GCP trained doctor. Inclusion and exclusion criteria will be checked using a tabulated format. Demographic and occupational data will be collected. Vital signs will be checked and bloods taken including FBC, clotting profile, U&Es, LFTs, HIV antibodies, HBsAg, HCV antibodies and IGRA. Spirometry will be performed according to local SOP. Volunteers will be counselled by one of the Investigators for HIV, Hepatitis B and Hepatitis C testing. Urine will be tested for the presence of clinically significant proteinuria, glycosuria or haematuria. A pregnancy test will be performed for female volunteers. Volunteers will then attend for a chest radiograph.

Laboratory parameters for inclusion/exclusion in the trial will be considered on an individual basis, with Investigator discretion for interpretation of results and the need for repeated tests. In general, volunteers will be excluded if a result at screening constitutes what would qualify as a grade 1 (or higher) laboratory AE, according to the site-specific laboratory AE tables found in the relevant SOP and filed in the TMF (Oxford), or Site Master File (Birmingham).

The total duration of the screening visit will be around 2 hours.

9.5 Randomisation and blinding

To reduce bias and the influence of any difference in the baseline characteristics between Groups 1C and 1D (Arm 1), as well as Groups 2E and 2F (Arm 2), we will randomise the allocation of volunteers by variable block randomisation using sequentially numbered sealed envelopes, prepared by an independent colleague at the Centre for Statistics in Medicine. This aims to achieve a balance between the two groups in any important confounding factors such as levels of pre-existing anti-mycobacterial immunity (influenced by, for example, country of origin). Randomisation will occur for Groups 1C and 1D (Arm 1), as well as Groups 2E and 2F (Arm 2), only (for Arm 1, the first volunteer will be assigned to Group 1C for safety).

Volunteers in randomised groups will be blinded to eliminate subject bias (either conscious or subconscious) using a paired placebo design, whereby volunteers randomised to groups 1C and 2E (inhaled aerosol) will receive a concurrent intradermal saline injection, while volunteers in groups 1D and 2F (intradermal) will receive a concurrent dose of inhaled saline. This design has the added benefit of allowing a distinction to be made between any adverse events attributable to the method (including nebuliser device) of challenge delivery and those attributable to the inhaled challenge product itself. At day 14 these volunteers will be unblinded as Groups 1D and

2F will undergo punch biopsy whereas Groups 1C and 2E will not. It would not be ethical to perform punch biopsy on Groups 1C and 2E volunteers to maintain blinding. There is potential impact on the reporting of AEs from this period hence, but as we anticipate the bulk of challenge-related AEs occurring in the first 2 weeks post challenge, this is a reasonable compromise in order to not subject these volunteers to an unnecessary procedure. A volunteer in Group 2E may become unblinded prior to day 14 if their induced sputum procedure falls at day 2 or day 7 follow up, but this will be in the minority of cases given not all Group 2E volunteers will be having induced sputum scheduled at these visits and not at all if they are enrolled at Birmingham.

The bronchoscopist performing the procedure at day 14 will be blinded to the route of challenge administration to eliminate any bias in the reporting of the appearance of the lung mucosa and extent of airway inflammation.

The laboratory staff analysing the immunology samples will be blinded to reduce any bias that could be introduced at the sample processing stage by anonymising all samples.

9.6 Follow up visits

D0 - Challenge

Any new medical issues or symptoms that have arisen will be assessed. Venepuncture and other samples will be taken according to the Schedule of Trial Procedures in section 4.2. Spirometry will be performed. The inclusion and exclusion criteria for the trial will be reviewed; provided that the volunteer still satisfies all inclusion criteria (and no exclusion criteria) and their consent remains valid, the volunteer will be allocated to a group. BCG challenge will be performed by the aerosol or intradermal route according to the site specific SOP. Placebo challenge will also be performed, according to their group. Volunteers will be kept under observation for around 60 minutes after challenge. The challenge visit will last approximately 2 hours. The e-diary will be set up during this visit. Birmingham volunteers will have transport to and from Oxford arranged for them.

D14 – Bronchoscopy

The bronchoscopy visit lasts several hours and takes place at the bronchoscopy suite in the OUH NHS Foundation Trust by the specialist team there. Blood will be taken as specified in the Schedule of Trial Procedures (section 4.2). Details of the procedure are outlined in section 6.1. Birmingham volunteers will have transport to and from Oxford arranged for them.

D14 – Biopsy (Groups 1D and 2F)

A 4mm x 3mm punch biopsy will be performed over the intradermal challenge site with local anaesthetic. This will be performed with a standard punch biopsy needle as used in outpatient dermatology clinics. This size is ideal for sampling the majority of the inflamed skin at the BCG challenge site. This procedure will occur following bronchoscopy and will add approximately 15 minutes to the visit.

Other follow-up visits

These will be performed as specified in the Schedule of Trial Procedures (section 4.2). Any new medical issues or symptoms that have arisen will be assessed, and the e-diary and any ongoing adverse events will be reviewed. Additional procedures or laboratory tests may be carried out at the discretion of the Investigators if deemed clinically necessary. Follow-up visits will last approximately 15-30 minutes, except for those having induced sputum where the visit will last approximately 1.5 hours. The maximum trial duration will be around 24 weeks after the date of enrolment.

Induced sputum collection will occur at 3 of the 5 follow up visits for Oxford volunteers in Groups 2A-2E. In order to collect samples at the maximum number of time points without overly burdening staff and volunteers with this time intensive procedure we are allocating volunteers to one of two schedules. Volunteers in Groups 2A-D will have induced sputum collection at the 4 week, 3 month and 6 month visit. The first 3 volunteers enrolled into Group 2E will also be allocated to the above the schedule. The final 6 volunteers enrolled into Group 2E will be allocated to sputum collection at day 2, day 7 and the 6 month follow up visit. This will ensure that 6 volunteers dosed with the maximal BCG aerosol dose will be allocated to the two schedules. Volunteers from Birmingham, while allocated to a schedule will not actually have the sputum collected due to time and logistic constraints. Induced sputum collection is an experimental aspect of this trial and we have devised the above schedule based on pragmatics and practicality given the limited resources of the clinic.

After the final trial visit, an End of Study letter will be sent to each volunteer's GP to inform them that the volunteer has either completed the trial and which intervention they received, or was not enrolled.

9.7 Timepoints

Screening – trial enrolment time window

Enrolment should take place no longer than 120 days following the date of screening appointment. If more than 120 days elapse, the screening visit should be repeated in full prior to enrolment in order to minimise the risk to participants of any new unidentified health problems having arisen during that period.

Challenge of first volunteer – subsequent challenge time window

Arm 1: The first volunteer, who will be allocated to Group 1A, will be challenged with BCG SSI by the aerosol inhaled route at least 7 days before any subsequent volunteers in this group. After safety review of the 3 Group 1A volunteers, the first volunteer in Group 1B will be challenged at least 7 days prior to further volunteer enrolment into Group 1B. After safety review of the 3 Group 1B volunteers, the first volunteer in Group 1C will be challenged at least 7 days prior to further volunteer enrolment into Groups 1C & 1D.

Arm 2: The first volunteer, who will be allocated to Group 2A, will be challenged with BCG Bulgaria (InterVax) by the aerosol inhaled route at least 7 days before any subsequent volunteers in this group. After safety review by the clinical team and the Chief Investigator of the 3 Group 2A volunteers, the first volunteer in Group 2B will be challenged at least 7 days prior to further volunteer enrolment into Group 2B. After similar safety review of the 3 Group 2B volunteers, the first volunteer in Group 2C will be challenged at least 7 days prior to further enrolment into Group 2C. After similar safety review of the 3 Group 2C volunteers, the first volunteer in Group 2D will be challenged at least 7 days prior to further enrolment into Group 2D. After similar safety review of the 3 Group 2D volunteers, and review of the results from Groups 2C and 2D to decide the optimal dose to take forwards, volunteers will be enrolled into Groups 2E and 2F.

Challenge – bronchoscopy and biopsy time window

In previous intradermal BCG challenge studies, two weeks has been established as the optimal time window for BCG recovery; this should also be the case for aerosol challenge.

Follow up period

The follow up period will be six months in accordance with findings from previous studies in which adequate safety data and reliable markers of immunogenicity have been obtained in this time interval.

9.8 Sample handling

Details regarding samples, volume and frequency of sampling are listed in the Schedule of Trial Procedures (section 4.2). Blood and other samples will be processed according to local laboratory SOPs. All samples will be in anonymised form at the CCVTM/OUH; samples at the NIHR-WTCRF may contain patient numbers and details as per NHS procedures.

Volunteers will be informed that there may be leftover samples of their blood, BAL, skin biopsies and sputum. With the volunteers' informed consent, any leftover cells and serum, plasma, sputum or BAL samples will be frozen for future analysis of *M.tb* and/or BCG-related responses. This may include human DNA and RNA analysis to search for correlates of TB risk and/or protection. Samples may be shipped to other parties involved in our research in anonymised form for immunological analysis. Volunteers will be able to decide if they will permit such future use of any leftover samples. If they elect not to permit this, all of those leftover samples will be discarded after the required period of storage to meet GCP and regulatory requirements.

9.9 Challenge Postponement Criteria

Challenge will not proceed on the scheduled day in any of the following situations:

- The volunteer has a current or recent upper respiratory tract infection, unless they have been symptom-free for at least one week
- The volunteer has a temperature > 37.5^{o}C
- The Investigator judges the volunteer to have an acute moderate or severe illness (whether febrile or not)
- The volunteer has received a live non-trial vaccine within the preceding 28 days
- The Investigator has any other concern that challenge may not be in the volunteer's best interests

In this case the volunteer may be challenged at a later date or withdrawn from the trial at the discretion of the Investigator.

9.10 Bronchoscopy Postponement criteria

Bronchoscopy will not proceed on the scheduled day in any of the following situations:

- The volunteer has a temperature > 37.5^{o}C
- The Investigator judges the volunteer to have an acute moderate or severe illness (whether febrile or not)
- The Investigator has any other concern that bronchoscopy may not be in the volunteer's best interests

In the event of postponement the bronchoscopy will be rescheduled when possible up to a 3 week window.

9.11 Discontinuation / Withdrawal Criteria

Every reasonable effort will be made to maintain protocol compliance and participation in the trial.

In accordance with the principles of the current revision of the Declaration of Helsinki and any other applicable regulations, a volunteer has the right to withdraw from the trial at any time and for any reason, and is not obliged to give his or her reasons for doing so. The Investigator may withdraw the volunteer at any time in the interests of the volunteer's health and well-being

(including on the advice of the LSC). In addition the volunteer may withdraw/be withdrawn for any of the following reasons:

- Administrative decision by the Investigator
- Ineligibility (either arising during the trial or retrospectively, having been overlooked at screening)
- Significant protocol deviation
- Volunteer non-compliance with trial requirements (failure to attend two or more follow-up visits)
- Any AE which requires discontinuation of trial involvement or results in inability to comply with trial procedures
- Confirmed pregnancy during the trial

Any volunteer who becomes pregnant during the trial will be followed up as per the protocol and until the end of the pregnancy. We will not routinely perform venepuncture in a pregnant volunteer.

The reason for withdrawal will be recorded in the CRF. Volunteers withdrawn from the trial may be replaced on the decision of the Investigator. If the volunteer is withdrawn due to an AE, the Investigator will arrange for appropriate specialist management or follow up visits or telephone calls until the AE has resolved or stabilised. The regulatory authorities will be informed in a timely manner. The extent of follow up after premature discontinuation will be determined by the Investigator but will be at least for the whole trial period, and if pregnant, until pregnancy outcome. An 'End of Study letter' will be sent to each withdrawn volunteer's GP.

If a volunteer withdraws from the trial, samples collected before their withdrawal from the trial will be used/stored unless the volunteer specifically requests otherwise. Long term safety data collection will continue as appropriate if a volunteer has received a challenge dose.

9.12 Safety

Discontinuation of the trial

The trial will be discontinued in the event of any of the following:

- New scientific information is published to indicate that volunteers in the trial are being exposed to undue risks as a result of administration of the challenge agent, or as a result of the trial procedures or follow-up schedule.
- Serious concerns about the safety of the challenge agent arise as a result of one or more challenge-related SAE(s) occurring in the volunteers enrolled in this or any other ongoing trial of BCG.
- For any other reason at the discretion of the Investigator.

9.13 End of Study Definition

The trial will be completed when the last volunteer enrolled into the trial has completed their final follow up visit. An end of study letter will be sent to each volunteer's GP.

10 CHALLENGE AGENT AND DEVICES

10.1 BCG Description

BCG SSI

BCG SSI contains live attenuated Danish strain 1331 Mycobacterium *bovis* BCG. It is supplied as a powder and solvent for suspension. Each vial contains 2-8 x 10^6 cfu (see SmPC).

BCG Bulgaria (InterVax)

BCG Bulgaria (Intervax) is a dried preparation containing live bacteria derived from an attenuated strain of Mycobacterium *bovis* BCG. It is supplied as a powder and diluent for suspension. Each vial contains 1.5-6 x 10^6 cfu (see SmPC).

Storage of BCG

BCG SSI will be supplied to the CCVTM in Oxford from the Churchill Hospital pharmacy. BCG Bulgaria (InterVax) will be shipped from the manufacturer InterVax in Bulgaria directly to the Clinical Biomanufacturing Facility (CBF) in the presence of a temperature logger. The vaccine will be re-labelled for local use in the TB041 trial.

Dispensed BCG vials are supplied in boxes containing multiple vials and each box is clearly labelled with the market product.

The challenge agent will be stored at +2 to +8°C (nominal temperature) in a secure, temperature-monitored refrigerator at the CCVTM, University of Oxford, Churchill Hospital.

10.2 Dispensing and administration

All movements of vials of the trial agent in or out of the locked refrigerator will be documented. Challenge agent accountability, storage, shipment and handling will be in accordance with local SOPs and other relevant local forms.

The challenge agent will be administered by ID injection over the deltoid region of the non-dominant upper arm, or by aerosol inhalation, according to the site-specific SOP. Volunteers will stay in the unit for 60 minutes (±10 minutes) after challenge. During the administration of the challenge agent, monitoring equipment, oxygen, medicines including bronchodilators and resuscitation equipment will be immediately available for the management of anaphylaxis and bronchospasm according to the site-specific SOP.

In order to minimise dissemination of the BCG bacteria into the environment, a number of measures will be instituted during and following challenge:

- The dilution process will be carried out in a sealed vial in accordance with the relevant SOP.
- The aerosol inhaled challenge will take place in a closed clinic room and not opened until 10 minutes after the end of nebulisation.
- During aerosol inhalation all clinical staff will wear appropriate protective clothing (PPE); gown, goggles, overshoes, gloves and FFP3 face mask. The volunteer will wear a hood in order to contain the aerosol.
- All disposable items including needles, vials, dressings, and protective clothing will be disposed of by following clinical waste and infection control policies, in accordance with the current approved SOP.

- Non-disposable parts of the MicroAIR NE-U22 nebuliser will be cleaned and disinfected in accordance with the device documentation.
- The clinic room will then undergo a terminal clean in accordance with local recommendations.

10.3 Saline

Sterile saline will be used for dilution (for BCG Bulgaria (InterVax) extra saline diluent will be supplied by the manufacturer) and placebo doses. The volume of saline used as placebo will be identical to the challenge volume by the same route.

Hypertonic saline will be delivered via nebuliser according to the site-specific SOP.

10.4 Salbutamol

Volunteers undergoing the induced sputum procedure will be pre-treated with a prescribed dose of salbutamol (200mcg delivered via spacer) in accordance with the European Respiratory Society's guidelines [33].

10.5 MicroAIR NE-U22

This nebuliser is used for aerosol delivery of the BCG. This is an approved electromedical device, CE0197, EAN code 40 15672 10142 1. Information about the specifications, usage, and maintenance of the nebuliser device can be found in the device documentation [34]. This is the same device used in studies TB026 and TB035.

10.6 Ultrasonic nebuliser NE-U780

This nebuliser is used for delivering the hypertonic saline during the induced sputum procedure. This is an approved electromedical device, CE0197, EN60601-1-2-2007. Information about the specifications, usage, and maintenance of the nebuliser device can be found in the device documentation [35].

10.7 Sedative & anaesthetic agents for bronchoscopy

Fentanyl is a licensed opioid used routinely to provide analgesia and sedation during medical procedures. It will be stored, dispensed and administered in accordance with standard NHS procedures and the Summary of Product Characteristics (SmPC).

Midazolam is a licensed benzodiazepine used routinely to provide sedation and amnesia during medical procedures. It will be stored, dispensed and administered in accordance with standard NHS procedures and the SmPC.

Lignocaine is a licensed local anaesthetic used routinely during medical procedures. It will be stored, dispensed and administered in accordance with standard NHS procedures and the SmPC.

Other licensed drugs may be used during bronchoscopy at the discretion of the Investigators.

11 ASSESSMENT OF SAFETY

Safety will be assessed by the frequency, incidence and nature of adverse events and serious adverse events arising during the trial. The safety profile of BCG given intradermally is well understood; therefore we will not be routinely collecting information regarding local reactions to ID administration (Groups 1D and 2F). We will however be recording other reactions since BCG is being given by a novel route (aerosol).

11.1 Interim Safety Review

Prior to dose escalation of the challenge agent, the clinical team and Chief Investigator will review the safety data and adverse events in volunteers before proceeding to the next challenge dose.

Dose escalation will not take place in the event of any of the following stopping criteria being met:

- **Any SAE deemed to be possibly, probably or definitely related to the challenge agent**
- **Solicited respiratory AEs:** If more than one dose of challenge agent (Groups 1A, 1B, 2A, 2B, 2C or 2D) are followed by the same Grade 3 solicited local AE beginning within 2 days after challenge (day of challenge and one subsequent day) and persisting at Grade 3 for >48 hours.
- **Solicited systemic AEs:** If more than one dose of challenge agent (Groups 1A, 1B, 2A, 2B, 2C or 2D) are followed by the same Grade 3 solicited systemic AE beginning within 2 days after challenge (day of challenge and one subsequent day) and persisting at Grade 3 for >48 hours.
- **Unsolicited AEs:** If more than one volunteer (Groups 1A, 1B, 2A, 2B, 2C or 2D) develops a Grade 3 unsolicited AE (including the same laboratory AE) that is considered possibly, probably or definitely related to challenge and persists at Grade 3 for >48 hours.

'Solicited respiratory AEs' and 'solicited systemic AEs' are those listed in Table 5 in Section 11.6. 'Unsolicited AEs' are any AE not described herein.

If a stopping rule has been met and following an internal safety review it is deemed appropriate to restart dosing, a request to restart dosing with pertinent data must be submitted to the regulatory authority as a request for a substantial amendment. The internal safety review will consider:

- The relationship of the AE or SAE to the challenge agent.
- The relationship of the AE or SAE to the challenge agent dose, or other possible causes of the event.
- If appropriate, additional screening or laboratory testing for other volunteers to identify those who may develop similar symptoms, and alterations to the current Participant Information Sheet (PIS).
- New, relevant safety information from ongoing research programs on the various components of the challenge agent.

The local ethics committee will also be notified if a holding rule is activated or released.

In addition to these pre-defined criteria, the study can be put on hold upon advice of the Local Safety Monitor, Chief Investigator, Study Sponsor, regulatory authority, Ethical Committee(s) or Local Safety Committee, for any single event or combination of multiple events which, in their professional opinion, jeopardise the safety of the volunteers or the reliability of the data.

11.2 Definitions

Adverse Event (AE)

An AE is any untoward medical occurrence in a volunteer, which may occur during or after administration of the challenge agent and does not necessarily have a causal relationship with

the intervention. An AE can therefore be any unfavourable and unintended sign (including an abnormal laboratory finding), symptom or disease temporally associated with the trial intervention, whether or not considered related to the trial intervention.

Each adverse event will be graded by the participant according to the table for grading severity of adverse events (see Section 11.7). Severity gradings may be reviewed and discussed with the participants at the clinic visits.

Adverse Reaction (AR)

An AR is any untoward or unintended response to the challenge agent. This means that a causal relationship between the agent and an AE is at least a reasonable possibility, i.e., the relationship cannot be ruled out. All cases judged by the reporting medical Investigator as having a reasonable suspected causal relationship to the challenge agent (i.e. possibly, probably or definitely related to it) will qualify as adverse reactions.

Unexpected Adverse Reaction

An adverse reaction, the nature or severity of which is not consistent with the applicable product information (e.g. SmPC).

Serious Adverse Event (SAE)

An SAE is an AE that results in any of the following outcomes, whether or not considered related to the trial intervention:

- Death.
- Life-threatening event (i.e., the volunteer was, in the view of the Investigator, at immediate risk of death from the event that occurred). This does not include an AE that, if it occurred in a more severe form, might have caused death.
- Persistent or significant disability or incapacity (i.e., substantial disruption of one's ability to carry out normal life functions).
- Hospitalisation or prolongation of existing hospitalisation, regardless of length of stay, even if it is a precautionary measure for continued observation. Hospitalisation (including inpatient or outpatient hospitalisation for an elective procedure) for a pre-existing condition that has not worsened unexpectedly does not constitute a serious AE.
- An important medical event (that may not cause death, be life threatening, or require hospitalisation) that may, based upon appropriate medical judgment, jeopardise the volunteer and/or require medical or surgical intervention to prevent one of the outcomes listed above. Examples of such medical events include allergic reaction requiring intensive treatment in an emergency room or clinic, blood dyscrasias, or convulsions that do not result in inpatient hospitalisation.
- Congenital anomaly or birth defect.

Serious Adverse Reaction (SAR)

An adverse event (expected or unexpected) that is both serious and, in the opinion of the reporting Investigator or Sponsors, believed to be possibly, probably or definitely due to the challenge agent or any other trial treatments, based on the information provided.

Suspected Unexpected Serious Adverse Reaction (SUSAR)

A serious adverse reaction, the nature and severity of which is not consistent with the information about the medicinal product in question set out in the SmPC.

NB: To avoid confusion or misunderstanding the following note of clarification is provided: "Severe" is often used to describe intensity of a specific event, which may be of relatively minor medical significance. "Seriousness" is the regulatory definition supplied above.

11.3 Foreseeable Adverse Reactions

Foreseeable adverse reactions are listed in section 6.3.

11.4 Expected Serious Adverse Events

No serious adverse events are expected in this trial.

11.5 Causality Assessment

For every unsolicited AE, an assessment of the relationship of the event to the administration of the challenge agent will be undertaken. An intervention-related AE refers to an AE for which there is a probable or definite relationship to administration of a challenge agent. An interpretation of the causal relationship of the intervention to the AE in question will be made, based on the type of event, the relationship of the event to the time of challenge administration and the known biology of the challenge agent, with reference to the SmPC (Table 4). Causality assessment will take place during interim analyses and at the final safety analysis.

0	**No Relationship**	No temporal relationship to trial product; ***and*** Alternate aetiology (clinical state, environment or other interventions); ***and*** Does not follow known pattern of response to challenge
1	**Unlikely**	Unlikely temporal relationship to trial product; ***and*** Alternate aetiology likely (clinical state, environment or other interventions); ***and*** Does not follow known typical or plausible pattern of response to challenge
2	**Possible**	Reasonable temporal relationship to trial product; ***or*** Event not readily produced by clinical state, environment or other interventions; ***or*** Similar pattern of response to that seen with other challenge agents
3	**Probable**	Reasonable temporal relationship to trial product; ***and*** Event not readily produced by clinical state, environment or other interventions; ***and*** Known pattern of response seen with other challenge agents
4	**Definite**	Reasonable temporal relationship to trial product; ***and*** Event not readily produced by clinical state, environment or other interventions; ***and*** Known pattern of response seen with the challenge agent

Table 4. Guidelines for assessing the relationship of challenge administration to an AE

11.6 Reporting Procedures for All Adverse Events

All AEs occurring in the 28 days following challenge observed by the Investigator or reported by the volunteer, whether or not attributed to trial medication, will be recorded on electronic diary cards. Data from the diary cards will be extracted following the last volunteer last visit (LVLV). Outside the diary card periods, respiratory and systemic AEs (listed in Table 5 below) will be specifically solicited at each visit, and graded by severity (as detailed in section 11.7). All AEs

starting after the diary card period, or persisting after this period, will be recorded in the AE line listing of the CRF.

The overall appearance of the lung mucosa will be assessed at the day 14 bronchoscopy.

All AEs that result in a volunteer's withdrawal from the trial will be followed up until a satisfactory resolution occurs, or until a non-trial related causality is assigned (if the volunteer consents to this). Serious adverse events (SAEs) will be collected throughout the entire trial period.

	Adverse event
Respiratory	Cough
	Sore throat
	Wheeze
	Shortness of breath
	Coughing up phlegm
	Coughing up blood
	Chest pain
Systemic	Documented fever (oral temperature > 37.5° C)
	Myalgia
	Arthralgia
	Feverishness
	Headache
	Fatigue
	Nausea
	Malaise

Table 5. Routinely solicited adverse events

Reporting Procedures for Serious AEs (see Safety Reporting SOP)
In order to comply with current regulations on serious adverse event reporting to regulatory authorities, the event will be documented accurately and notification deadlines respected. SAEs will be reported on the SAE forms to members of the trial team immediately, once the Investigators become aware of their occurrence, as described in the site-specific SOP. Copies of all reports will be forwarded for review to the Chief Investigator and Principal Investigator (as the Sponsor's representatives) within 24 hours of the Investigator being aware of the suspected SAE. The local safety committee (LSC) will be notified of SAEs which are deemed possibly, probably or definitely related to trial interventions; the LSC will be notified immediately (within 24 hours) of the Investigators' being aware of their occurrence. SAEs will not normally be reported immediately to the ethical committee(s) unless there is a clinically important increase in occurrence rate, an unexpected outcome, or a new event that is likely to affect safety of trial volunteers, at the discretion of the Chief Investigator and/or LSC.

Reporting Procedures for SARs
These will be reported as per any SAE.

Reporting Procedures for SUSARs
The Chief Investigator will report all SUSARs to the MHRA and ethical committee(s) within required timelines (15 days for all SUSARs, unless life threatening in which case 7 days, with a final report within a further 8 days (total 15)). The Chief Investigator will also inform all

Investigators concerned of relevant information about SUSARs that could adversely affect the safety of participants.

All SUSARs and deaths occurring during the trial will be reported to the Sponsor. For all deaths, available autopsy reports and relevant medical reports will be made available for reporting to the relevant authorities.

11.7 Assessment of Severity

The severity of clinical AEs will be assessed according to the scale in Tables 5-6:

	Grade 1 (mild)	**Grade 2 (moderate)**	**Grade 3 (severe)**
Fever (oral)	37.6°C - 38.0°C	38.1°C – 39.0°C	>39.0°C
Tachycardia (bpm)*	101 - 115	116 – 130	>130
Bradycardia (bpm)**	50 – 54	40 – 49	<40
Systolic hypertension (mmHg)	141 - 159	160 – 179	≥180
Diastolic hypertension (mmHg)	91 - 99	100 – 109	≥110
Systolic hypotension (mmHg)***	85 - 89	80 – 84	<80

Table 6. Severity grading criteria for physical observations.

*Taken after ≥10 minutes at rest; **When resting heart rate is between 60 – 100 beats per minute. Use clinical judgement when characterising bradycardia among some healthy volunteer populations, for example, conditioned athletes; ***Only if symptomatic (e.g. dizzy/ light-headed)

GRADE 0	None
GRADE 1	Mild: Transient or mild discomfort (< 48 hours); no medical intervention/therapy required
GRADE 2	Moderate: Mild to moderate limitation in activity – some assistance may be needed; no or minimal medical intervention/therapy required
GRADE 3	Severe: Marked limitation in activity, some assistance usually required; medical intervention/therapy required, hospitalisation possible

Table 7. Severity grading criteria for respiratory and systemic AEs

11.8 Procedures to be followed in the event of abnormal findings

Eligibility for enrolment in the trial in terms of laboratory findings will be assessed as detailed in Section 9.4. Abnormal clinical findings from medical history, examination or blood tests will be assessed as to their clinical significance throughout the trial. Laboratory adverse events will be assessed using the site-specific tables in the Site Master File/TMF. If a test is deemed clinically significant, it may be repeated, to ensure it is not a single occurrence. If a test remains clinically significant, the volunteer will be informed and appropriate medical care arranged as appropriate and with the permission of the volunteer. Specific details regarding findings, discussion with volunteers and resulting actions will be recorded in the CRF. Decisions to exclude the volunteer

from enrolling in the trial or to withdraw a volunteer from the trial will be at the discretion of the Investigator.

11.9 Local Safety Committee

A Local Safety Committee (LSC) will be appointed to provide real-time safety oversight. The LSC will be notified within 24 hours of the Investigators' being aware of the occurrence of SAEs. The LSC has the power to place the trial on hold if deemed necessary following a trial intervention-related SAE. At the time of writing the LSC will be chaired by Professor Brian Angus, Clinical Tutor in Medicine, Honorary Consultant Physician and Director of the Oxford Centre for Clinical Tropical Medicine at Oxford University. There will be a minimum of two other appropriately qualified committee members. All correspondence between Investigator and LSC will be conveyed by the Investigator to the trial Sponsor. The Chair of the LSC will be contacted for advice and independent review by the Investigator or trial Sponsor in the following situations:

- Following any SAE deemed to be possibly, probably, or definitely related to the trial challenge agent.
- Any other situation where the Investigator or trial Sponsor feels independent advice or review is important.

Safety Profile Review

The safety profile will be assessed on an on-going basis by the Investigators. The Chief Investigator, Principal Investigator, and relevant Investigators (as per the trial delegation log) will also review safety issues and SAEs as they arise.

12 STATISTICS

This is primarily an exploratory and safety trial with descriptive endpoints. For Arm 1 of the trial, following the first six volunteers enrolled into Groups 1A and 1B, 12 volunteers will be recruited into each experimental group of the trial (Groups 1C and 1D). Similarly for Arm 2, following enrolment of the first 12 volunteers into Groups 2A, 2B, 2C and 2D, subsequent volunteers will be randomised into Groups 2E (9 volunteers) and 2F (12 volunteers); data from volunteers given the optimal dose selected for 2E (ie 2C or 2D) will be analysed together with those from that optimal dose Group (2C or 2D); total 12. Our previous experience with clinical studies suggests that this sample size is a feasible number to recruit, screen, enrol, and follow up in practical terms, whilst also allowing the determination of any substantial differences in the outcome measures between the two experimental groups in each Arm. The sample size has not been determined with the aim of achieving statistical significance.

Differences between means or medians between the two groups will be presented along with their 95% confidence intervals. Within each group, paired data will be analysed using either a paired t-test or the Wilcoxon matched pairs test depending on the normality of the data.

13 DATA MANAGEMENT

13.1 Source Data

Source documents are where data are first recorded, and from which participants' CRF data are obtained. These include the CRF itself (history and examination), the volunteer consent form, blood and microbiology results, radiology report, GP response letter, copy of bronchoscopy report and any further correspondence relating to the volunteer regarding medical/clinical issues. The CRF will be electronic and paper (CCVTM/OUH) or paper (NIHR-WTCRF) together with electronic diary cards (both sites).

CRF entries will be considered source data if the CRF is the site of the original recording (e.g. there is no other written or electronic record of data). All documents will be stored safely in confidential conditions. On all trial-specific documents, other than the signed consent, the participant will be referred to by the trial participant number/code, not by name.

13.2 Access to Data

Direct access will be granted to authorised representatives from the Sponsor, host institution and the regulatory authorities to permit trial-related monitoring, audits and inspections. All information relating to the trial and its volunteers will be held in strict confidence, and in accordance with GCP and institutional requirements.

13.3 Data Recording and Record Keeping

The CI will be responsible for collecting, recording, analysing, and storing all the data accruing from the trial. These tasks may be delegated to other Investigators. Paper CRFs will be stored in a key-locked cabinet at the NIHR-WTCRF and CCVTM, and electronic CRFs on the OpenClinica™ database, which is stored electronically on secure servers that are outsourced by OpenClinica™. Data from paper CRFs will be transcribed onto the OpenClinica™ database. Some data may be duplicated anonymously into an electronic Microsoft Excel™ file on the CCVTM secure server for clinical monitoring through the trial.

Trial records will be held by the Investigator for as long as required by legislation as a minimum (currently until at least 2 years after the last marketing authorisation for the product) and in order to enable dissemination of trial results after publication, and to enable decoding and destruction of anonymised samples if subsequently requested by a volunteer. Data will subsequently be transferred to a secure archive in accordance with the Data Protection Act. Volunteers will be assigned individual unique trial numbers for identification on all trial records, except where the use of identifiable information is unavoidable (including on GP correspondence, registration documents, and consent forms).

14 QUALITY ASSURANCE PROCEDURES

14.1 Quality Assurance

Investigator procedures

Approved site-specific SOPs will be used at all clinical and laboratory sites.

Modification to protocol

No substantial amendments to this protocol will be made without consultation with, and agreement of, the Sponsor. Any substantial amendments to the trial that appear necessary during the course of the trial must be discussed by the Investigator and Sponsor concurrently. If agreement is reached concerning the need for an amendment, it will be produced in writing by the CI and will be made a formal part of the protocol following ethical and regulatory approval.

An administrative change to the protocol is one that modifies administrative and logistical aspects of a protocol but does not affect the volunteers' safety, the scientific value of the trial, the conduct of the trial or safety of the challenge agent. An administrative change is a non-substantial amendment and does not require REC approval. The CI is responsible for ensuring that changes to an approved trial, during the period for which REC approval has already been given, are not initiated without REC review and approval except to eliminate apparent immediate hazards to the volunteer.

Protocol deviation

All deviations from the protocol will be documented in a protocol deviation form and filed in the trial master file and site file accordingly.

14.2 Monitoring

Data will be evaluated for compliance with the protocol and accuracy in relation to source documents. The trial will be conducted in accordance with procedures identified in the protocol. Regular monitoring will be performed according to ICH GCP. According to applicable SOPs, the Monitors will verify that the clinical trial is initiated, conducted and completed, and data are generated, documented and reported in compliance with the protocol, GCP and the applicable regulatory requirements.

15 SERIOUS BREACHES

The Medicines for Human Use (Clinical Trials) Regulations contain a requirement for the notification of "serious breaches" to the MHRA within 7 days of the Sponsor becoming aware of the breach. A serious breach is defined as “A breach of GCP or the trial protocol which is likely to affect to a significant degree:

- the safety or physical or mental integrity of the volunteers of the trial
- the scientific value of the trial”

In the event that a serious breach is suspected the Sponsor must be contacted within 1 working day. In collaboration with the CI, the serious breach will be reviewed by the Sponsor and, if appropriate, the Sponsor will report it to the REC committee, Regulatory Authority and the NHS host organisation within 7 calendar days.

16 ETHICAL AND REGULATORY CONSIDERATIONS

16.1 Declaration of Helsinki

The Investigator will ensure that this trial is conducted in accordance with the principles of the Declaration of Helsinki as agreed by the World Medical Association General Assembly (Washington 2002).

16.2 Good Clinical Practice

The Investigator will ensure that this trial is conducted in accordance with ICH Good Clinical Practice (GCP), and local regulatory requirements.

16.3 Approvals

A copy of the protocol, proposed informed consent form, other written volunteer information and the proposed advertising material will be submitted to an independent REC for written approval. The Investigators will submit and, where necessary, obtain approval from the REC for all subsequent substantial amendments to the protocol and informed consent document. The Investigators will notify deviations from the protocol or SAEs occurring at the site to the Sponsor and will notify the REC of these in accordance with local procedures.

16.4 Reporting

The CI shall submit once a year throughout the clinical trial, or on request, an Annual Progress Report to the REC, host organisation and Sponsor. In addition, an End of Study notification and final report will be submitted to the MHRA, the REC, host organisation and Sponsor. An annual DSUR will be submitted to the Regulatory Authority, if required by the MHRA.

16.5 Volunteer Confidentiality

The trial staff will ensure the volunteers' anonymity is maintained. All documents will be stored securely and only accessible by trial staff and authorised personnel. No information concerning the trial or the data will be released to any unauthorised third party, without prior written approval of the sponsor. The trial will comply with the Data Protection Act, which requires data to be anonymised as soon as it is practical to do so.

16.6 Expenses and Benefits

Volunteers will be compensated *pro rata* for their time, travel and for trial procedures while participating in the trial, amounting to a total of approximately £515-£635, depending on the exact number of visits, site of recruitment and whether any repeat or additional visits are necessary.

17 FINANCE AND INSURANCE

17.1 Funding

This trial will be financed by research grants from The Gates Foundation and The Wellcome Trust, held by Professor Helen McShane.

17.2 Indemnity

If any volunteer is harmed as a result of this trial, medical care will be provided under the NHS.

Negligent Harm

Indemnity and/or compensation for negligent harm arising specifically from an accidental injury for which the University is legally liable as the Research Sponsor will be covered by the University of Oxford.

Non-Negligent Harm

Indemnity and/or compensation for harm arising specifically from an accidental injury, and occurring as a consequence of the Research volunteers' participation in the trial for which the University is the Research Sponsor will be covered by the University of Oxford.

17.3 Insurance

The University has a specialist insurance policy in place - Newline Underwriting Management Ltd, at Lloyd's of London - which would operate in the event of any volunteer suffering harm as a result of their involvement in the research.

18 PUBLICATION POLICY

When the trial is complete, a manuscript describing the primary trial results will be written and published in a peer-reviewed, open access journal. International guidelines will be followed regarding authorship. There may also be secondary publications on more exploratory results.

19 REFERENCES

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Appendix B

TB043 Clinical Trial Protocol

CLINICAL TRIAL PROTOCOL

A human challenge study to evaluate innate and adaptive immune responses to a controlled human infection with BCG administered by the aerosol inhaled route in healthy, BCG-naïve, UK adult volunteers

Short title: Investigating immune responses to aerosol BCG challenge in healthy UK adults

Trial Reference:	TB043
REC Reference:	18/SC/0307
IRAS Reference:	246087
Date and Version Number	21 May 2019, v3.0
Chief Investigator:	Professor Helen McShane
Sponsor:	University of Oxford
Safety Monitoring Committee:	Dr Hassan Mahomed
	Professor Stephen Gordon
	Dr Francesca Little
Funding body:	The Wellcome Trust
Author:	Dr Julia Marshall and Dr Rebecca Powell Doherty

Confidentiality Statement

This document contains confidential information that must not be disclosed to anyone other than the Sponsor, the Investigator Team, and members of the Research Ethics Committee, unless authorised to do so. This information cannot be used for any purpose other than the evaluation or conduct of the clinical investigation without the prior written consent of Professor Helen McShane.

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1 STATEMENT OF COMPLIANCE

Investigator Agreement

"I have read this protocol and agree to abide by all provisions set forth therein. I agree to comply with the International Conference on Harmonisation Tripartite Guideline on Good Clinical Practice."

Professor Helen McShane
21.05.2019

Chief Investigator	Investigator Signature	Date

Conflict of Interest

"According to the Declaration of Helsinki, 2008, I have read this protocol, and declare no conflict of interest with any Investigators"

Professor Helen McShane
21.05.2019

Chief Investigator	Investigator Signature	Date

2 AMENDMENT HISTORY

Amendment No.	Protocol Version No.	Date issued	Author(s) of changes	Details of Changes made
SA02	3.0	21 May 2019	Dr Rebecca Powell Doherty	In section 4.2 (table 1- schedule of visits) and section 6.3 (risks and benefits), we have removed all references to the use of disposable duckbill masks, as we will no longer utilise these masks in our trial.
SA01	2.0	15 January 2019	Dr Rebecca Powell Doherty and Dr Julia Marshall	Throughout the protocol, we have amended references to BCG Bulgaria to reflect our switch to BCG Danish 1331. We have provided a detailed explanation for the switch. We have revised our volunteer compensation to account for added pulmonary function tests, an additional £20. The range is now £685-725. We have added details (throughout) about the disposable duckbill mask we will ask volunteers to wear at D0, day of bronchoscopy, and at the 3 and 6 month visits. We have added further detail (throughout) regarding the pulmonary function tests we will ask the volunteers to undergo at various points in the study. We have clarified our randomisation language and process to reflect that the process is actually partial randomisation, as detailed by the lead statistician for the study. We have updated the background information related to the previous trial, TB041, as this trial is ending, and additional data is now available.

				Specifically, volunteer numbers have been updated regarding those who have received various doses of aerosol BCG.

3 KEY STUDY CONTACTS AND ROLES

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4 SYNOPSIS

4.1 Synopsis

Trial Title	A human challenge study to evaluate innate and adaptive immune response to a controlled human infection with BCG administered by the aerosol inhaled route in healthy, BCG-naïve, UK adult volunteers	
Trial Identifier	TB043	
Chief Investigator	Professor Helen McShane	
Trial Centres	Centre for Clinical Vaccinology and Tropical Medicine (CCVTM), Churchill Hospital, Old Road, Headington, Oxford, OX3 7LE Oxford University Hospital NHS Foundation Trust (OUH), Headington, Oxford, OX3 7LE	
Trial participants	Healthy adult volunteers aged 18-50 years	
Planned Sample Size	65 subjects Group 1: 10 volunteers will receive 1 x 10^7 cfu aerosol inhaled BCG and 3 volunteers will receive aerosol inhaled normal saline placebo. All Group 1 volunteers will have a bronchoscopy 2 days post challenge Group 2: 10 volunteers will receive 1 x 10^7 cfu aerosol inhaled BCG and 3 volunteers will receive aerosol inhaled normal saline placebo. All Group 2 volunteers will have a bronchoscopy 7 days post challenge Group 3: 10 volunteers will receive 1 x 10^7 cfu aerosol inhaled BCG and 3 volunteers will receive aerosol inhaled normal saline placebo. All Group 3 volunteers will have a bronchoscopy 14 days post challenge Group 4: 10 volunteers will receive 1 x 10^7 cfu aerosol inhaled BCG and 3 volunteers will receive aerosol inhaled normal saline placebo. All Group 4 volunteers will have a bronchoscopy 28 days post challenge Group 5: 10 volunteers will receive 1 x 10^7 cfu aerosol inhaled BCG and 3 volunteers will receive aerosol inhaled normal saline placebo. All Group 5 volunteers will have a bronchoscopy 56 days post challenge	
Challenge Schedule	Single BCG challenge at day 0	
Follow-up Duration	24 weeks from challenge day	
Blood Sampling	See visit schedule	
Trial Interventions	Pulmonary function tests BCG challenge by aerosol inhaled route Saline placebo by aerosol inhaled route Venepuncture Bronchoscopy, BAL and endobronchial biopsy Chest x-ray Induced sputum	
Trial Duration	Estimated end date is June 2020	
Planned Trial Period	Planned start date is July 2018	
	Objectives	Outcome Measures
Primary	To define the systemic and mucosal innate and adaptive immune responses induced by aerosol inhaled BCG infection in healthy BCG and *M.tb* naïve UK adults	Laboratory markers of innate and adaptive immunity, including *ex-vivo* ELISpot and ELISAs; and RNA sequence analysis, flow cytometry and intracellular cytokine staining with blood, BAL and biopsy samples
Secondary	To identify laboratory markers of the immune response that correlate with the protection as defined by PBMC MGIA conducted at D56 post challenge	Established and exploratory markers of innate, cell mediated and humoral immunity in blood, BAL and biopsy samples; MGIA on PBMCs collected at Day 56

Tertiary	To describe the human clinical response to BCG challenge by the aerosol inhaled route in healthy, BCG-naïve UK adult volunteers	Actively and passively collected data on adverse events; detailed participant symptom profiles; Laboratory parameters including BCG CFU counts in induced sputum
Challenge agent	*Mycobacterium bovis* BCG	
Dose	1×10^7 cfu BCG	
Route of challenge	Aerosol inhalation by nebuliser	
Allocation Method	Sequential enrolment into groups (Groups 1-5), followed by within-group randomisation by sequentially numbered sealed envelopes (BCG or placebo)	

4.2 Schedule of visits and procedures

Table 1. Schedule of study procedures

Visit number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Timeline (days)	Screening	0	2	7	14	28	56	84	168
Timeline (weeks)	Screening	0		1	2	4	8	12	24
Time windows (days)			±1	±2	±5	±7	±14	±21	±28
Inclusion/exclusion criteria	X	X							
Review contra-indications	X	X	G1	G2	G3	G4	G5		
Informed consent	X	X	G1	G2	G3	G4	G5		
Medical history	X	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)
Physical examination	X	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)
Vital signs	X	X	X	X	X	X	X	X	X
Urinalysis	X								
Pulmonary Function Tests[+]	X	X	X	X	X	X	X	X^	X^
β-HCG urine test (females)	X	X	G1	G2	G3	G4	G5		
BCG challenge		X							
Bronchoscopy			G1	G2	G3	G4	G5		
Chest radiograph	X								
Induced sputum*								X	X
Local & systemic events/reactions		X	X	X	X	X	X	X	X
E-diary #1 setup		X							
E-diary #1 final review					X				
7-day E-diary #2 for bronchoscopy symptoms					G3	G4	G5		
Biochemistry (mL)	4					4			
Haematology (mL)	2					2			
Coagulation (mL)	4								
HBV, HCV, HIV (mL)	10								
HLA typing (mL)		4							
Exploratory immunology +/- IGRA (mL)	10	60	60	60	60	60	60	60	60
Blood vol per visit (mL)	30	64	60	60	60	66	60	60	60
Cumulative blood vol (mL)	30	94	154	214	274	340	400	460	520

X Event scheduled to occur
(X) If considered necessary, emphasising any complaint or change in medications
G1-G5 Designates Group number
+ Allowable window for formal pulmonary function tests is +/- 7 days on top of specified time-point windows
^ If clinically indicated
* No induced sputum for controls (Arm 2 volunteers)

5 ABBREVIATIONS

AE	Adverse Event
AR	Adverse Reaction
BAL	Bronchoalveolar Lavage
BCC	Basal cell carcinoma
BCG	Bacille Calmette-Guérin
BCG SSI	BCG Danish strain 1331 produced by Statens Serum Institute
β-HCG	Beta - Human Chorionic Gonadotrophin
CCVTM	Centre for Clinical Vaccinology and Tropical Medicine
CFU	Colony-forming unit
CI	Chief Investigator
CIS	Carcinoma *in situ*
CRF	Case Report Form
DGE	Differential Gene Expression
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked Immunospot
FBC	Full Blood Count
GCP	Good Clinical Practice
GP	General Practitioner (Family Doctor)
HBsAg	Hepatitis B Surface Antigen
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
ICH	International Committee on Harmonisation
IFN-ɣ	Interferon Gamma
IGRA	Interferon-Gamma Release Assay
IM	Intramuscular
LFT	Liver Function Test
LSC	Local Safety Committee
MAIT cell	Mucosal associated invariant T cell
MGIA	Mycobacteria Growth Inhibition Assay
MHRA	Medicines & Healthcare Regulatory Authority
M.tb	*Mycobacterium tuberculosis*
MVA	Modified vaccinia Virus Ankara
NK cells	Natural Killer cells
NHS	National Health Service
NIHR	National Institute for Health Research
OUH	Oxford University Hospitals
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
REC	Research Ethics Committee
SAE	Serious Adverse Event
SAR	Serious Adverse Reaction
SMC	Safety Monitoring Committee
SmPC	Summary of Product Characteristics
SOP	Standard Operating Procedure
SUSAR	Suspected Unexpected Serious Adverse Reaction
TB	Tuberculosis

TLCO	Transfer factor of the Lung for Carbon Monoxide
TMF	Trial Master File

6 BACKGROUND AND RATIONALE

6.1 Context

Mycobacterium tuberculosis (*M.tb*) is a pathogen with worldwide preponderance that infects humans and causes the transmissible disease tuberculosis (TB). An estimated one-third of the world's population is latently infected with *M.tb*, carrying a 10% lifetime risk of developing active life-threatening disease (1). In 2016, there were 10 million new cases worldwide and 1.7 million people died of TB (2). Co-infection with human immunodeficiency virus (HIV) greatly increases the risk of TB reactivation and death (3, 4). Diagnosis is challenging and drug treatment is often harmful, costly and complex. For these reasons, it is essential to develop a more effective vaccine against TB.

The Bacille Calmette-Guérin (BCG) vaccine is the only licensed *M.tb* vaccine and has been administered globally to several billion people over a 90 year period, by the intradermal route (5). Although it is effective in preventing disseminated TB disease including tuberculous meningitis in childhood, it does not protect against pulmonary TB in endemic areas (4, 6, 7).

Recent advances in TB vaccine development have primarily been in the area of viral-vectored vaccines, given in prime-boost regimes with BCG as the priming vaccine. The most advanced of these vaccine candidates, MVA85A, had promising phase I results in the UK when given by the intradermal route, but subsequently showed significantly lower immunogenicity in phase II efficacy trials in South Africa (8, 9). The failure of the MVA85A vaccine to improve efficacy in BCG-vaccinated infants and adults highlights our inability to predict which candidate TB vaccines might work in humans. The predictive value of preclinical animal models remains uncertain, and we do not have a validated immunological correlate of protection with which to guide vaccine design and the selection of which candidate vaccines should progress to efficacy trials.

An improved understanding of the nature of protective immunity in humans would significantly improve rational vaccine development. Whilst host immunity, particularly systemic adaptive immunity, has been well characterised in murine models, our understanding of the immunological events that occur in humans during acute infection is limited. In particular, our knowledge of human mucosal responses to *M.tb.* is limited. This is primarily due to the difficulties in studying early disease processes in the lung. Consequently, the majority of human studies have investigated immune responses *ex-vivo* in peripheral blood or after *in-vitro* infection of cell lines (10). A better understanding of the adaptive immune components that exist at the respiratory mucosal surfaces in humans could lead to interventions that prevent infection at the point of entry (11).

It is, however, well established that in both humans and mice, an effective Th1 cell-mediated immune response, characterised by the secretion of IFN-ɣ and TNF from antigen-specific CD4+ T-cells, is necessary but insufficient for protective immunity (12-17). Other components of host adaptive immunity for which there is some evidence of a role in protection include MHC class I-restricted CD8+ T-cells, CD1-restricted T-cells, IL-17 secreting T-cells and antibodies (18-21).

An innate immune response is essential for protective immunity (22). Up to 48% of people who are heavily exposed to *M.tb* do not develop any evidence of immune sensitisation (23). Innate immune cells such as dendritic cells, neutrophils and natural killer (NK) cells all play a role in the control of mycobacterial infection (24-26), as might tissue-resident T-cells, such as mucosal-associated invariant T-cells (MAIT), which possess immediate effector functions such as cytokine

expression and cytotoxicity (27). Some of these immune pathways differ between animal models and humans. For example, humans express group 1 CD1a,b and c molecules, which present foreign lipid antigens (28). These molecules do not express in mice. Additionally, gamma delta T-cells are more important in the non-human primate model than in mice (29, 30).

The innate response also shapes the subsequent adaptive response. We found that TLR-1 expression on the day of vaccination predicted the magnitude of the cell-mediated immune response to a candidate TB vaccine (31). BCG vaccination resulted in NOD2-dependent epigenetic changes in monocytes which then enhanced cytokine production and increased non-specific pathogen clearance *in-vitro*, suggesting that innate immunity can be trained (32).

Although it is clear that the innate response is necessary for protective immunity, the balance, timing and coordination needed for an effective innate immune response in humans is unknown (11).

Challenge models

Observational field studies are of limited value in the study of human protective immunity, not least because the timing of exposure cannot be defined. Controlled human infection studies utilised in studies related to malaria, for example, to define the immunology and identify immune mechanisms of protection. Similar studies are now needed in TB to determine whether observations in the murine model translate to humans and to determine which vaccines and interventions will be effective in preventing human TB (33). These studies should focus on the mucosal route of infection (the lung) because systemic and lung immune responses may differ. It is not, however, ethical to administer virulent *M.tb* to humans, but BCG, a live replicating mycobacterial strain, is very closely related to *M.tb,* and is suitable for use in a human infection model. BCG elicits a similar host gene expression signature to *M.tb* (O'Shea unpublished data; Figure 1), and genetic studies demonstrate only 61 (of 3924) open reading frames (ORFs) present in *M.tb* are absent in BCG (34).

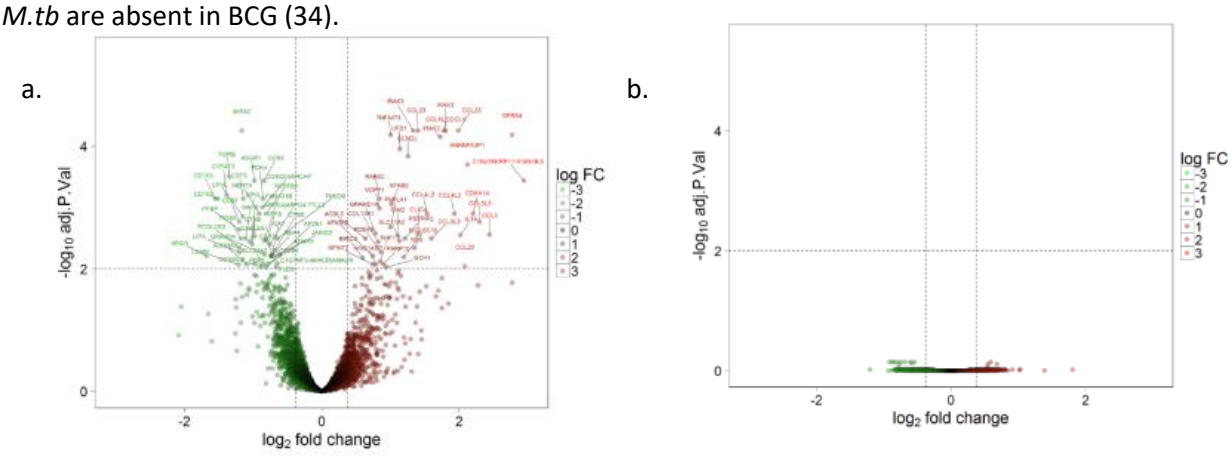


Figure 1. Changes in gene expression after PBMC from BCG-vaccinated subjects are stimulated with BCG or M.tb strain H37Rv. (a) BCG/H37Rv vs unstimulated; (b) BCG vs H37Rv.

Mycobacterial growth inhibition assay and correlates of protection

We have optimised a functional mycobacterial growth inhibition assay (MGIA) in which the degree of *M.tb* growth inhibition *in-vitro* in splenocytes from BCG-vaccinated and naïve mice directly correlates with the protective effect of BCG against *M.tb* infection *in-vivo* (35). BCG

growth in PBMC from BCG-vaccinated non-human primates inversely correlates with lung pathology (Tanner, unpublished data; Figure 2). Furthermore, both whole blood and PBMC from recently BCG-vaccinated volunteers inhibit BCG growth *in-vitro* (36). This MGIA offers a controlled and tractable system that should allow us to identify the critical protective components of host immunity.

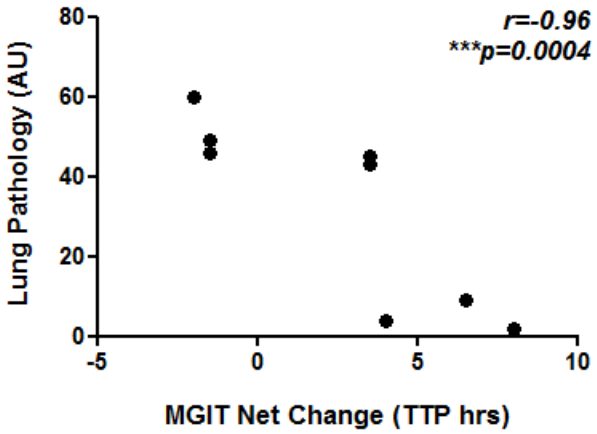


Figure 2. Net change in growth inhibition following BCG vaccination of non-human primates compared with lung pathology following in vivo challenge with M. Tb; (TTP: Time To Positivity; AU: Arbitrary units)

Basis for the aerosol inhaled route

A limitation of the BCG challenge work conducted to date is the intradermal route of delivery (37). The natural route of infection for *M.tb* is by inhalation of aerosolised infectious droplets containing tubercle bacilli, leading to the establishment of primary infection in the lung. The lung has a distinct mucosal immune system which is well adapted to encounter and process antigens. (38) Administering BCG challenge via the airway should therefore have the advantage over other routes of more correctly reflecting the mucosal response to infection with *M.tb*. This will aid our understanding of the evolution of early mycobacterial infection in the lung.

The inhaled route is a well-established route of drug delivery. Aerosolised droplets of broncho-dilating, anti-inflammatory and antimicrobial drugs are administered by inhalation to patients worldwide (39, 40). In the 1960s, BCG was safely delivered by aerosol in a small study in healthy subjects and in two studies in patients with lung cancer (41, 42).

We have established a new experimental medicine paradigm in which aerosolised BCG or candidate TB vaccines are delivered to the lungs. This is followed by bronchoscopic assessment of mucosal immunity. We have conducted 127 bronchoscopies on 91 healthy human subjects, of whom 22 have received aerosolised BCG in a dose-finding safety study without clinically significant adverse events ((43); clinicaltrials.gov NCT02709278, NCT01954563, NCT02532036). We are therefore well-placed to use state-of-the-art immuno-monitoring to dissect the human pulmonary innate and adaptive immune responses to aerosol BCG challenge.

Aerosol delivery devices

Currently the World Health Organisation (WHO) is making a major investment in developing new aerosol devices, providing a portable, low cost method of vaccine delivery (44). These devices are small, lightweight and aerosolise through a mesh to provide small and consistent particle size for vaccine or challenge agent delivery to the distal airway mucosa more accurately and precisely than conventional jet mechanisms.

One such mesh nebuliser is the MicroAIR NE-U22 (Omron® Healthcare Limited, Japan) which uses ultrasound to push liquid through a fine metal mesh. This generates an aerosol mist with a particle diameter of about 4µm. It is in current use with licensed drugs such as bronchodilating and antimicrobial agents (e.g. salbutamol, ipratropium bromide and tobramycin) and achieves good bioavailability. BCG particles are typically around 2-4µm in size and can therefore be aerosolised with minimal damage (45-47). We have used this device in our previous human aerosol TB vaccine clinical trials, TB026 and TB035 (43); Clinicaltrials.gov NCT01954563), and the current TB041 clinical trial (Clinicaltrials.gov NCT02709278) with a good usability and safety profile.

TB041: A clinical challenge trial to evaluate controlled human infection with BCG administered by the aerosol-inhaled route compared with the intradermal route in healthy, BCG-naïve, UK adult volunteers. Clinicaltrials.gov NCT02709278

TB041 is an ongoing trial in our group that delivers BCG via the aerosol route. The aims of the study are to determine tolerability of BCG delivered by this route, and feasibility as a challenge model, by assessing BCG recovery from the BAL fluid. Volunteers undergo bronchoscopy with bronchoalveolar lavage 14 days following inhalation of BCG. The study initially used BCG SSI, the licenced BCG vaccine in the UK for intradermal vaccination, and 10 volunteers inhaled BCG SSI with no safety concerns. In 2016 Statens Serum Institute ceased production of BCG SSI Danish strain. A substantial amendment was submitted and approved by MHRA and OxREC A, to start a new arm of the study using BCG Bulgaria, the BCG vaccine recommended for use in the UK by Public Health England (48).

We have now completed enrolment into Arm 2; delivered BCG Bulgaria via the aerosol route to all 21 volunteers and repeated the dose escalation with no safety concerns (Appendix A). Three volunteers received $1x10^4$cfu BCG Bulgaria; three volunteers received 1 $x10^5$cfu, three volunteers received 1 x 10^6cfu, and twelve volunteers have received the target dose of 1 x 10^7cfu BCG Bulgaria via aerosol delivery.

In total in TB041 31 volunteers have received BCG via the aerosol route.

BCG Danish 1331 production, manufactured by AJ vaccines (formerly SSI), has now recommenced. As this BCG strain is the sole UK licenced vaccine for intradermal delivery and is recommended by Public Health England for vaccine programmes(49), we plan to use this strain for TB043.

In TB041 six volunteers received BCG aerosol at a dose of 1 x 10^4 cfu (three volunteers BCG SSI and three volunteers BCG Bulgaria) and seven volunteers received BCG aerosol at a dose of 1 x 10^5 cfu (four volunteers BCG SSI and three volunteers BCG Bulgaria). While numbers are small, adverse events were not deemed to be notably different between the strains (Appendix A).

Bronchoscopy and bronchoalveolar sampling

Obtaining BAL samples allows us to evaluate the local innate and adaptive immune response induced by aerosol BCG. This also allows us to determine whether we can detect BCG using culture and PCR from the BAL fluid. Protection from TB induced by intra-pulmonary vaccination

is associated with cellular immune responses detected in the BAL samples of immunised mice (50). In macaques receiving aerosolised MVA85A, we detected significant levels of antigen-specific cellular immune responses in BAL samples (51), and in 12 UK adults who received aerosol MVA85A, we also detected significant levels of antigen-specific cellular immune responses in these BAL specimens, with higher frequency compared to adults who received intradermal MVA85A (43). Both CD4+ and CD8+ antigen-specific T-cells were detectable in the BAL. In macaques receiving aerosolised BCG, poly-functional T-cells and significant levels of antigen-specific cellular immune responses were detected in BAL specimens (47). These studies indicate the utility of BAL samples in determining correlates of protection.

BAL samples are obtained by performing a bronchoscopy, a widely and safely used procedure. The short procedure involves the insertion of a narrow flexible fibre-optic tube into the airway under light intravenous sedation and topical local anaesthesia. Under direct vision, saline is delivered to a section of lung mucosa and then recollected by suction. In all clinical trials using bronchoscopy, TB026 (43), TB035 and TB041 (Clinicaltrials.gov NCT01954563 and NCT02709278) bronchoscopies have been well tolerated by volunteers. During bronchoscopy other samples may be taken such as using a swab to absorb airway fluid ("bronchosorption") or bronchial brushings. Collecting these samples will not impact on total length of the bronchoscopy procedure, the risk to the volunteer or the overall experience by the volunteer of the bronchoscopy.

Cellular immune responses from BAL only describe immune responses in the mucosa and airways and not the lung parenchyma. It is now well recognised that localisation of immune cells into distinct compartments through homing markers influences protective immunity. In patients with active TB, peripheral blood is depleted of *M.tb*-specific MAIT cells while BAL is enriched (52). In murine adoptive transfer studies, $CXCR3^{hi}$CD4+ T-cells preferentially localise to the lung parenchyma and are better at controlling *M.tb* infection than their $CX3CR1^{hi}KLRG1^{hi}$ counterparts which localise to the lung intravasculature (53). Therefore, in this study, we will also obtain lung tissue samples by endobronchial biopsy during the bronchoscopy. This is a routine procedure where forceps on the end of a wire are passed into the airway and a small sample is collected. Due to the small size of the biopsy this procedure results in minimal bleeding, and we foresee it to be no less tolerated than performing a bronchoscopy with BAL only as in previous studies (54, 55). Importantly this procedure will allow us to characterise for the first time the motility and extravasation of immune cells to the local site of infection.

Data from human BCG aerosol studies are lacking; therefore, time points have been extrapolated from human TB viral vector aerosol studies (MVA85A) or animal BCG aerosol studies to capture the immune response as broadly as possible.

6.2 Rationale

Hypothesis

We hypothesise that following Bacille Calmette-*Guérin* (BCG) aerosol challenge, components of the innate immune response will correlate with the adaptive immune response and subsequent protection as demonstrated by *in-vitro* PBMC mycobacterial growth inhibition at Day 56.

BCG and dosage

We will deliver a dose of $1x10^7$cfu BCG Danish 1331 by the aerosol route. This is the same dose that was used for Arm 2 of TB041. TB041 closed for enrolment October 2018 and five volunteers remain in follow up. Preliminary safety data is shown in Appendix A.

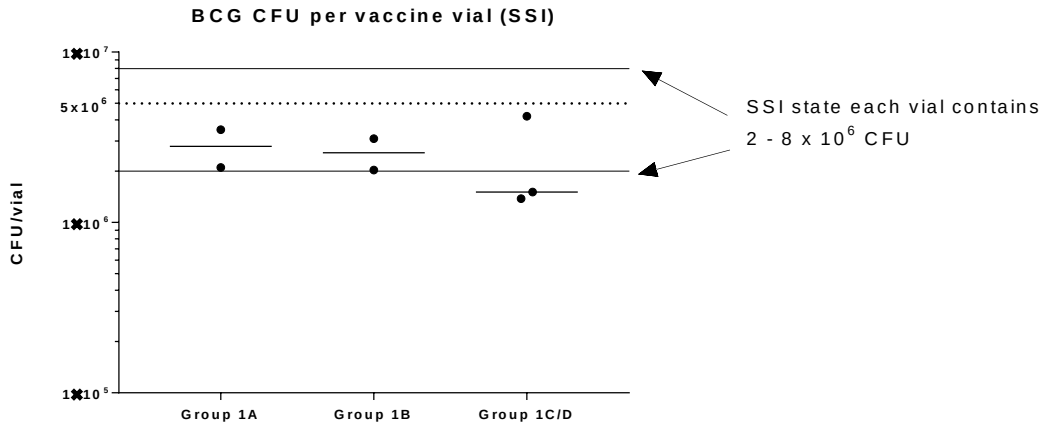
Rationale for dose

The intradermal licensed dose of BCG Danish 1331 for adults is 2-8 x 10^5cfu in 0.1ml.(56)

We calculated the dose for TB043 based upon an average of this: 5x10^5cfu in a 0.1ml adult standard dose, which translates into a BCG concentration of 5x10^6cfu/ml.

We aim to deliver this standard dose of 5x10^5cfu into the lung by loading 1x10^7cfu BCG into the nebuliser. This accounts for the losses of BCG due to method of delivery as follows:

1. The amount of viable BCG cfu recoverable from a vial of BCG is often lower than the expected amount based on the Summary of Product Characteristics (SmPC). The viable BCG cfu from the vials of BCG Danish 1331 reconstituted in TB041 is shown below (Figure 3).



2.

Figure 3. Viable BCG cfu from BCG Danish vials reconstituted in the TB041

3. The vaccine vial is diluted to achieve the required dose and we have plated out these dilutions to obtain an estimate of the dose that is loaded onto the nebuliser (*Figure **4***). This loaded dose is consistently one-half to one log lower than our intended dose.

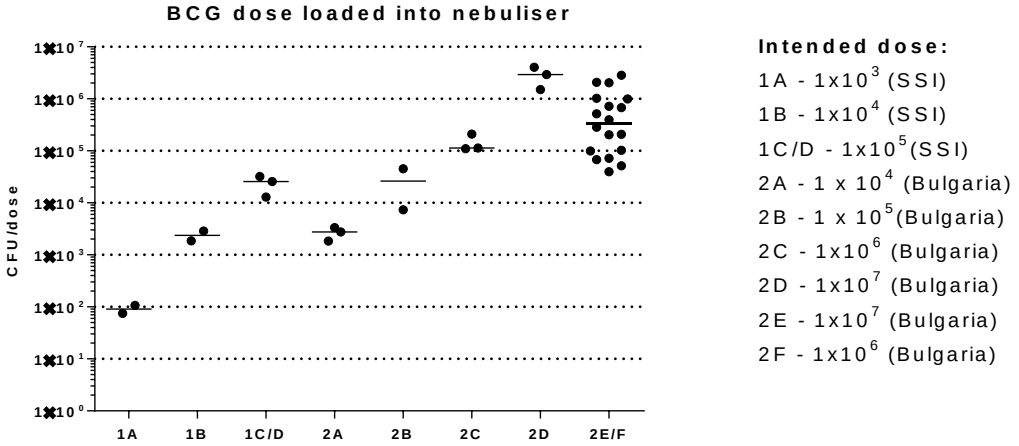


Figure 4. BCG cfu intended dose following dilution of either BCG SSI and BCG Bulgaria

4. We have developed an *in-vitro* model to quantify the actual delivered dose of nebulised BCG. This involved using a vacuum pump attached to an impinger to collect the aerosol expelled by the Omron nebuliser. The amount of BCG in this collected sample was quantified by culture plating. This model consistently measured an approximately 50% loss of BCG compared to the amount of BCG loaded into the nebulizer (*Figure **5***). This model does not take into account the further potential loss of BCG that occurs in a clinical setting through loss of aerosol into the environment, as the volunteer does not breathe in continuously, and the nebuliser continues to aerosolise the BCG between breaths.

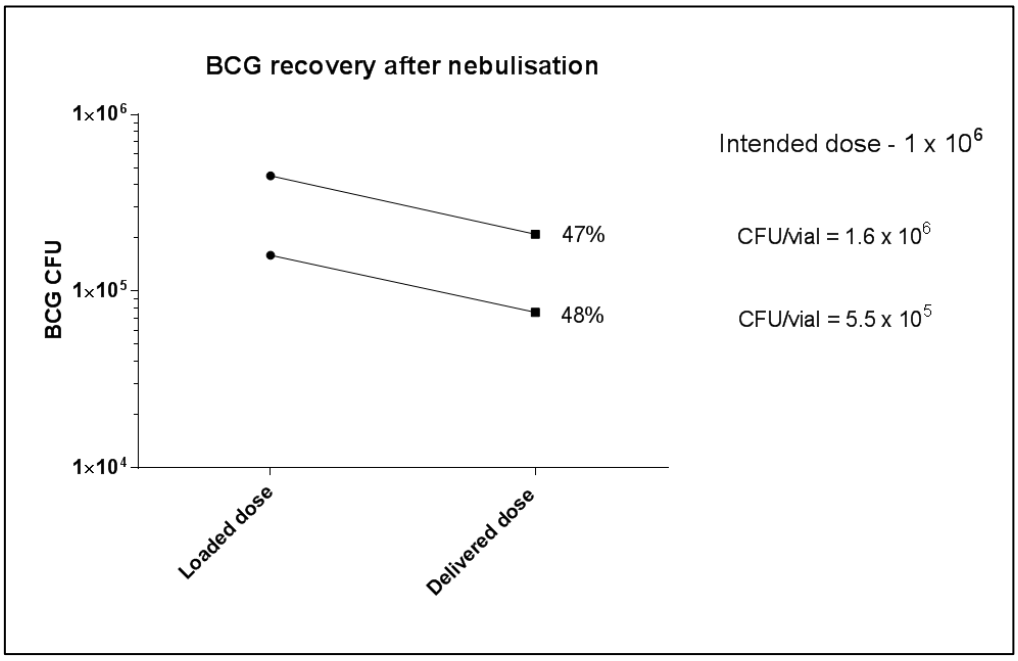


Figure 5. BCG recovery after nebulisation using vacuum model

Given these documented losses, we ensure the appropriately delivered dose of ~$5x10^5$cfu by utilising a prepared dose of $1x10^7$cfu.

6.3 Risks and Benefits

Potential risks

The potential risks to participants in this study include risks associated with:

1. Venepuncture and intravenous cannulation

Localised bruising and discomfort can occur at the site of venepuncture. Infrequently fainting may occur. The total volume of blood drawn over a six-month period will be 520ml which should not compromise these otherwise healthy volunteers, as they would donate 470mL during a single blood donation for the National Blood transfusion Service over a 3-4 month period. Volunteers will be asked to refrain from blood donation for the duration of their involvement in the study.

An intravenous cannula is routinely inserted into a peripheral (usually forearm) vein prior to bronchoscopy in order to administer intravenous sedation. This is removed once the procedure is completed. The risks of cannulation are identical to those associated with venepuncture but include an additional small risk of soft tissue infection. This risk will be minimised by an aseptic insertion technique and is easily recognisable and treatable. The short duration of cannulation (a few hours) further minimises this risk.

2. BCG challenge

BCG Danish 1331 is on the WHO list of pre-qualified vaccines and has a well-defined side effect profile. Full details are given in the SmPC.(56)BCG is licensed for delivery via the intradermal route. It is not licensed for delivery via the aerosol route.

Anticipated local adverse events following aerosol challenge include mild throat discomfort and cough. Transient bronchospasm causing wheezing, shortness of breath, chest tightness or chest pain are also possible (Appendix A).

Systemic reactions

Systemic reactions to aerosolised BCG could include transient fever or feverishness, malaise, nausea, headache, myalgia, arthralgia or fatigue. In the aerosol BCG trial TB041 (Clinicaltrials.gov NCT02709278), approximately 1/3 of participants experienced systemic symptoms, with an average duration of 24 hours.

Following intradermal BCG, disseminated complications of BCG, such as bone infections, have been reported, but are extremely rare and usually reported in immunocompromised individuals. No such complications have occurred in any trial delivering aerosolised BCG. Volunteers are screened to ensure they are immunocompetent prior to enrolment. BCG Danish 1331 is fully susceptible to isoniazid, rifampicin, ethambutol and all second line anti-tuberculous drugs. (56)

Allergic reactions

Allergic reactions from mild to severe may occur in response to any constituent of a medicinal product's preparation. Anaphylaxis is extremely rare (less than 1/1000 people) but can occur in response to any vaccine or medication.

3. Bronchoscopy

Bronchoscopy is a widely and safely used investigative procedure in clinical research studies involving both healthy volunteers and patients with respiratory conditions such as asthma and interstitial lung disease (59, 60). Clinical guidelines for performing investigative bronchoscopy in research studies are well established (61).

The bronchoscopies will be carried out in a dedicated NHS bronchoscopy suite with an excellent safety record by highly skilled and experienced consultant respiratory physicians. Intravenous sedation and topical local anaesthesia are administered prior to bronchoscopy to reduce discomfort, facilitate the procedure, and remove memory of the event. (In 98% of cases, subjects have no memory of the procedure). Trained, experienced staff and facilities for resuscitation and drugs for reversal of sedation will be available. To further minimise risk, volunteers will be excluded if they have an abnormal chest radiograph, increased bleeding risk, a significant smoking history, a clinically significant history of atopy or any evidence of lung disease, including asthma (as defined by: a clinical diagnosis of asthma; prescription of asthma medication; airflow obstruction on spirometry; history of nocturnal or exercise-induced wheeze).

The risks of bronchoscopy are discussed at the time of consent and comprise: adverse reaction to sedation or local anaesthetic, sore throat and /or transient hoarse voice, blood-stained phlegm, laryngospasm/bronchospasm, hypoxia, post-procedure flu-like symptoms (1-2 days), damage to airways and risk of death (<1 in 100,000).

- Bronchoalveolar lavage and endobronchial biopsy are routine procedures in investigative bronchoscopy and carry a minimum bleeding risk. The risk of infection or febrile reactions will be minimised by full bronchoscope asepsis.
- Respiratory depression secondary to sedation is rare. No rescue medication for oversedation has been required for volunteers participating in any of our aerosol clinical trials to date (43)), Clinicaltrials.gov NCT02532036, NCT01954563 and NCT02709278).
- Allergic reactions from mild to severe may occur in response to any constituent of the local anaesthetic or sedative agents. Anaphylaxis is extremely rare but can occur. The Summary of Product Characteristics (SmPC) for the local anaesthetic and sedative agents contain full details of the indications and side effects of these licensed medications.

4. Chest radiograph

A chest radiograph is a painless radiological investigation that exposes volunteers to approximately 0.02 milliSieverts of radiation, equivalent to around 3 days of natural background radiation. The additional risk of cancer due to one chest radiograph is insignificant (1/900,000).

5. Spirometry and Transfer factor of the Lung for Carbon Monoxide (TLCO)

Vigorous respiratory manoeuvres such as forced expiration through the spirometer can occasionally lead to coughing or light-headedness, but these symptoms are mild and rapidly self-limiting.

6. Induced sputum

Inhalation of hypertonic saline to induce sputum production can occasionally result in excessive bouts of coughing or sensation of shortness of breath but these symptoms are mild and rapidly self-limiting. Rarely, hypertonic saline can induce bronchoconstriction but this is very unlikely to occur in our cohort of patients who have no clinically significant history of atopy and no asthma. Hypertonic saline-induced bronchospasm is quickly reversed by treatment with an inhaled short-acting B_2 agonist. Volunteers will be pre-treated with nebulised salbutamol and their lung function will be monitored throughout the procedure. This procedure has been used in TB041 Clinicaltrials.gov NCT02709278 with no adverse events and has been well tolerated by volunteers.

Potential benefits

Volunteers are not expected to benefit directly from participation in this study. Volunteers will gain some information about their general health as a result of the screening history, examination, blood tests, urine tests, chest radiograph and pulmonary function. They may also gain health information from the bronchoscopy. Volunteers found to have a previously undiagnosed condition thought to require further medical attention will be referred appropriately to their GP or an NHS specialist service for further investigation and treatment, with their permission.

It is hoped that their contribution will further the development of a safe and successful vaccine for TB and our knowledge about TB infection and protection.

7 OBJECTIVES AND OUTCOME MEASURES

Table 2. Objectives and Outcome Measures

Objectives	Outcome Measures	Timepoint(s) of evaluation of this outcome measure
Primary Objective To define the systemic and mucosal innate and adaptive immune responses induced by aerosol inhaled BCG infection in healthy BCG and *M.tb* naïve UK adults	Laboratory markers of innate and adaptive immunity, including *ex-vivo* ELISpot and ELISAs; and RNA sequence analysis and intracellular cytokine staining or chip cytometry with blood, BAL and biopsy samples	At each visit
Secondary Objective To identify laboratory markers of the immune response that correlate with protection as defined by PBMC MGIA at D56 post challenge	Established and exploratory markers of innate, cell mediated and humoral immunity in blood, BAL and biopsy samples; Culture (colony counting) and PCR quantification of BCG in BAL; MGIA on PBMCs collected at Day 56	At each visit
Tertiary Objective To describe the human clinical response to BCG challenge by the aerosol inhaled route in healthy, BCG-naïve UK adult volunteers	Actively and passively collected data on adverse events; detailed participant symptom profiles; Laboratory parameters including BCG CFU counts in induced sputum	At each visit and via e-diary(s)

8 PARTICIPANT IDENTIFICATION

8.1 Study Participants

Healthy, BCG and *M.tb.*-naïve UK adults have been chosen because of their low baseline level of anti-mycobacterial immunity. The aim is to identify a primary innate and adaptive immune response after aerosol BCG infection in people who are mycobacterially naïve.

Inclusion Criteria

Volunteers must meet all of the following criteria to enter the study:

- Healthy adult aged 18-50 years
- Resident in or near Oxford for the duration of the study period
- Screening IGRA negative
- No relevant findings in medical history or on physical examination
- Allow the Investigators to discuss the individual's medical history with their GP
- Use effective contraception (see below) for the duration of the study period (females only)
- Refrain from blood donation during the study
- Give written informed consent
- Allow the Investigator to register volunteer details with a confidential database (The Over-volunteering Protection Service) to prevent concurrent entry into clinical studies/trials

- Able and willing (in the Investigator's opinion) to comply with all the study requirements

Exclusion Criteria

Volunteers must meet none of the following criteria to enter the study:

- Previously resident for more than 12 months concurrently in a tropical climate where significant non-tuberculous mycobacterial exposure is likely
- Participation in another research study involving receipt of an investigational product in the 30 days preceding enrolment, or planned use during the study period[a]
- Prior vaccination with BCG or any candidate TB vaccine
- Administration of immunoglobulins and/or any blood products within the three months preceding the planned study challenge date
- Clinically significant history of skin disorder, allergy, atopy, immunodeficiency (including HIV), cancer (except BCC or CIS), cardiovascular disease, gastrointestinal disease, liver disease, renal disease, endocrine disorder, neurological illness, psychiatric disorder, drug or alcohol abuse
- Concurrent oral, inhaled or systemic steroid medication or the concurrent use of other immunosuppressive agents
- Shares a household with someone with clinically significant immunodeficiency (either from infection or medication) who is deemed to be at risk of developing disseminated BCG infection if exposed to BCG
- History of anaphylaxis to vaccination or any allergy likely to be exacerbated by any component of the study agent, sedative drugs, or any local or general anaesthetic agents
- Pregnancy, lactation or intention to become pregnant during study period
- Any respiratory disease, including asthma
- Current smoker (defined as any smoking including e-cigarettes in the last 3 months)
- Clinically significant abnormality on screening chest radiograph[b]
- Clinically significant abnormality of pulmonary function
- Any nasal, pharyngeal, or laryngeal finding which precludes bronchoscopy
- Current use of any medication taken through the nasal or inhaled route including cocaine or other recreational drugs
- Clinical, radiological, or laboratory evidence of current active TB disease
- Past treatment for TB disease
- Any clinically significant abnormality of screening blood or urine tests[b]
- Positive HBsAg, HCV or HIV antibodies[b]
- Any other significant disease, disorder, or finding, which, in the opinion of the Investigator, may either put the volunteer at risk, affect the volunteer's ability to participate in the study or impair interpretation of the study data

[a] Volunteers will be excluded from the study if they are concurrently involved in a study or trial that involves regular blood tests or an investigational medicinal product. In order to check this, volunteers will be asked to provide their National Insurance or Passport number (if they are not

entitled to a NI number) and will be registered on a national database of participants in clinical trials (www.tops.org.uk).

[b] Volunteers who are excluded from the study because they have been discovered during screening procedures to be suffering from a previously undiagnosed condition thought to require further medical attention will be referred appropriately to their GP or an NHS specialist service for further investigation and treatment.

Effective contraception for female volunteers

Female volunteers are required to use an effective form of contraception during the course of the study.

Acceptable forms of contraception for female volunteers include:

- Established use of oral, injected on implanted hormonal methods of contraception
- Placement of an intrauterine device (IUD) or intrauterine system (IUS)
- Permanent sterilisation or bilateral tubal occlusion
- Barrier methods of contraception (condom; or occlusive cap with spermicide)
- Male sterilisation, if the vasectomised partner is the sole partner for the subject
- True abstinence, when this is in line with the preferred and usual lifestyle of the subject (periodic abstinence and withdrawal are not acceptable methods of contraception)

9 STUDY DESIGN AND PROCEDURES

This is a partially randomised controlled clinical challenge study of BCG administered by the aerosol-inhaled route in healthy UK volunteers.

9.1 Study Numbers and Groups

65 volunteers will be enrolled; the first thirteen to Group 1, the next thirteen to Group 2, the next thirteen to Group 3, the next thirteen to Group 4 and the final thirteen to Group 5. Once allocated to groups, the volunteer will be randomly allocated to either BCG challenge (Arm A) or placebo saline control (Arm B); Table 3.

In the event of bronchoscopy postponement, (see postponement criteria 9.10) the bronchoscopy will be rescheduled where possible within the allowable window for that follow up visit. If possible the rest of the procedures for that follow up visit will occur on the scheduled day (e.g. observations, e-diary check, blood sample collection), with the bronchoscopy to follow. If the bronchoscopy occurs outside of the allowable time window (as is likely for deferred bronchoscopies in Groups G1, G2 or G3 due to the tight time windows) the volunteer may be reallocated to a later Group in order to fit in with schedule, as per the discretion of the investigator. A new volunteer will then be enrolled into that original group using additional randomisation envelopes, where needed, to replace the volunteer whose bronchoscopy was postponed.

Table 3. Study groups

Group	Intervention	Sample size
Group 1: Bronchoscopy Day 2 post challenge	**Arm A BCG**: Aerosol inhaled BCG	10
	Arm B Control: Aerosol inhaled normal saline placebo	3
	Arm A BCG: Aerosol inhaled BCG	10

Group 2: Bronchoscopy Day 7 post challenge	***Arm B Control*:** Aerosol inhaled normal saline placebo	3
Group 3: Bronchoscopy Day 14 post challenge	**Arm A BCG**: Aerosol inhaled BCG	10
	Arm B Control: Aerosol inhaled normal saline placebo	3
Group 4: Bronchoscopy Day 28 post challenge	**Arm A BCG**: Aerosol inhaled BCG	10
	Arm B Control: Aerosol inhaled normal saline placebo	3
Group 5: Bronchoscopy Day 56 post challenge	**Arm A BCG**: Aerosol inhaled BCG	10
	Arm B Control: Aerosol inhaled normal saline placebo	3

9.2 Recruitment

Volunteers may be recruited by use of an advertisement formally approved by the ethics committee and distributed or posted in the following places:

- In public places (including NHS hospitals and university buildings) with the agreement of the owner or proprietor
- In newspapers or other literature for circulation
- On radio via announcements
- On a website operated by our group or with the agreement of the owner or operator
- By e-mail distribution to a group or list only with the express agreement of the network administrator or with equivalent authorisation
- On stalls or stands at exhibitions or fairs
- Direct mail-out: This will involve obtaining names and addresses of adults via the most recent Electoral Roll. The contact details of individuals who have indicated that they do not wish to receive postal mail-shots would be removed prior to the Investigators being given this information. The company providing this service is registered under the Data Protection Act 1998. Investigators would not be given dates of birth or ages of individuals, but the list supplied would only contain names of those aged between 18-50 years (as per the inclusion criteria).
- Oxford Vaccine Centre databases: We may contact individuals from databases of groups within the CCVTM (including the Oxford Vaccine Centre database) of previous trial participants who have expressed an interest in receiving information about future studies for which they may be eligible.

A copy of intended advertising will be submitted with the initial application for ethical approval and any significant changes to this advertisement will be submitted as an amendment to the ethics committee for approval before use.

Volunteers who express an interest in the study will be given a Volunteer Summary Sheet and asked to register for the study through the website. In addition, and with their permission, their details will be passed to the study team to arrange a pre-screening call and screening appointment.

9.3 Informed Consent

Written informed consent will be obtained at screening by a GCP trained Investigator.

The volunteer must sign and date the latest approved version of the consent form before any study specific procedures are performed.

The Volunteer Information Sheet (VIS) will be made available to the volunteer no less than 24 hours prior to attending for screening. The details enclosed will be discussed with the volunteer, including:

- the aims and nature of the study
- the agents used
- the schedule of visits and tests to be carried out
- the implications and constraints of the protocol
- compensation for the volunteer
- known side effects and risks of taking part in the study

The following general principles will be emphasised:

- Participation is entirely voluntary
- The volunteer may withdraw from the study at any time for any reason
- Withdrawal or refusal to participate involves no penalty or loss of medical benefits
- The volunteer is free to ask questions at any time to allow him or her to understand the purpose of the study and the procedures involved
- There is no direct benefit from participating
- The volunteer's GP will be contacted to corroborate their medical history and confirm that the volunteer does not meet any of the exclusion criteria
- The volunteer will be registered on the TOPS database (The Over-Volunteering Protection System)
- With permission, the volunteer's samples taken as part of the study will be stored indefinitely for further research.

The volunteer will then have time to consider whether or not to participate. If the volunteer decides to participate, they will sign and date two copies of the consent form, one for them to take away and keep, and one for the Investigator that will be retained at the study site. These forms will also be signed and dated by the Investigator. This will occur before any study specific procedures are performed.

9.4 Screening and Eligibility Assessment

Once the volunteer has given their consent to undergo screening, a baseline medical history (including concomitant medication and allergy to medication) and physical examination will be performed by a GCP trained doctor. Inclusion and exclusion criteria (listed below) will be checked using a tabulated format. Demographic and occupational data will be collected. Vital signs will be checked and bloods taken including FBC, clotting profile, U&Es, LFTs, HIV antibodies, HBsAg, HCV antibodies and IGRA. Spirometry will be performed according to local SOP. Volunteers will be counselled by one of the Investigators for HIV, Hepatitis B and Hepatitis C testing. Urine will be tested for the presence of clinically significant proteinuria, glycosuria or haematuria. A pregnancy test will be performed for female volunteers. Volunteers will then attend for a chest radiograph and pulmonary function test (spirometry and Transfer factor of the Lung for Carbon Monoxide (TLCO)).

Laboratory parameters for inclusion/exclusion in the study will be considered on an individual basis, with Investigator discretion for interpretation of results and the need for repeated tests. In general, volunteers will be excluded if a result at screening constitutes what would qualify as a grade 1 (or higher) laboratory AE, according to the laboratory AE tables found in the relevant SOP and filed in the trial master file (TMF).

The total duration of the screening visit will be around 2 hours. Depending upon availability and scheduling of the volunteer and NHS trust radiography staff, the screening may be split across two visits.

9.5 Randomisation and blinding

Volunteers will be allocated to a study group (Groups 1 - 5) in a sequential manner. To reduce bias and the influence of any difference in the baseline characteristics between the BCG and control groups, we will randomise the allocation of volunteers using sequentially numbered sealed envelopes, prepared by an independent statistician at the Department of Primary Care.

Volunteers will be blinded to eliminate subject bias (either conscious or subconscious) using a 10:3 randomised control design (BCG:placebo) whereby volunteers randomised to the BCG arms (Arm A) will inhale aerosolised BCG mixed with normal saline and those randomised to the placebo arms (Arm B) will inhale aerosolised normal saline. This design has the added benefit of allowing a distinction to be made between any adverse events attributable to the method (including nebuliser device) of challenge delivery and those attributable to the inhaled challenge product itself. The volunteers will be unblinded just prior to the 3 month visit when those in Arm A will have sputum collected and those in Arm B will not.

In the event that a volunteer is not fit for their scheduled bronchoscopy they will be reallocated to a later group. A new volunteer will then be allocated to the earlier group and extra randomisation envelopes added as needed. In this case, as the allocation ratio for each group may be altered, this will technically be a “partial randomisation”, with allocations being made dependent on what has occurred already.

The bronchoscopist performing the procedure will also be blinded to eliminate any bias in the reporting of the appearance of the lung mucosa and extent of airway inflammation.

All samples will be anonymised and the subject number will be allocated sequentially and therefore not identifiable with the allocated Arm. The senior immunologist will be blinded to reduce any bias that could be introduced at the sample processing stage.

9.6 Follow up visits

D0 - Challenge

Any new medical issues or symptoms that have arisen will be assessed. Venepuncture and other samples will be taken according to the Schedule of Study Procedures in section 4.2. Vital signs and spirometry will be performed. The inclusion and exclusion criteria for the study will be reviewed; provided that the volunteer still satisfies all inclusion criteria (and no exclusion criteria) and their consent remains valid, the volunteer will be allocated to a group and randomised to study intervention. BCG challenge or placebo will be administered by aerosol using the Omron nebuliser according to the study-specific SOP. Volunteers will be kept under observation for around 60 minutes after challenge. The challenge visit will last approximately 3 hours. The e-diary will be set up during this visit.

E-diaries

All volunteers will complete an e-diary from Day 0 through to Day 13 following challenge. The e-diary will ask for recorded temperature and will solicit respiratory and systemic symptoms as well as capturing any unsolicited symptoms. A second 7 day e-diary will be completed by volunteers in Groups 3-5, starting at time of bronchoscopy, in order to capture bronchoscopy-related symptoms. For Groups 1 and 2 this data will be captured within their first e-diary as their bronchoscopy will fall within the first e-diary period.

Bronchoscopy

The bronchoscopy visit lasts several hours and takes place at the bronchoscopy suite in the OUH NHS Foundation Trust by the specialist team there. Blood will be taken as specified in the Schedule of Study Procedures (Table 1). Details of the procedure are outlined in Section 6.3.

Other follow-up visits

These will be performed as specified in the Schedule of Study Procedures (Table 1). Any new medical issues or symptoms that have arisen will be assessed, and the e-diary (if applicable) and any ongoing adverse events will be reviewed. Additional procedures or laboratory tests may be carried out at the discretion of the Investigators if deemed clinically necessary. Follow-up visits will last approximately 15-30 minutes, except for D84 and D168 visits when induced sputum is collected for volunteers in Arm A. For these volunteers, the visit will last approximately 1.5 hours. The study duration will be approximately 24 weeks from the date of enrolment.

Volunteers will be advised of the intervention they received (BCG infection or saline control) prior to their 3 month visit (or earlier if they withdraw).

After the final study visit, an End of Study letter will be sent to each volunteer's GP to inform them that the volunteer has either completed the study and which intervention they received, or was not enrolled.

9.7 Timepoints

Screening – study enrolment time window

Enrolment should take place no longer than 120 days following the date of screening appointment. If more than 120 days elapse, the screening visit should be repeated in full, prior to enrolment, in order to minimise the risk to participants of any new unidentified health problems having arisen during that period.

Follow up period

The follow up period will be six months in accordance with findings from previous studies in which adequate safety data and reliable markers of immunogenicity have been obtained in this time interval.

9.8 Sample handling

Details regarding samples, volume and frequency of sampling are listed in the Schedule of Study Procedures (Table 1). Blood and other samples will be processed according to local laboratory SOPs. All samples will be anonymised at the CCVTM/OUH.

Volunteers will be informed that there may be leftover samples of their blood, BAL, biopsy and sputum. With the volunteers' informed consent, any leftover samples will be frozen for future analysis. This may include human DNA and RNA analysis to search for correlates of TB risk and/or protection. Samples may be shipped to other parties involved in our research in anonymised form for immunological analysis. Volunteers will be able to decide if they will permit such future use of any leftover samples. If they elect not to permit this, leftover samples will be discarded.

9.9 Challenge Postponement Criteria

Challenge will not proceed on the scheduled day in any of the following situations:

- The volunteer has a current or recent upper respiratory tract infection, unless they have been symptom-free for at least one week
- The volunteer has a temperature > 37.5^{o}C

- The Investigator judges the volunteer to have an acute moderate or severe illness (whether febrile or not)
- The volunteer has received a live vaccine within the preceding 28 days
- The Investigator has any other concern that challenge may not be in the volunteer's best interests

In these cases, the volunteer may be challenged at a later date or withdrawn from the study at the discretion of the Investigator.

9.10 Bronchoscopy Postponement criteria

Bronchoscopy will not proceed on the scheduled day in any of the following situations:

- The volunteer has a temperature > 37.5^{o}C
- The Investigator judges the volunteer to have an acute moderate or severe illness (whether febrile or not)
- The Investigator has any other concern that bronchoscopy may not be in the volunteer's best interests

9.11 Discontinuation / Withdrawal Criteria

Every reasonable effort will be made to maintain protocol compliance and participation in the study.

In accordance with the principles of the current revision of the Declaration of Helsinki and any other applicable regulations, a volunteer has the right to withdraw from the study at any time and for any reason, and is not obliged to give his or her reasons for doing so. The Investigator may withdraw the volunteer at any time in the interests of the volunteer's health and well-being (including on the advice of the LSC). In addition, the volunteer may withdraw/be withdrawn for any of the following reasons:

- Administrative decision by the Investigator
- Ineligibility (either arising during the study or retrospectively, having been overlooked at screening)
- Significant protocol deviation
- Volunteer non-compliance with study requirements (failure to attend two or more follow-up visits)
- Any AE which requires discontinuation of study involvement or results in inability to comply with study procedures
- Confirmed pregnancy during the study

Any volunteer who becomes pregnant during the study will be followed up as per the protocol and until the end of the pregnancy. We will not routinely perform venepuncture on a pregnant volunteer.

The reason for withdrawal will be recorded in the Case Report Form (CRF). Volunteers withdrawn from the study may be replaced on the decision of the Investigator. If the volunteer is withdrawn due to an AE, the Investigator will arrange for appropriate specialist management or follow up visits or telephone calls until the AE has resolved or stabilised. The extent of follow up after premature discontinuation will be determined by the Investigator but will be at least

for the whole study period, and if pregnant, until pregnancy outcome. An 'End of Study letter' will be sent to each withdrawn volunteer's GP.

If a volunteer withdraws from the trial, samples collected before their withdrawal from the study will be used/stored unless the volunteer specifically requests otherwise. Long term safety data collection will continue as appropriate if a volunteer has received a challenge dose.

9.12 Safety

Discontinuation of the study

The study will be discontinued in the event of any of the following:

- New scientific information is published to indicate that volunteers in the study are being exposed to undue risks as a result of administration of BCG, or as a result of the study procedures or follow-up schedule.
- Serious concerns about the safety of BCG arise as a result of one or more challenge-related SAE(s) occurring in the volunteers enrolled in this or any other ongoing study of BCG.
- For any other reason at the discretion of the Investigator.

9.13 End of Study Definition

The study will be completed when the last volunteer enrolled into the study has completed their final follow up visit.

10 STUDY AGENTS AND DEVICES

10.1 BCG Description

BCG Danish 1331

BCG Danish 1331 is a dried preparation containing live bacteria derived from an attenuated strain of *Mycobacterium bovis* BCG. It is supplied as a powder and diluent for suspension. Each vial contains 2-8x10^6cfu (see SmPC).

Storage of BCG

BCG Danish will be shipped to the CCVTM, University of Oxford, Churchill Hospital, in the presence of a temperature logger. The vaccine will be re-labelled for local use.

Dispensed BCG vials are supplied in boxes containing multiple vials and each box is clearly labelled with the market product.

BCG will be stored at +2 to +8°C (nominal temperature) in a secure, temperature-monitored refrigerator at the CCVTM, University of Oxford, Churchill Hospital.

10.2 Dispensing and administration

All movements of vials of the study agent in or out of the locked refrigerator will be documented. BCG accountability, storage, shipment and handling will be in accordance with local SOPs and other relevant local forms.

BCG will be administered by aerosol inhalation, according to the study-specific SOP. Volunteers will stay in the unit for 60 minutes (±10 minutes) after challenge. During the administration of BCG, monitoring equipment, oxygen, medicines including bronchodilators and resuscitation

equipment will be immediately available for the management of anaphylaxis and bronchospasm according to the study-specific SOP.

In order to minimise dissemination of the BCG bacteria into the environment and to ensure the protection of staff, measures will be instituted during and following challenge to fully comply with local infection control and Occupational Health regulations.

10.3 Saline

Sterile saline will be mixed with the reconstituted BCG to add to the nebuliser. Sterile saline will also be used for placebo doses. The volume of saline used as placebo will be identical to the challenge volume.

Hypertonic saline will be delivered via nebuliser during the induced sputum procedure according to the study-specific SOP.

10.4 Salbutamol

Volunteers undergoing the induced sputum procedure will be pre-treated with a prescribed dose of salbutamol (200mcg delivered via spacer) in accordance with the European Respiratory Society's guidelines [33].

10.5 MicroAIR NE-U22

This nebuliser is used for aerosol delivery of the BCG or placebo. This is an approved electromedical device, CE0197, EAN code 40 15672 10142 1. Information about the specifications, usage, and maintenance of the nebuliser device can be found in the device documentation (62). This is the same device used in studies TB026, TB035 and TB041 ((40), Clinicaltrials.gov NCT01954563 and NCT02709278).

10.6 Ultrasonic nebuliser NE-U780

This nebuliser is used for delivering the hypertonic saline during the induced sputum procedure. This is an approved electromedical device, CE0197, EN60601-1-2-2007. Information about the specifications, usage, and maintenance of the nebuliser device can be found in the device documentation [35].

10.7 Sedative & anaesthetic agents for bronchoscopy

Fentanyl is a licensed opioid used routinely to provide analgesia and sedation during medical procedures. It will be stored, dispensed and administered in accordance with standard NHS procedures and the SmPC.

Midazolam is a licensed benzodiazepine used routinely to provide sedation and amnesia during medical procedures. It will be stored, dispensed and administered in accordance with standard NHS procedures and the SmPC.

Lignocaine is a licensed local anaesthetic used routinely during medical procedures. It will be stored, dispensed and administered in accordance with standard NHS procedures and the SmPC.

Other licensed drugs may be used during bronchoscopy at the discretion of the Respiratory consultant performing the bronchoscopy and/or the Investigators.

11 ASSESSMENT OF SAFETY

Safety will be assessed by the frequency, incidence and nature of adverse events and serious adverse events arising during the study, as defined in Schedule of Visits and Procedures Section 4.2.

11.1 Definitions

Although the BCG challenge agent does not constitute an IMP, definitions for safety purposes are based on those used for IMPs.

Adverse Event (AE)

An AE is any untoward medical occurrence in a volunteer, which may occur during or after administration of BCG and does not necessarily have a causal relationship with the intervention. An AE can therefore be any unfavourable and unintended sign (including an abnormal laboratory finding), symptom or disease temporally associated with the study intervention, whether or not considered related to the study intervention.

Each adverse event will be graded by the participant according to the table for grading severity of adverse events (see Section 11.6). Severity gradings may be reviewed and discussed with the participants at the clinic visits.

Adverse Reaction (AR)

An AR is any untoward or unintended response to BCG. This means that a causal relationship between the agent and an AE is at least a reasonable possibility, i.e., the relationship cannot be ruled out. All cases judged by the reporting medical Investigator as having a reasonable suspected causal relationship to BCG (i.e. possibly, probably or definitely related to it) will qualify as adverse reactions.

Unexpected Adverse Reaction

An adverse reaction, the nature or severity of which is not consistent with the applicable product information (e.g. SmPC).

Serious Adverse Event (SAE)

An SAE is an AE that results in any of the following outcomes, whether or not considered related to the study intervention:

- Death.
- Life-threatening event (i.e., the volunteer was, in the view of the Investigator, at immediate risk of death from the event that occurred). This does not include an AE that, if it occurred in a more severe form, might have caused death.
- Persistent or significant disability or incapacity (i.e., substantial disruption of one's ability to carry out normal life functions).
- Hospitalisation or prolongation of existing hospitalisation, regardless of length of stay, even if it is a precautionary measure for continued observation. Hospitalisation (including inpatient or outpatient hospitalisation for an elective procedure) for a pre-existing condition that has not worsened unexpectedly does not constitute a serious AE.
- An important medical event (that may not cause death, be life threatening, or require hospitalisation) that may, based upon appropriate medical judgment, jeopardise the volunteer and/or require medical or surgical intervention to prevent one of the outcomes listed above. Examples of such medical events include allergic reaction

requiring intensive treatment in an emergency room or clinic, blood dyscrasias, or convulsions that do not result in inpatient hospitalisation.

- Congenital anomaly or birth defect.

Serious Adverse Reaction (SAR)

An adverse event (expected or unexpected) that is both serious and, in the opinion of the reporting Investigator or Sponsors, believed to be possibly, probably or definitely due to BCG or any other study treatments, based on the information provided.

Suspected Unexpected Serious Adverse Reaction (SUSAR)

A serious adverse reaction, the nature and severity of which is not consistent with the information about the medicinal product in question set out in the SmPC.

NB: To avoid confusion or misunderstanding the following note of clarification is provided: "Severe" is often used to describe intensity of a specific event, which may be of relatively minor medical significance. "Seriousness" is the regulatory definition supplied above.

11.2 Foreseeable Adverse Reactions

Foreseeable adverse reactions are listed in section 6.3.

11.3 Expected Serious Adverse Events

No serious adverse events are expected in this study.

11.4 Causality Assessment

For every AE, an assessment of the relationship of the event to the administration of BCG will be undertaken. An intervention-related AE refers to an AE for which there is a probable or definite relationship to administration of BCG. An interpretation of the causal relationship of the intervention to the AE in question will be made, based on the type of event, the relationship of the event to the time of BCG administration and the known biology of BCG, with reference to the SmPC (Table 4). Causality assessment will take place during any interim analyses and at the final safety analysis.

Table 4. Guidelines for assessing the relationship of challenge administration to an AE

0	**No Relationship**	No temporal relationship to BCG; ***and*** Alternate aetiology (clinical state, environment or other interventions); ***and*** Does not follow known pattern of response to BCG
1	**Unlikely**	Unlikely temporal relationship to BCG; ***and*** Alternate aetiology likely (clinical state, environment or other interventions); ***and*** Does not follow known typical or plausible pattern of response to BCG
2	**Possible**	Reasonable temporal relationship to BCG; ***or*** Event not readily produced by clinical state, environment or other interventions; ***or*** Similar pattern of response to that seen with other challenge agents
3	**Probable**	Reasonable temporal relationship to BCG; ***and*** Event not readily produced by clinical state, environment or other interventions; ***and*** Known pattern of response seen with other challenge agents
4	**Definite**	Reasonable temporal relationship to BCG; ***and***

		Event not readily produced by clinical state, environment or other interventions; ***and*** Known pattern of response seen with BCG

11.5 Reporting Procedures for All Adverse Events

All AEs occurring in the 14 days following challenge and the 7 days following bronchoscopy observed by the Investigator or reported by the volunteer, whether or not attributed to BCG, will be recorded on electronic diary cards. Data from the diary cards will be extracted following the last volunteer last visit (LVLV) or at any time prior to this in order to perform an interim safety analysis. Outside the diary card periods, respiratory and systemic AEs (listed in Table 5 below) will be specifically solicited at each visit, and graded by severity (as detailed in section 11.6). All AEs starting after the diary card period(s), or persisting after this period, will be recorded in the AE line listing of the CRF.

The overall appearance of the lung mucosa will be assessed at the bronchoscopy.

All AEs that result in a volunteer's withdrawal from the study will be followed up until a satisfactory resolution occurs, or until a non- study related causality is assigned (if the volunteer consents to this). Serious adverse events (SAEs) will be collected throughout the entire study period.

Table 5. Routinely solicited adverse events

	Adverse event
Respiratory	Cough
	Sore throat
	Tickly throat
	Wheeze
	Shortness of breath
	Coughing up phlegm
	Coughing up blood
	Chest tightness
	Chest pain
Systemic	Documented fever (oral temperature > 37.5° C)
	Myalgia
	Arthralgia
	Feverishness
	Headache
	Fatigue
	Nausea
	Malaise

Reporting Procedures for Serious AEs (see Safety Reporting SOP)

SAEs will be reported on the SAE forms to members of the study team immediately, once the Investigators become aware of their occurrence, as described in the study-specific SOP. Copies of all reports will be forwarded for review to the Chief Investigator (as the Sponsor's representatives) within 24 hours of the Investigator being aware of the suspected SAE. The Safety Monitoring Committee (SMC) will be notified of SAEs which are deemed possibly,

probably or definitely related to study interventions; the SMC will be notified immediately (within 24 hours) of the Investigators' being aware of their occurrence. SAEs will not normally be reported immediately to the ethical committee(s) unless there is a clinically important increase in occurrence rate, an unexpected outcome, or a new event that is likely to affect safety of study volunteers, at the discretion of the Chief Investigator and/or SMC.

Reporting Procedures for SARs
These will be reported as per any SAE.

Reporting Procedures for SUSARs
The Chief Investigator will report all SUSARs to the ethical committee(s) within required timelines (15 days for all SUSARs, unless life threatening in which case 7 days, with a final report within a further 8 days (total 15)). The Chief Investigator will also inform all Investigators concerned of relevant information about SUSARs that could adversely affect the safety of participants.

All SUSARs and deaths occurring during the study will be reported to the Sponsor. For all deaths, available autopsy reports and relevant medical reports will be made available for reporting to the relevant authorities.

Any serious adverse event considered by the CI to be related to the challenge agent and unexpected will be reported to the REC. As the challenge agent has a Marketing Authorisation, the mechanism for reporting any SAEs to the MHRA is via yellow card.

11.6 Assessment of Severity

The severity of clinical AEs will be assessed according to the scale in Tables 6 &7:

Table 6. Severity grading criteria for physical observations.

	Grade 1 (mild)	**Grade 2 (moderate)**	**Grade 3 (severe)**
Fever (oral)	37.6°C - 38.0°C	38.1°C – 39.0°C	>39.0°C
Tachycardia (bpm)*	101 - 115	116 – 130	>130
Bradycardia (bpm)**	50 – 54	40 – 49	<40
Systolic hypertension (mmHg)	141 - 159	160 – 179	≥180
Diastolic hypertension (mmHg)	91 - 99	100 – 109	≥110
Systolic hypotension (mmHg)***	85 - 89	80 – 84	<80

*Taken after ≥10 minutes at rest; **When resting heart rate is between 60 – 100 beats per minute. Use clinical judgement when characterising bradycardia among some healthy volunteer populations, for example, conditioned athletes; ***Only if symptomatic (e.g. dizzy/ light-headed)

Table 7. Severity grading criteria for respiratory and systemic AEs

GRADE 0	None
GRADE 1	Mild: Transient or mild discomfort (< 48 hours); no medical intervention/therapy required

GRADE 2	Moderate: Mild to moderate limitation in activity – some assistance may be needed; no or minimal medical intervention/therapy required
GRADE 3	Severe: Marked limitation in activity, some assistance usually required; medical intervention/therapy required, hospitalisation possible

11.7 Procedures to be followed in the event of abnormal findings

Eligibility for enrolment in the study in terms of laboratory findings will be assessed as detailed in Section 9.4. Abnormal clinical findings from medical history, examination or blood tests will be assessed as to their clinical significance throughout the study. Laboratory adverse events will be assessed using the TMF. If a test is deemed clinically significant, it may be repeated, to ensure it is not a single occurrence. If a test remains clinically significant, the volunteer will be informed and medical care arranged as appropriate and with the permission of the volunteer. Specific details regarding findings, discussion with volunteers and resulting actions will be recorded in the CRF. Decisions to exclude the volunteer from enrolling in the study or to withdraw a volunteer from the study will be at the discretion of the Investigator.

11.8 Safety Monitoring Committee

An independent Safety Monitoring Committee (SMC) will be appointed to provide real-time safety oversight. The SMC will be notified within 24 hours of the Investigators' being aware of the occurrence of SAEs. The SMC has the power to place the study on hold if deemed necessary following a study intervention-related SAE. The SMC will be chaired by Dr Hassan Mahomed with Professor Stephen Gordon and statistician Dr Francesca Little as committee members. All correspondence between Investigator and SMC will be conveyed by the Investigator to the study Sponsor. The Chair of the SMC will be contacted for advice and independent review by the Investigator or study Sponsor in the following situations:

- Following any SAE deemed to be possibly, probably, or definitely related to BCG.
- Any other situation where the Investigator or study Sponsor feels independent advice or review is important.

Safety Profile Review

The safety profile will be assessed on an on-going basis by the Investigators. The Chief Investigator and relevant Investigators (as per the study delegation log) will also review safety issues and SAEs as they arise.

12 STATISTICS

Statistical support will be provided by an ongoing collaboration with the statistical team at the Nuffield Department of Primary Care Health Sciences Clinical trials Unit (safety and non-transcriptomic data) and with the Jenner Transcriptomic Core Facility (transcriptomic data).

This is an exploratory study with descriptive endpoints. Our previous experience with clinical studies suggests that this sample size is a feasible number to recruit, screen, enrol, and follow up in practical terms, whilst also allowing the determination of any substantial and clinically significant differences in safety and immunity between the BCG and control arms. Previous studies looking at differential gene expression (DGE) in vaccination studies have demonstrated significant levels of DGE in 1100 genes with 10-12 subjects/group (31). Studies using controlled human malaria infection have successfully identified a cellular immune response, CD38+CD4+ T cells with cytotoxic potential, which associates with protection in 7-10 subjects/group(63). In our intradermal BCG infection studies, we were able to show that mycobacterial recovery was inversely correlated with PPD-specific ELISpot responses using 12 subjects per group (37). Gene

signatures which defined protective antibody responses to an influenza vaccine could be established using 24 subjects (64). In a similar study with a candidate HIV vaccine, 35 subjects in total were sufficient to define a gene signature of innate responses that predicted subsequent CD8+ T cell responses (65). We will evaluate 10 infected subjects at each of 5 time points (N=50) and, assuming control subject responses are negative, expect to pool data from the 3 saline controls at each point (N=15).

A detailed statistical analysis plan will be prepared prior to the data lock at the end of the study. Any deviations from this plan will be detailed in the analysis report.

Primary objective

The laboratory markers of innate and adaptive immunity will be summarised separately for the BCG and control arms using means and standard deviations. Log transformations will be considered where data are not normally distributed, and if this fails, the median and interquartile range will instead be presented. Statistical comparisons between groups will be made using t-tests or Mann-Whitney tests where appropriate, although it will be remembered that the study was not powered to detect significant statistical differences.

We will select key independent variables across all non-transcriptomic assays and combine into one model to determine if a combined model including both innate, cellular and humoral data is better able to predict control of mycobacterial growth than each outcome alone (66, 67).

To capture the dynamic transcriptional responses over time versus baseline, a functional principal component based analysis will be applied to all time points (68). We will identify the minimum number of transcripts required to accurately classify protected and non-protected individuals, using the PBMC MGIA data to define protection.

We will attempt in an exploratory analysis to combine the non-transcriptomic and transcriptomic datasets in order to improve the predictive value of our model.

Secondary objective

The secondary objective will be assessed by correlating different components of the innate immune response with the adaptive response using Spearman's rho. We will also correlate mycobacterial growth in PBMC at D56 with these innate and adaptive immune responses.

Tertiary objective

The tertiary objective, describing the human response to aerosol BCG challenge, will be assessed through collection of AEs as described in Section 4.2. The following parameters will be assessed:

- Occurrence of local signs and symptoms for 14 days following BCG (or placebo); for 7 days following bronchoscopy; and at each visit
- Occurrence of systemic signs and symptoms for 14 days following BCG (or placebo); for 7 days following bronchoscopy; and at each visit
- Bronchoscopy report
- Change from baseline for safety laboratory measures for 28 days following BCG (or placebo)
- Occurrence of serious adverse events during the whole study duration

This AE data will be tabulated and frequency, duration and severity of AEs compared between the two groups (BCG N=50: placebo N=15). Proportions in each group will be compared using

the Chi-squared test or Fisher's exact test for small numbers. Continuous outcomes such as duration will be compared between groups using t-tests or Mann-Whitney tests where appropriate.

13 DATA MANAGEMENT

13.1 Source Data

Source documents are where data are first recorded, and from which participants' CRF data are obtained. These include, but are not limited to, the CRF itself (history and examination), the volunteer consent form, blood and microbiology results, radiology report, GP response letter, copy of bronchoscopy report and any further correspondence relating to the volunteer regarding medical/clinical issues. The CRF will be electronic and paper together with electronic diary cards.

CRF entries will be considered source data if the CRF is the site of the original recording (e.g. there is no other written or electronic record of data). All documents will be stored safely in confidential conditions. On all study-specific documents, other than the contact form, signed consent, the chest X-ray report, GP/medical correspondence and the bronchoscopy report, the participant will be referred to by the study participant number/code, not by name.

13.2 Access to Data

Direct access will be granted to authorised representatives from the Sponsor, host institution and the regulatory authorities to permit study-related monitoring, audits and inspections. All information relating to the study and its volunteers will be held in strict confidence, and in accordance with GCP and institutional requirements.

13.3 Data Recording and Record Keeping

The CI will be responsible for collecting, recording, analysing, and storing all the data accruing from the study. These tasks may be delegated to other Investigators. Paper CRFs will be stored in a key-locked cabinet at the CCVTM, and electronic CRFs on the OpenClinica™ or the e-diary ITCRC LTD databases, which are stored electronically on secure servers that are outsourced by OpenClinica™ or ITCRC respectively. Data from paper CRFs will be transcribed onto the OpenClinica™ database. Some data may be duplicated anonymously into an electronic Microsoft Excel™ file on the CCVTM secure server for clinical monitoring through the study.

Study records will be held by the Investigator for as long as required by anticipated legislation as a minimum (2 years) in order to enable dissemination of study results after publication and to enable decoding and destruction of anonymised samples if subsequently requested by a volunteer. Data will subsequently be transferred to a secure archive in accordance with the Data Protection Act. Data is archived for 25 years and then destroyed. This does not include the retention of bank details, which are destroyed following the completion of volunteer compensation. Volunteers will be assigned individual unique study numbers for identification on all study records, except where the use of identifiable information is unavoidable (including on GP correspondence, registration documents, and consent forms).

14 QUALITY ASSURANCE PROCEDURES

14.1 Quality Assurance

Investigator procedures

Approved SOPs will be used at all clinical and laboratory sites.

Modification to protocol

No substantial amendments to this protocol will be made without consultation with, and agreement of, the Sponsor. Any substantial amendments to the study that appear necessary during the course of the study must be discussed by the CI and Sponsor concurrently. If agreement is reached concerning the need for an amendment, it will be produced in writing by the CI and will be made a formal part of the protocol following ethical approval.

An administrative change to the protocol is one that modifies administrative and logistical aspects of a protocol but does not affect the volunteers' safety, the scientific value of the study, the conduct of the study or safety of BCG. An administrative change is a non-substantial amendment and does not require REC approval. The CI is responsible for ensuring that changes to an approved study, during the period for which REC approval has already been given, are not initiated without REC review and approval except to eliminate apparent immediate hazards to the volunteer.

Protocol deviation

Any deviations from the protocol will be documented in a protocol deviation form and filed in the trial master file accordingly.

14.2 Monitoring

Data will be evaluated for compliance with the protocol and accuracy in relation to source documents. The study will be conducted in accordance with procedures identified in the protocol. Regular monitoring will be performed according to ICH GCP. According to applicable SOPs, the Monitors will verify that the clinical study is initiated, conducted and completed, and data are generated, documented and reported in compliance with the protocol, GCP and the applicable regulatory requirements.

15 SERIOUS BREACHES

A serious breach is defined as "A breach of GCP or the study protocol which is likely to affect to a significant degree:

- the safety or physical or mental integrity of the subjects of the study
- the scientific value of the study"

In the event that a serious breach is suspected, the Sponsor will contact the REC within 7 days within becoming aware of the breach of GCP.

16 ETHICAL AND REGULATORY CONSIDERATIONS

16.1 Declaration of Helsinki

The Investigator will ensure that this study is conducted in accordance with the principles of the Declaration of Helsinki as agreed by the World Medical Association General Assembly (Washington 2002).

16.2 Good Clinical Practice

The Investigator will ensure that this study is conducted in accordance with ICH Good Clinical Practice (GCP), and local regulatory requirements.

16.3 Approvals

A copy of the protocol, proposed informed consent form, other written volunteer information and the proposed advertising material will be submitted to an independent REC for written approval. The Investigators will submit and, where necessary, obtain approval from the REC for all subsequent substantial amendments to the protocol and informed consent document. The Investigators will notify deviations from the protocol or SAEs occurring at the site to the Sponsor and will notify the REC of these in accordance with local procedures.

16.4 Reporting

The CI shall submit once a year throughout the study, or on request, an Annual Progress Report to the REC, host organisation and Sponsor. In addition, an End of Study notification and final report will be submitted to the REC, host organisation and Sponsor.

16.5 Volunteer Confidentiality

The study staff will ensure the volunteers' anonymity is maintained. All documents will be stored securely and only accessible by study staff and authorised personnel. No information concerning the study or the data will be released to any unauthorised third party, without prior written approval of the Sponsor. The study will comply with the Data Protection Act, which requires data to be anonymised as soon as it is practical to do so.

16.6 Expenses and Benefits

Volunteers will be compensated *pro rata* for their time, travel and for study procedures while participating in the study, amounting to a total of between £685 and £725 depending on the exact number of visits, and whether any repeat or additional visits are necessary.

17 FINANCE AND INSURANCE

17.1 Funding

This study will be financed by a research grant from The Wellcome Trust, held by Professor Helen McShane.

17.2 Indemnity

If any volunteer is harmed as a result of this study, medical care will be provided under the NHS.

Negligent Harm

Indemnity and/or compensation for negligent harm arising specifically from an accidental injury for which the University is legally liable as the Research Sponsor will be covered by the University of Oxford. NHS indemnity operates in respect to clinical treatment that is provided.

Non-Negligent Harm

Indemnity and/or compensation for harm arising specifically from an accidental injury, and occurring as a consequence of the Research volunteers' participation in the study for which the University is the Research Sponsor will be covered by the University of Oxford.

17.3 Insurance

The University has a specialist insurance policy in place - Newline Underwriting Management Ltd, at Lloyd's of London - which would operate in the event of any volunteer suffering harm as a result of their involvement in the research.

18 PUBLICATION POLICY

When the study is complete, a manuscript describing the primary study results will be written and published in a peer-reviewed, open access journal. International guidelines will be followed regarding authorship. There may also be secondary publications on more exploratory results.

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Appendix C

R packages used for single cell sequencing analysis

R packages used for single cell sequencing analysis (performed by Dr. Mirvat Surakhy)
R version 4.0.2 (2020-06-22)
Platform: x86_64-apple-darwin17.0 (64-bit)
Running under: macOS Mojave 10.14.6

Matrix products: default
BLAS:
/System/Library/Frameworks/Accelerate.framework/Versions/A/Frameworks/vecLib.framework/Versions/A/libBLAS.dylib
LAPACK: /Library/Frameworks/R.framework/Versions/4.0/Resources/lib/libRlapack.dylib

locale:
[1] en_GB.UTF-8/en_GB.UTF-8/en_GB.UTF-8/C/en_GB.UTF-8/en_GB.UTF-8

attached base packages:
[1] grid parallel stats4 stats graphics grDevices utils datasets
[9] methods base

other attached packages:
[1] clustermole_1.1.0 sctransform_0.3.1
[3] RCurl_1.98-1.2 cowplot_1.1.0
[5] scales_1.1.1 Seurat_3.2.2
[7] caret_6.0-86 lattice_0.20-41
[9] ISLR_1.2 ggvenn_0.1.8
[11] ggVennDiagram_0.3.3 devtools_2.3.2
[13] usethis_1.6.3 XML_3.99-0.5
[15] tinytex_0.28 forcats_0.5.0
[17] stringr_1.4.0 purrr_0.3.4
[19] readr_1.4.0 tibble_3.0.4
[21] tidyverse_1.3.0 ggpubr_0.4.0
[23] tidyr_1.1.2 ashr_2.2-47
[25] apeglm_1.10.0 calibrate_1.7.7
[27] MASS_7.3-51.6 org.Bt.eg.db_3.11.4
[29] RColorBrewer_1.1-2 edgeR_3.30.3
[31] limma_3.44.3 magrittr_2.0.1
[33] DESeq2_1.28.1 SummarizedExperiment_1.18.2
[35] DelayedArray_0.14.1 matrixStats_0.57.0
[37] GenomicRanges_1.40.0 GenomeInfoDb_1.24.2
[39] pheatmap_1.0.12 corrplot_0.84
[41] GGally_2.0.0 ggplot2_3.3.2
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[45] org.Hs.eg.db_3.11.4 AnnotationDbi_1.50.3
[47] IRanges_2.22.2 S4Vectors_0.26.1
[49] Biobase_2.48.0 BiocGenerics_0.34.0
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[53] tmod_0.46.2

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[10] backports_1.2.0 reprex_0.3.0 GOSemSim_2.14.2
[13] rlang_0.4.8 XVector_0.28.0 ROCR_1.0-11
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