Evaluating aerosol administration of a candidate TB vaccine, MVA85A, as a way to induce potent local cellular Immune responses and avoid anti-vector immunity



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Evaluating aerosol administration of a candidate TB vaccine, MVA85A, as a way to induce potent local cellular immune responses and avoid anti-vector immunity

There is an urgent need for a better vaccine against TB than BCG, which confers variable protection against pulmonary TB. Heterologous prime-boost regimens with viral vector vaccines are a leading strategy for TB vaccine development. MVA85A is a viral vector candidate TB vaccine designed to boost BCG. This phase I clinical trial was designed to evaluate whether alternating aerosol and intradermal routes of vaccination can improve Ag85A immunogenicity; maybe by circumventing local anti-MVA immunity. Thirty-six BCG-vaccinated healthy UK adults received two MVA85A vaccinations one month apart as heterologous (aerosol-intradermal or intradermal-aerosol) or homologous prime-boost (intradermal-intradermal). Bronchoscopies with bronchoalveolar lavages (BAL) were performed 7 days after each vaccination. Delivering MVA85A by aerosol as a priming immunisation was well tolerated and highly immunogenic. Boosting an intradermal MVA85A prime with an aerosolised MVA85A vaccination led to transient respiratory and systemic adverse events which resulted in vaccinations in this group ceasing. Aerosolised MVA85A induced potent Ag85A-specific T cell responses in mucosal and systemic compartments; and suggested a potential for dose sparing with this route. The intradermal-aerosol regimen boosted Ag85A-specific cellular immune responses in systemic and lung mucosal compartments. Serum antibodies to Ag85A and MVA were only detected after intradermal vaccination. Anti-Ag85A antibodies in serum were boosted by a second intradermal vaccination. The findings of this trial are important for the development of aerosolised TB vaccines but also for new viral vector vaccines for other respiratory pathogens.

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Abbreviations

Ag85A	Antigen 85A
α4β1	Alpha 4 Beta 1 integrin
BAL	Bronchoalveolar lavage
BCG	Bacille Calmette–Guérin vaccine
CD	Cluster of differentiation
CD4+ T cells	CD4 T helper cells
CD8+ T cells	CD8 T helper cells
CFP-10	10 kDa culture filtrate antigen
CONSORT	Consolidated Standards of Reporting Trials
CRP	C-reactive protein
CT	Computed tomography
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked ImmunoSpot
EPI	Expanded Programme on Immunization
ESAT-6	6 kDa early secretory antigenic target produced by *M.tb*
FACS	Fluorescence activated cell sorting
FEV1	Forced Expiratory Volume in the first second
FP85A	Fowl-poxvirus vector vaccine expressing antigen 85A
FSC-A	Forward Scatter-Area
FSC-H	Forward Scatter-Height
FVC	Forced vital capacity
γδ T cells	Gamma Delta T cells
GCP	Good clinical practice

GM-CSF	Granulocyte macrophage colony stimulating factor
GP	General Practitioner
HP	Hypersensitivity pneumonitis
ICS	Intracellular cytokine staining
IFN-γ	Interferon-Gamm
IGRA	IFN-γ release assay
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
iNKT	invariant Natural Killer cells
LTBI	Latent tuberculosis infection
M.tb	Mycobacterium tuberculosis
MAIT	Mucosal associated invariant T cells
MDR	Multi drug resistant
MGIA	Mycobacterial growth inhibition assay
MHC	Major Histocompatibility complex
MHRA	Medicines and Healthcare Products Regulatory Agency
MVA	Modified Vaccinia Ankara
MVA85A	Modified Vaccinia Ankara expressing antigen 85A
NHS	National Health Service
NICE	The National Institute for Health and Care Excellence
NTM	Non-tuberculous mycobacteria

NYVAC	Attenuated strain of vaccinia virus
OD	Optical Density
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
pfu	Plaque forming units
SEB	Staphylococcal Enterotoxin B
Spo2	Peripheral capillary oxygen saturation
SSC	Side Scatter
T regs	Regulatory T cells
TB	Tuberculosis
TCM	Central Memory T cells
TEM	Effector Memory T cells
Th	T helper cell
TNF-α	Tumour necrosis factor alpha
TST	Tuberculin skin test
VIS	Volunteer Information Sheet
vs.	*versus*
WB	Whole blood
WHO	World Health organisation
XDR	Extremely drug resistant

1 Introduction

1.1 Tuberculosis

1.1.1 Mycobacterium tuberculosis

Mycobacteria are a group of aerobic, non-motile, acid-fast organisms which can be divided into the *Mycobacterium tuberculosis* complex and non-tuberculous mycobacteria (NTM). Species of the *Mycobacterium tuberculosis* complex include *Mycobacterium tuberculosis (M.tb)*, *Mycobacterium bovis* and *Mycobacterium africanum* all of which can cause Tuberculosis (TB).

M.tb was first identified by Robert Koch in 1882[1, 2]. Ziehl and Neelsen refined a method (originally developed by Koch) to visualise *M.tb* using acid-fast alcohol stains[2]. Humans are the main host for *M.tb*. *Mycobacterium bovis* can cause TB in cattle and humans and the derived *Bacille Calmette-Guérin* (BCG) is the only licensed vaccine against TB[3, 4].

One of the characteristic features of *M.tb* is its intricate cell wall, which permits it to survive in dry soil environments[2]. This cell wall, compared to other Gram-positive bacteria, contains an additional layer rich in lipids, glycolipids and polysaccharides. The mycolic acid containing outer layers render it hydrophobic, i.e. difficult for water soluble antibiotics to penetrate[5]. Important in cell wall synthesis is the so called antigen 85 complex, including antigens 85A (Ag85A), B and C, which has mycolyl transferase activity[5, 6].

NTM are found ubiquitously in the environment and most people are presumed to have been exposed to them[4]. NTM include *M.smegmatis* or species of the *Mycobacterium avium* complex[7]. Disseminated clinical disease is mostly seen in immunocompromised individuals[4, 7].

1.1.2 Epidemiology

TB remains a significant global health problem and in 2014 was reported to have been the leading global cause of death from a single infectious disease[8]. According to the WHO Global TB report 2015, there were 9.6 million new cases including 1 million children and an estimated 1.5 million deaths, including 390 000 deaths among people infected with HIV in the year prior[8-10]. In addition, an estimated one third of the world's population is thought to be latently infected with *M.tb* (LTBI), which can reactivate, leading to TB disease[8]. This LTBI population represents a large potential reservoir of *M.tb* that is often undiagnosed. There is a 10% lifetime risk of reactivation of LTBI, which increases to an annual risk of 10% in people who are co-infected with HIV[11]. This increased risk is only partially abrogated by anti-retroviral therapy[8].

The scale of the TB disease burden is further compounded by diagnostic and therapeutic challenges, such as the emergence of increasingly drug-resistant strains of *M.tb*[8, 9, 12, 13]. Globally, approximately 480 000 people are thought to have multidrug resistant (MDR) TB, with only an estimated quarter of these reported. MDR TB describes resistance to two first-line anti-TB drugs (rifampicin and isoniazid). Extensively drug resistant (XDR) TB, which is MDR TB plus additional resistance to at least one fluoroquinolone and a second-line anti-TB injectable drug, was widely reported across 105 countries. Russia accounted for over half of the reported XDR TB cases[8, 12]. The rate of decline in TB incidence worldwide has remained slow at approximately 1.5% per year[8]. Interestingly, a recent report comparing the TB incidence in the US and the UK from 2000 to 2011, found that in the US there was a decline in the number of cases in native-born as well as in people born abroad.

However, in the UK there was an increase in cases diagnosed in foreign-born, with cases in UK-born remaining stagnant[14]. This reported trend highlights the need for improved TB control measures in the UK and globally, but also that any progress made needs to be sustained.

Resource constraints, ongoing conflicts and the HIV epidemic are still hindering efforts towards diagnosis and treatment targets[8]. The Millennium Development Goals (MDG), which were set out by the United Nations Development Programme, included control of TB in one of their 8 MDGs[15]. The final MDG report in 2015 celebrated progress in prevention, diagnosis and treatment that had been made through this initiative; but also recognised the need for sustained efforts which are required to maintain the development and progress in these fields[15]. The WHO End TB strategy called to eradicate the global TB epidemic by 2030 through a reduction in the incidence of TB by 90%, and reduction in the mortality associated with TB by 95%[16]. The Stop TB partnership, hosted by the United Nations, echoed these aims and recently set out their 4th Global Plan to control the TB epidemic[17]. The partnership called for a concerted effort to reach their so-called 90-90-90 targets: reaching at least 90% of all people with TB, including 90% of the vulnerable target population, and accomplishing 90% of treatment success in all people diagnosed with TB[17]. In order to reach these goals the Stop TB Partnership advocated a "paradigm shift" including a change in mindset and innovative TB programmes[17]. It also called for research and development investment into vaccines, improved diagnostics and treatment regimens.

1.1.3 Clinical disease: diagnosis and treatment

The lungs are the port of entry of *M.tb.* Pulmonary TB disease can either be due to a primary infection, or secondary as reactivation of LTBI[18, 19]. Pulmonary TB is characterised by fever, weight loss and a productive cough[19, 20]. Active pulmonary TB leads to infiltration and consolidation of the affected lung and consequently tissue destruction and cavitation. Radiological examination assists the diagnosis of active pulmonary TB[9]. The gold standard diagnostic test for active pulmonary TB is positive pulmonary *M.tb* sputum culture which takes several weeks[9]. The diagnosis of extra-pulmonary disease is made by radiological examination and culture of microbiological sampling from the affected site[9, 21]. Magnetic resonance imaging is the preferred choice for suspected TB in the central nervous system, whereas computed tomography (CT) is the preferred choice for suspected abdominal TB[21]. The value of 18 F-fluorodeoxyglucoase positron emission tomography/CT, which can detect metabolic changes, is being evaluated as a tool for TB diagnosis or to monitor response to anti-TB treatment[21]. Advances in TB diagnosis have included DNA-based automated detectors of *M.tb* components such as Gene Xpert which also allows the detection of rifampicin resistance[9].

The standard treatment for active pulmonary TB recommended by the National Institute for Health and Care Excellence (NICE) is a 6 months regimen with isoniazid and rifampicin, supplemented with pyrazinamide and ethambutol in the first 2 months[9]. Commonly fixed-dose combinations rather than single-drug formulations are prescribed to increase compliance. A recent Cochrane review found the effectiveness of these two to be comparable[22]. NICE guidance recommends that any patient clinically suspected to have TB, is risk assessed for drug resistance and considered

for testing. It is recommended that confirmed drug-resistant TB is managed by an experienced multi-disciplinary team[9].

1.1.4 Subclinical *M.tb* infection

LTBI is considered as a clinical state where the host immune response is controlling mycobacterial replication to a point which renders the host asymptomatic and not contagious[18, 23]. Microbiological testing in infected individuals is negative and the diagnosis is made by detection of a host immune response to *M.tb*-specific antigens. Tuberculin skin testing (TST) was developed based on Robert Koch`s observation that infection with *M.tb* caused cutaneous reactivity to culture filtrate protein (tuberculin). It was later named the Mantoux test after Charles Mantoux who described the intradermal delayed type hypersensitivity reaction[2]. The TST has imperfect sensitivity and specificity: Immunosuppressed individuals can have a false-negative TST; and conversely sensitisation to tuberculin from BCG vaccination or exposure to NTM can lead to false-positive testing[24]. More specific Interferon-gamma release assays (IGRA) measure the number of *M.tb* antigen-specific reactive T lymphocytes by means of an enzyme-linked immunosorbent spot assay (ELISPOT) or quantify *M.tb* antigen-specific Interferon-gamma (IFN-γ) using an enzyme-linked immunosorbent assay (ELISA) based method. The *M.tb* antigens used in these assays are the early secreted antigen target 6 (ESAT-6) and culture filtrate protein (CFP-10) and for the ELISA based assay also a short peptide from antigen TB7.7[9, 18, 23]. ESAT-6 and CFP-10 are relatively but not absolutely *M.tb*-specific as orthologues are also present in the genome of NTM including *M.leprae* and *M. smegmatis.* The specificity and hence the utility of an IGRA in developing countries with a high TB burden is an issue as these countries also have a high prevalence of NTM[25, 26].

Eradication treatment for LTBI with 6 months of isoniazid or 3 months of isoniazid plus rifampicin is recommended in individuals who are below 35 years of age, healthcare workers or those co-infected with HIV (6 months of isoniazid)[9].

Control of the TB disease burden and ultimately eradication requires improved diagnosis, better treatments and an efficacious vaccine. Only these measures combined will, in a first phase, eliminate all cases of active TB cases, and then in a second phase the LTBI reservoir[8, 9, 27]. The focus of this thesis is a novel route of TB vaccine delivery. Therefore, the remaining part of this introduction will focus on TB vaccination and protective vaccine-induced immune responses.

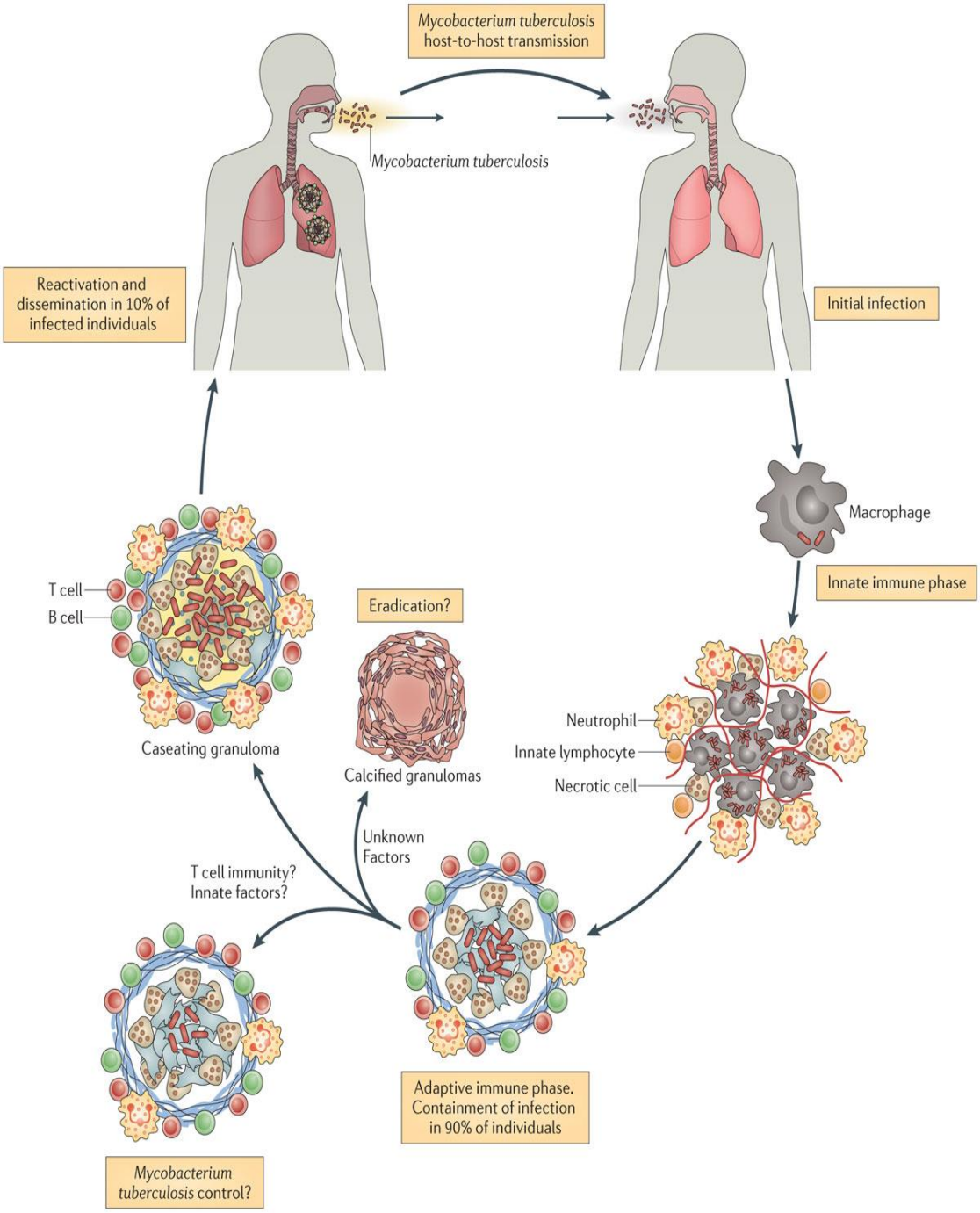
1.1.5 Pathogenesis and host immune response

Infection with *M.tb* is via inhalation of aerosolised droplets containing the infectious bacilli (Figure 1.1)[28, 29]. On reaching the human lung, innate immune responses are triggered by components of the *M.tb* cell wall recognised by alveolar macrophages and dendritic cells[28, 30]. Bacilli are phagocytosed by lung alveolar macrophages leading to recruitment of granulocytes and mononuclear cells[28, 30]. *M.tb* can reside intracellularly in activated macrophages[31]. In the draining lymph nodes, presentation of *M.tb* antigens by dendritic cells and macrophages leads to priming and expansion of antigen-specific effector T cells[28]. Secretion of Interleukin-12 (IL-12) leads to a Th1 biased pro-inflammatory cytokine profile[28, 30, 32]. In the infected lung, effector T cells producing cytokines including IFN-γ, TNF-α and IL-17 stimulate the formation of granulomata. These are formed collections of activated macrophages, leukocytes and fibroblasts containing *M.tb* [23, 33-35]. As the granuloma differentiates, it becomes more organised with a distinct centre of an increasingly more caseous necrotic core of debris. Granulomata contain *M.tb* but at the same time provide a location for

persistence of *M.tb* in the latent state (Figure 1.1)[34]. Rupture of an active granuloma results in the release of infectious bacilli into the airway which facilitates airborne spread of *M.tb* in form of a productive cough in the host[28, 30]. In patients with established pulmonary TB, collagenases such as matrix metalloproteinases are upregulated, leading to lung matrix destruction and cavitation which is associated with a high bacillary burden[20].

Outcomes of significant *M.tb* exposure can be a spectrum from clearance or containment of *M.tb* at one end of the spectrum, to TB disease at the other end of the spectrum (Figure 1.1)[18, 23]. The host immune response can facilitate or contain *M.tb* replication and therefore the outcome of an *M.tb* infection is influenced by virulence of the pathogen but also by the balance of host immune responses[18, 36].

Figure 1.1 TB Pathogenesis. M.tb transmission occurs by inhalation of aerosolised droplets. Initial infection triggers innate and then adaptive immune responses. Recruitment of macrophages, lymphocytes and neutrophils leads to formation of granulomata in the lung which can contain M.tb, or rupture releasing M.tb. Reproduced with permission for use in this DPhil thesis: Nunes-Alves et al. In search of a new paradigm for protective immunity to TB. Nature Reviews Microbiology 12, 289–299 (2014) doi:10.1038/nrmicro3230



1.1.6 Protective immunity to TB

1.1.6.1 Innate Immune responses

Innate immune responses are the first line of defence. Some integrated key roles of this arm of the immune system have already been mentioned. Alveolar macrophages are the first cell to encounter *M.tb* and recognise these by means of receptors that can identify pathogen-specific patterns[31]. Once recognised, *M.tb* is internalised into phagosomes for proteolytic degradation as well as antigen processing and presentation to cells of the adaptive arm of the immune response. *M.tb* is able to block phagosome maturation and thereby can arrest phago-lysosomal fusion and subsequent degradation[31, 37]. This is one of the many mechanisms by which *M.tb* evades the immune system.

A number of cytokines related to macrophage function have been linked to improved TB disease outcome. For instance, Interleukin-1beta (IL-1β) is a pro-inflammatory cytokine produced by macrophages. Mice with IL-1β deficiency were shown to be more susceptible to *M.tb* infection[38]. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is produced by leukocytes and epithelial cells. *In vitro*, GM-CSF treatment of murine or human macrophages restricted intracellular *M.tb* growth[39]. In accordance with this, *in vivo,* mice lacking GM-CSF were highly susceptible to TB. In humans, impaired GM-CSF function is associated with opportunistic infections: a retrospective analysis performed in patients with cryptococcoal meningitis demonstrated that some of these patients had autoantibodies to GM-CSF[40].

Another innate cell type important in *M.tb* infection is the neutrophil, which is thought to be involved in granuloma formation. When neutrophil-deplete mice were challenged

with *M.tb*, granuloma formation was impaired but this did not affect mycobacterial burden in the lung[41]. In zebrafish experiments, neutrophils were recruited to granulomatous sites triggered by signals from infected macrophages, which they were able to phagocytose[35]

Dendritic cells are the major antigen presenting cells of the immune system, positioned on the interface of innate and adaptive immune responses. *In vitro, M.tb* was shown to be capable of manipulating dendritic cells by blocking their maturation and hence impairing their function[42].

1.1.6.2 Adaptive Immune responses

A number of studies have demonstrated that a strong cell-mediated immune response is essential for protective immunity against TB[28, 43-45].

M.tb peptides from the phagosome presented on MHC class II on the macrophage cell surface are recognised by CD4+ T cells. MHC class II restricted CD4+ effector T cells are necessary for protection[46-48]. It is well recognised that in humans infected with HIV, a low CD4+ T cell count is associated with an increased risk of TB[47, 49]. In murine studies, lack of MHC class II or CD4+ T cells was associated with increased bacterial burden and increased susceptibility to *M.tb*[50-52]. In non-human primates, depletion of CD4+ T cells led to reactivation of latent infection or more severe TB disease[53, 54].

CD4+ T helper (Th) cells operate in distinct functional phenotypes. Activation of the T cell receptor drives differentiation of naïve CD4+ T cells into different Th lineages depending on the cytokine milieu[55]. Interleukin-2 (IL-2) is required for T cell growth and differentiation. IL-2 is produced by CD4+ T cells themselves when these have been activated by antigen; but to a lesser extent IL-2 is also produced by dendritic cells[55,

[56]. *M.tb* infected dendritic cells also secrete another cytokine IL-12, which promotes differentiation into the Th1 lineage. IL-12 is required to activate, expand and sustain IFN-γ T cell responses in *M.tb* infection[32, 55, 57]. IFN-γ is one of the characteristic cytokines produced by effector cells of the Th1 lineage[57]. IL-12 knock-out mice were shown to produce reduced levels of IFN-γ and to have less lymphocytes present at the site of infection. In challenge experiments these IL-12 knock-out mice had a reduced ability to control *M.tb* growth[58]. Humans, with mutations in signalling between IL-12 and IFN-γ are predisposed to disseminated infection with mycobacteria. This inherited susceptibility in families is known as Mendelian susceptibility to mycobacterial disease[59, 60]. Inborn errors of IFN-γ immunity in humans due to mutations in the receptor also increase susceptibility to mycobacterial infections[61]. Several experiments using IFN-γ knock-out mice confirmed susceptibility to TB associated with lack of this cytokine[62]. The functional importance of IFN-γ in protective immunity to TB is attributed to its ability to activate macrophages for killing of intracellular mycobacteria[31].

Tumour necrosis factor- alpha (TNF-α) is the other main Th1 cytokine considered pivotal in the immune response to TB[57]. Humans treated with anti-TNF-α monoclonal antibodies for rheumatoid arthritis or inflammatory bowel disease demonstrate an increased incidence of TB[63, 64]. Therefore, routine screening for evidence of LTBI is recommended prior to anti-TNF-α therapy[9, 65]. Pre-clinical animal models reiterate the importance of TNF-α in TB, especially in granuloma integrity. In a murine model of LTBI, blocking TNF-α using a monoclonal antibody resulted in TB reactivation[66]. Another study compared mice with either a disrupted TNF-α receptor or mice treated with monoclonal anti-TNFα antibodies to wildtype untreated controls. Both experimental TNF-α deficient mice demonstrated delayed and less well organised

granulomata with more acid-fast bacilli in some organs. Both groups of mice formed granulomata in their lungs, but necrotic areas with high *M.tb* burden were only found in the treated mice.[67]. Mice immunised with BCG prior to anti-TNF-α antibody treatment reached humane endpoints later but demonstrated higher *M.tb* burden in their lungs *post-mortem*[67].

Another pro-inflammatory cytokine implicated in protective immunity to TB is Interleukin-17 (IL-17). Th17 cells are another subset of Th cells and produce IL-17 as one of their effector cytokines[68]. Immunising mice with TB vaccine candidates was shown to induce Th17 responses, which correlated with improved protection upon *M.tb* challenge[69, 70]. Th17 cells require IL-23 for differentiation into this lineage[71-73]. *In vitro, M.tb* was shown to induce production of IL-23 by human monocyte-derived dendritic cells[72]. A murine model studying the role of IL-23 and IL-17 after vaccination, used IL-23 deficient mice combined with subsequent addition or depletion of IL-17[74]. This loss- and then subsequent gain-of-function models revealed that in vaccinated mice challenged with *M.tb*, IL-23 was necessary for a productive IFN-γ CD4+ T cell response and Th17 population in the lung[74]. IL-17 preceded IFN-γ responses by CD4+ T cells. There was a concurrent reduction in *M.tb* growth[74]. The earlier mentioned IL-1β produced by infected macrophages can recruit Th17 cells and neutrophils to the lung and induce tissue inflammation[75, 76]. Th17 cells are also able to recruit neutrophils to mucosal sites themselves. The inflammatory responses induced by Th17 and neutrophils at the site might on the one hand aid granuloma formation but on the other can lead to tissue damage[34, 68]. IL-17 has also been implicated in animal models of autoimmune disease. Therefore whilst IL-17 could augment a proinflammatory environment to curtail intracellular *M.tb* growth, the balance of Th1 and Th17 responses is crucial in order to avoid immunopathology due to an excessive

inflammatory response[75, 77] The realisation that a fine balance between pro-inflammatory and anti-inflammatory responses are important was also reported in relation to eicosanoids. Zebrafish experiments revealed polymorphisms in the leukotriene pathway to influence inflammatory responses mediating susceptibility to TB. Parallel observations in humans showed that TB patients were less likely to be heterozygous[78, 79].

A Th lineage contributing to regulation of inflammation are regulatory T cells (T_{regs}), which are characterised by expression of the transcription factor Foxp3[80]. T_{reg} pathways limit excess host-induced immune pathology[80]. Inhibitory cytokines, IL-10 and TGF-β as well as inhibitory surface molecule CTLA-4, are thought to be involved in this regulatory capacity. Excess IL-10 *in vitro* was shown to suppress macrophages, impair their function and therefore attenuate antigen-specific CD4+ and CD8+ T cell responses[81]. Inappropriate levels of T_{reg} activity could inadvertently promote *M.tb* persistence. In mice, depletion of Foxp3 expressing cells led to reduced *M.tb* growth in the lung when these mice were challenged with *M.tb*[80]. Adoptive transfer experiments in mice demonstrated the presence of T_{regs} reduced the clearance of *M.tb*[82]. Elevated numbers of T_{regs} as well as increased levels of IL-10 and TGF-β were previously demonstrated in TB patients[83].

The emphasis so far has been on the role of CD4+ T cells. MHC class I restricted CD8+ T cells are also thought to play a role in protective immunity to TB. In terms of relative contribution of CD4+ and CD8+ T cells, in a murine model mimicking active TB and LTBI, depletion of CD4+ T cells in the active phase led to increased bacterial burden in the lung, whereas depletion of CD8+ T cells in the latent phase led to a more marked increase in bacterial burden. This suggests that the relative significance of

CD8+ T cells might depend on the phase of *M.tb* infection[84]. In mice, following *M.tb* challenge, CD8+ T cells were found in the lung and draining lymph nodes[85]. Phenotypically these were either cytotoxic or IFN-γ secreting[85]. *In vitro*, these CD8+ T cells recognised *M.tb* infected macrophages and lysed them[86]. In a murine model deficient in MHC class I (beta 2 microglobulin knock-out), *M.tb* challenge led to increased morbidity and increased pathology[87, 88]. In BCG vaccinated non-human primates, CD8+ T cell depletion led to a reduction in vaccine-induced TB immunity[89]. Human *M.tb* specific CD8+ T cells *in vitro* were shown to be capable of secreting IFN-γ and lyse infected cells killing intracellular mycobacteria[90].

In the context of TB vaccination, the key immunological concept is that the immune system remembers this first encounter when later exposed to *M.tb.* This requires immunological memory. An important cytokine involved in T cell memory is IL-2. Apart from being important in T cell growth and differentiation, as mentioned earlier, IL-2 is also necessary for memory T cell responses[91]. In BCG vaccinated mice that were able to control *M.tb* initially, regrowth of *M.tb* coincided with waning of IL-2 secreting central memory T cells[91]. Antigen-specific memory T cells have been described in terms of central or effector memory T cell populations (TCM and TEM respectively)[92]. TEM were shown to predominantly produce IFN-γ and TCM predominantly IL-2[92, 93]. In a study of TB patients comparing antigen-specific CD4+ T cells secreting IFN-γ or IL-2 before and after treatment, a reduction of IFN-γ and a concurrent increase in IL-2 was observed following treatment[94]. Whilst a mechanism cannot be concluded from a change in levels of these markers in TB patients following treatment, the changes could relate to a higher *M.tb* burden before treatment and a subsequent reduction afterwards.

In the context of memory, homing integrins are thought to be important in facilitating circulation of memory T cells back to their first site of antigen encounter. In animal models α4β1 (alpha 4 beta 1) integrins were shown to play a role in recruiting lymphocytes to the lung[95]. One study reported CD4+ T cells found in BAL of both healthy controls and asymptomatic latently *M.tb* infected patients had a higher proportion of cells expressing α4β1 integrins compared to blood[96]. Upon bronchoscopic PPD challenge, in the latently infected patients, there was an increase in total BAL CD4+ T cells enriched for α4β1 integrin[96]. The majority of PPD-specific IFN-γ+ BAL Th1 cells also stained positively for α4β1[96]. The ability of *M.tb* specific CD4+ T cells to migrate to infected lung parenchyma is key to exerting their protective effector function there. Experiments using intravascular staining by injection of traceable antibody demonstrated the different location, phenotype and function of Th1 cell populations in lung parenchyma and the intravascular space[97]. Characterisation of these two phenotypes showed that the parenchyma-homing phenotype expressed high levels of chemokine CXCR3, was highly activated and secreted lower amounts of IFN-γ than their intravascular counterparts[97]. In contrast the Th1 phenotype retained in the lung intravascular space expressed high levels of chemokine CX3CR1 and produced comparatively high levels of IFN-γ[97]. In adoptive transfer experiments mice that received Th1 cells with lung-homing markers were better able to control *M.tb* infection compared to mice who had received the intravascular-homing cell type[46, 97]. These experiments also observed migration of Th1 cells with these lung-homing markers to the lung parenchyma of the recipient mice[46, 97]. In humans, with LTBI, *M.tb* antigen-specific memory Th1 cells were biased towards a phenotype that was CXCR3 positive[98].

T cell types not discussed thus far include unconventional T cells. A presence of unconventional T cells in TB patients has been demonstrated in several studies but the relevance of their involvement is not fully understood[99-101]. The CD1 family are antigen presenting molecules which present lipids rather than peptide to CD1 restricted T cells[102]. *M.tb* contains glycolipids in cell wall and membrane [2]. In humans there are two groups of CD1 molecules[102]. Group 1 CD1 molecules include CD1a, 1b and 1c; and group 2 CD1 molecules CD1d and CD1e[102]. Group 1 CD1-restricted T cells, which recognise mycobacterial antigen, have been found in higher frequency in people exposed or infected with *M.tb* compared to controls[103-107]. *In vitro*, group 1 CD1 restricted T cells when stimulated with mycobacterial antigens are cytotoxic and produce IFN-γ and TNF-α[105, 106]. Mice do not possess group 1 CD1 molecules. However, CD1 transgenic mice infected with BCG or *M.tb* generate CD1 restricted T cell responses[108]. Invariant Natural Killer T cells (iNKT) recognise lipid and glycolipid antigens presented on CD1d. Activation of iNKT cells in mice experiments led to a better outcome following *M.tb* challenge[109]. Adoptive transfer of iNKT cultured with *M.tb* infected macrophages was shown to reduce mycobacterial growth *in vivo*[110]. *In vitro*, stimulated human iNKT were shown to be able to kill *M.tb* infected macrophages[111]. Gamma Delta T cells (γδ T cells) recognise phosphoantigens. In the murine model, γδ T cells appeared not essential for protection, but were shown to be a significant source of IL-17 production in the lung during *M.tb* infection[68]. In non-human primates infected with *M.tb*, activation of γδ T cells was associated with decreased mycobacterial burden in the lung[112]. γδ T cells also generated a recall responses in *M.tb* challenge experiments performed in BCG-vaccinated non-human primates[113]. Mucosal-associated invariant T cells (MAIT) are MR1 restricted recognising riboflavin metabolites. *In vitro*, MAIT cells were observed to recognise *M.tb*

infected lung epithelial cells. Less MAIT cells were found in the blood of patients with active TB compared to LTBI[114].

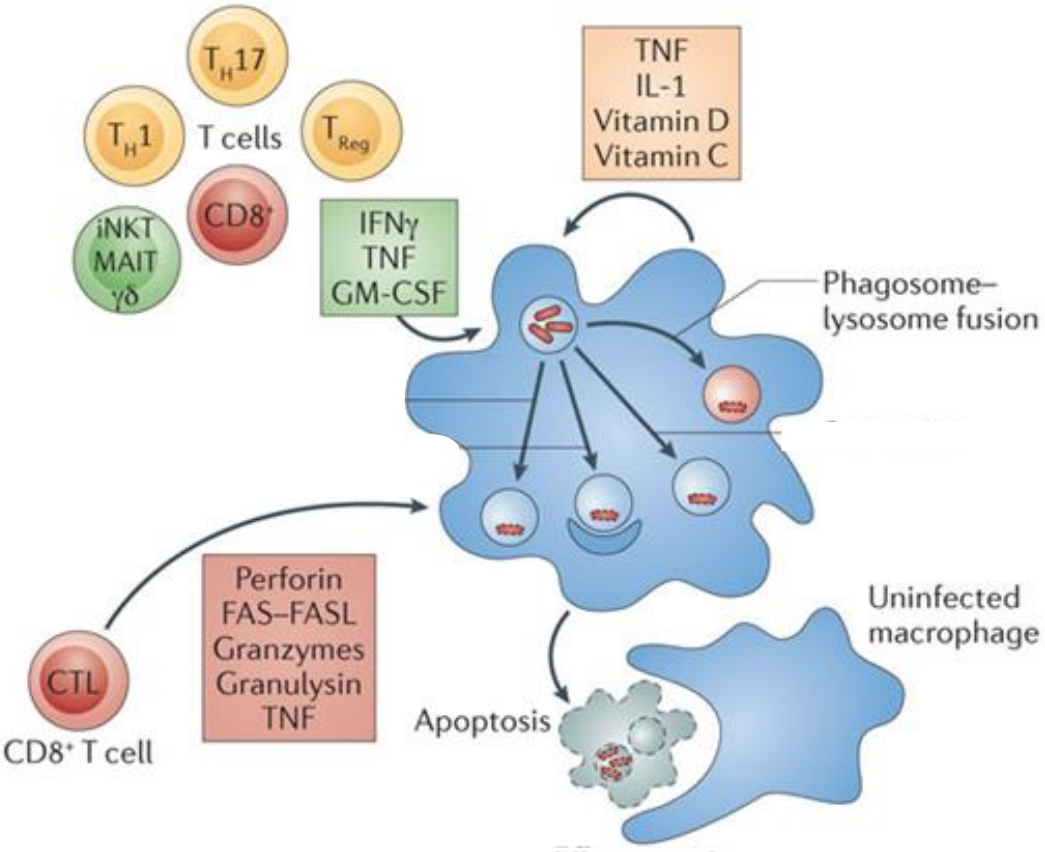
So called non-classical HLA class I molecules include HLA-E. HLA-E was shown to present mycobacterial derived antigen [115]. Human CD8+ T cells from BCG vaccinated infants and PPD responsive adults were shown to recognise *M.tb* peptides with HLA-E binding motifs and respond with cytotoxic and regulatory activity [116].

The other T cell type not mentioned yet is the Th2 axis of CD4+ T cell responses. The role of Th2 cells is to control extracellular pathogens. Cytokines characteristic of the Th2 axis are IL-4, IL-5, and IL-13. Th2 cells also interact with B cells and promote their differentiation and antibody production. Eosinophils, basophils and mast cells are other effector cells recruited by Th2 type responses. A predominantly Th2 biased response is thought to be more counterproductive in TB. In a study of patients with active TB, serum markers indicative of Th1 responses were reduced and markers of Th2 responses increased (compared to healthy controls)[117]. On successful completion of TB treatment, cured patients had higher levels of Th1 serum markers and lower levels of Th2 markers than patients who did not complete curative treatment[117]. Interestingly, exposed household contacts of these TB patients also exhibited a high ratio of Th1 to Th2 markers[117]. Helminth co-infection in humans triggers a Th2 biased response and TB patients who are co-infected were shown to have lower total frequencies of CD4+ and CD8+ T cells in their whole blood compared to TB patients with no evidence of helminth co-infection[118]. The reduction in total T cell frequency was accompanied by low levels of IFN-γ[118]. These co-infected patients also demonstrated more severe TB disease radiologically and worse treatment outcomes[118]. Experimental models of shifts in the Th1/Th2 balance reiterate the negative impact Th2 cells appear to have in TB

immunity. In a murine model of latent *M.tb*, reactivation was associated with a shift from Th1 to Th2 biased cytokines[119]. Conversely control of mycobacterial growth was marked by reversal to a Th1 biased phenotype[119].

Due to the intracellular nature of *M.tb*, the role of B lymphocytes was not considered essential for protective immunity to TB. However there is a resurgence of interest in the role of B cells in TB[120]. B cells were identified in animal models with TB. In non-human primates challenged with *M.tb*, presence of B cell and plasma cell clusters were noticed within the lung granulomata[121]. These plasma cells produced *M.tb*-specific antibodies and there was evidence of *M.tb*-specific IgG in infected tissue[121]. B cells could play a role in the extracellular stages in the host following *M.tb* exposure or as antigen presenting cells in T cell priming[120].

Figure 1.2 Overview of T cell mediated immunity in TB. Antigen presentation by the macrophage to CD4+ T cells of several T helper lineages and cytotoxic CD8+ T cells. Reproduced with permission for use in this DPhil thesis: Nunes-Alves et al. In search of a new paradigm for protective immunity to TB. Nature Reviews Microbiology 12, 289–299 (2014) doi: 10.1038/nrmicro3230



Several other factors thought to be involved in the immune response to TB were not discussed here. For instance, nutrition is considered important. Malnutrition as well as diabetes influence the immune system and both predispose to TB[122, 123]. Also, Vitamin D is involved in phagosome-lysosome function and found to be reduced in patients with TB, but whether this is cause or effect is not known[124]. Also, Vitamin C was shown to sterilise *M.tb* cultures *in vitro*[125].

Collectively, the range of studies to date have demonstrated that the immune response to *M.tb* is complex and the magnitude as well as the quality and location of the response are important.

1.1.7 Correlates of protection

TB vaccine design has focussed on ways to induce T cell mediated immunity on the experimental knowledge basis that this arm of the immune response is important in controlling intracellular *M.tb*. Whilst there is an increased understanding of the components of the immune response which are important for protection from TB, none of the described immunological parameters alone were shown to correlate well with protection[27, 126-128]. Although immunosuppression is associated with reactivation of *M.tb* infection, it is not established which factors precisely determine progression from infection to disease, either[23, 126]. Measurement of IFN-γ reflects the functional potential of the Th1 axis, which is important in TB immunity, but by itself could not be validated as a correlate of protection in TB. In a recent report of a case-control analysis from the MVA85A infant efficacy trial for the identification of immune correlates of risk of TB disease, the frequency of activated HLA-DR+CD4+Tcells was associated with an increased risk; and the magnitude of BCG-specific IFN-γ secreting T cells and Ag85A-specific IgG with reduced risk[129, 130]. In contrast, a cohort study of BCG-vaccinated

infants, demonstrated that the frequency of BCG- specific CD4+ T cells and their Th1 biased cytokine expression profile did not correlate with protection[128]. The different findings might be attributable to differences in study designs. For instance the readout in the infant efficacy trial were IFN-γ ELISPOT responses using peripheral blood mononuclear cells (PBMC). The readout in the other trial was frequency of polyfunctional T cells as measured in whole blood by intracellular cytokine staining (WB ICS).

Cells with polyfunctional phenotypes have the capability to secrete multiple cytokines concurrently. Polyfunctional Th1 biased T cells co-express multiple cytokines including IFN-γ, TNFα and IL-2[131]. Studies in different disease models demonstrated the significance of polyfunctional T cells: correlation with protection in murine model of *Leishmania major*; correlation with protective immunity against *M.tb* challenge in mice; and an inverse correlation with viral load in HIV patients who were able to control their disease or received HIV treatment[132-134]. In HIV negative patients diagnosed with active TB, anti-TB treatment was shown to induce a shift from single cytokine positive CD4+ T cells to CD4+ T cells with polyfunctional phenotypes[94].

As there is currently no validated correlate of protection from *M.tb* infection or TB disease, pre-clinical animal models and *in vitro* mycobacterial growth inhibition assays (MGIA) have been used as surrogate indicators to guide vaccine selection[27, 135].

MGIAs measure inhibition of *M.tb* growth by co-culturing host cells or whole blood with *M.tb* for a period of time before lysing the cells to quantify the amount of viable *M.tb*. MGIAs are therefore an unbiased holistic measure of the summative ability of the studied host to control *M.tb* growth *in vitro.* MGIAs have been used to measure *in vitro* bactericidal activity of anti-TB drugs but also to measure inhibition of mycobacterial

growth following BCG vaccination in mice and humans[136-138]. MGIAs using murine splenocytes demonstrated growth inhibition of *M.tb in vitro* using cells harvested from BCG-vaccinated mice compared to cells from unvaccinated mice[137]. In humans use of the MGIA demonstrated inhibition of mycobacterial growth *in vitro* following primary but not repeat BCG vaccination[138].

Animal models have been used to study tuberculosis as a disease model but also as the first step in TB vaccine development[139, 140]. To study TB disease, animal models permitted the evaluation of aspects of pathology and immunology using experimental loss- or gain-of-function *in vivo*[139]. Safety, immunogenicity and efficacy of potential TB vaccine candidates and routes of vaccine delivery were studied in mice and other animal models to guide selection of promising vaccines and to support the development of biomarkers [141-143] The cattle and non-human primate model are particularly important in TB vaccine development[143]. In cattle an effective vaccine is important to control TB disease and eliminate the zoonotic transmission by *M.bovis*[139, 143]. The advantages of studies in cattle include its parallels with human disease which can be studied in a natural host[139, 143]. The advantage of non-human primate models is also its similarity to the evolution of TB observed in humans and the wide availability of immunological reagents[139, 143]. Toxicology testing in animal models remains a pre-requisite for clinical development[139, 140]. Immunogenicity data generated in animal models has been used to inform design of an optimal vaccine regimen; and to determine the dose prior to challenge experiments[140]. Protection in several animal species following *M.tb* challenge together with safety and immunogenicity examined in clinical trials are essential to justify large scale human efficacy trials whereby protection is considered to be improvement in a readout thought to reflect disease, e.g. mycobacterial load in an organ at a specific timepoint[140]. Data generated from

animal studies are necessary for an increased understanding of vaccine induced immune responses and to identify correlates of protection[140].

Animal challenge models have been increasingly refined to mimic natural acquisition of *M.tb.* For example a guinea pig model of *M.tb* challenge with air from a ward with TB inpatients contained more representative strains and doses of *M.tb*[144]. Also, a model of ultra-low dose aerosol *M.tb* challenge has recently been described in non-human primates[145].

Whilst MGIA and pre-clinical models are essential in TB vaccine development they might not always predict what happens in humans *in vivo*. A human challenge model, using BCG as the mycobacterial challenging agent to measure anti-mycobacterial control *in vivo* is being developed[146]. Human challenge models in the development of vaccines for other disease have demonstrated the usefulness of this approach. For instance, rather than testing efficacy of several malaria vaccine candidates in field trials, human malaria challenges expedited this phase of vaccine development[147]. The results of such human challenge experiments informed development of improved vaccination regimens and identification of biomarkers[147]. For TB vaccine development expensive large efficacy trials are required to test vaccine efficacy and advance our understanding about correlates of risk and protection[27, 130].

1.2 Tuberculosis vaccines

The aim of TB vaccination is to deliver one or more immunodominant antigens prophylactically, in order to initiate and establish a durable protective immune response, so that upon subsequent encounter with *M.tb* the individual is protected or at least able to deal with the infection through rapid recall of a vaccine-induced

acquired immune responses. Vaccines developed for TB include live attenuated vaccines (sections 1.2.1 and 1.2.2, p.39-42) or subunit vaccines which contain purified *M.tb* antigens (section 1.2.3, p.42-45)[135, 148-151].

1.2.1 BCG vaccine

The only licensed vaccine against TB is BCG, which was developed after 13 years of continuous *in vitro* passage of *M.bovis*[152]. BCG has been included in the Expanded Programme of Immunisation (EPI) since 1973[153].

Complications of BCG vaccination are rare in immunocompetent individuals, with local reactions observed in less than 1 in 1000 and disseminated BCG disease in less than 1 in 500000[154]. Given the more frequent complication of disseminated BCG disease in immunocompromised individuals, vaccination with BCG is avoided in HIV-infected children or HIV-exposed infants until the HIV status is known[155].

When administered at birth, BCG was shown to be effective in protecting against disseminated paediatric TB and TB meningitis[156-159]. However, the protection conferred by BCG against pulmonary TB in adults is highly variable[156, 159]. There is also geographical variation of BCG efficacy with higher rates of protection in Northern Europe and North America and lower rates in tropical countries: A trial in South India showed no efficacy over 15 years follow-up, whereas in a trial in the UK efficacy averaged 77% over a 20 year follow-up period[160-162]. There are various hypotheses on the variable efficacy of BCG. One of which is the geographical variation in exposure to NTM. Two mechanisms for NTM and BCG interaction have been proposed. Firstly, masking by NTM, whereby BCG cannot further improve on the anti-mycobacterial immunity previously induced by NTM exposure. Secondly, pre-existing immunity to

NTM, which blocks BCG replication and thereby inhibits BCG-specific immune responses. In populations with a higher prevalence of NTM exposure and reduced BCG efficacy, individuals with lower pre-existing NTM immunity demonstrated greater IFN-γ responses to vaccination[163]. Also, responses to purified protein derivative from *M.tb* (PPD) in Malawi infants were found to be nearly half that of infants in the UK[164]. Another hypothesis for BCG variability is chronic helminth infection. Areas of higher prevalence of helminth infections coincide with areas of reduced BCG efficacy[165]. As mentioned, helminth infections favour a shift towards a Th2 biased immune responses. Therefore, reduced BCG efficacy might be attributable to an impaired Th1 type immune responses in these areas. Helminth infected individuals in Ethiopia were demonstrated to display lower immune responses to BCG compared to controls who had been treated for helminth infections[165].

A safer and more effective vaccine that will provide robust protection against pulmonary tuberculosis is urgently needed. Revaccination with BCG provides no substantial additional protection and might be associated with an increased frequency of adverse events[166-168]. In the last decade several approaches have been proposed to improve on BCG[135, 150, 151, 169]. One approach to improve vaccine-induced protective immunity to TB is to use novel whole mycobacterial vaccines designed to replace or boost BCG. The advantage of such regimens would be retaining the benefits of BCG against disseminated paediatric TB and TB meningitis; and also the possible non-specific effects of BCG reducing infant mortality [156, 158, 159]. [170]. For these reasons a clinical trial without BCG in some form would be difficult to justify.

1.2.2 BCG replacement vaccines

Two BCG replacement vaccines are currently being evaluated clinically: MTBVAC and recombinant BCG VPM1002[171-173]. MTBVAC is an attenuated strain of *M.tb* which has deletions in the genes encoding phoP and fadD26 (both genes involved in *M.tb* virulence)[171]. Pre-clinical evaluation of MTBVAC showed safety to be comparable to BCG in experiments with mice, guinea pigs and more recently newborn mice. MTBVAC mediated protection was superior to BCG in several animal models challenged with *M.tb*[171, 174]. MTBVAC has recently been evaluated in a phase I trial as the first live attenuated *M.tb* vaccine; and was shown to be as safe and immunogenic as BCG[175]. The other BCG replacement vaccine in clinical trials is VPM 1002, which is a live recombinant BCG strain expressing listeriolysin with the urease gene deleted. Listeriolysin perforates the phagosomal membrane and the urease deletion ensures optimum pH for listeriolysin activity[172]. The rationale for this design is to enhance release of BCG-derived antigens into the cytosol and consequently enhance MHC class I presentation of these antigens[172]. VPM1002 within the phagosome is also presented to CD4+ T cells on MHC class II. *In vivo,* VPM1002 administered to mice was shown to be more immunogenic and safer than the wild type BCG[172]. In mice it also appeared to confer superior protection from an aerosol challenge with *M.tb*[172]. For studies of VPM 1002 in larger animal models acceptable safety profiles were reported[176]. Phase I trials in Germany and South Africa were recently completed and a phase IIa trial is underway (NCT 02391415)[173, 176].

Another example of a recombinant BCG vaccine was AERAS-422, a recombinant BCG, expressing Ag85A, Ag85B and the latency antigen Rv3407. AERAS-422 contained a mutant perfringolysin, designed for escape from endosomes into the

cytoplasm to trigger MHC class-I restricted immune responses[177]. The vaccine was withdrawn from development after a phase I safety trial where 2 out of 8 vaccine recipients developed serologically confirmed shingles in the high dose group[178].

1.2.3 Subunit vaccines

The other approach on improving on protective immunity conferred by BCG is to boost BCG with a subunit vaccine. The ultimate goal of an effective TB vaccine regimen is to establish a permanent reservoir of strong *M.tb*-specific immune responses, which have the required magnitude and quality to protect from subsequent encounters with *M.tb;* and can mount such a response timely and effectively. Heterologous prime-boost immunisation regimens, where two different vaccines expressing common antigens are given consecutively, are an effective way to induce strong cellular immune responses and polyfunctional T cells[179-184]. In such prime-boost regimens for TB, BCG is the logical priming vaccine, to retain the protective effects of BCG in childhood[156-159]. Several prophylactic subunit vaccines have been designed as BCG booster vaccines[135, 151, 169]. These display one or more selected mycobacterial antigens e.g. Antigen 85A or B, ESAT-6, and TB10.4[135, 169]. There is some interest in so-called latency antigens such as Rv2660c, particularly for post-exposure vaccines to prevent reactivation in people with LTBI[135, 169].

The main categories of subunit vaccines are adjuvanted recombinant proteins or recombinant viral vectors[148, 149].

1.2.3.1 Protein adjuvant vaccines

In contrast to whole organisms, purified protein antigens delivered by themselves are not very immunogenic and therefore require an adjuvant to stimulate immune

responses[148-151]. Protein adjuvant vaccines that have completed phase I trials include: Hybrid 1, a fusion protein consisting of antigen 85B fused to ESAT-6; Hybrid 56, consisting of antigens 85B, ESAT-6 and latency antigen Rv2660c; and HyVaC 4, consisting of antigens 85B and TB10.4. All these fusion proteins are being evaluated in clinical trials with adjuvant IC31, an activator of a Toll-like receptor[185-187]. M72, another fusion protein, used with the GSK adjuvant AS01, was evaluated in several phase I/IIa trials and recently entered phase IIb efficacy testing with expected completion in November 2018 (ClinicalTrials.gov Identifier:NCT01755598)[188, 189]. The vaccine candidate of the Infectious Disease Research Institute (IDRI), ID93+GLA-SE, is composed of a recombinant fusion protein of 4 *M.tb* antigens combined with an oil in water adjuvant. ID93+GLA-SE has entered phase I clinical trials[190].

1.2.3.2 Recombinant viral vector vaccines

In subunit vaccines that use recombinant viral vectors, the encoded antigen is expressed and presented to the immune system[148, 149]. Recombinant viral vectors as a vaccine platform are attractive due to their safety profile and their ability to induce potent acquired immune responses against the expressed antigen. Viral vector vaccines developed for TB are based on replication deficient virus variants expressing various immunodominant TB antigens.

Adenoviral vector based vaccines have been developed for a variety of pathogens and shown to induce robust antigen-specific T cell responses when given alone or in a heterologous prime-boost regimen[191-196]. A candidate TB vaccine utilising a recombinant human type 5 adenoviral vector is AdHu5Ag85A, expressing Ag85A. A phase I study of intramuscular immunisation with AdHu5Ag85A has recently been completed demonstrating the vaccine to be safe, well tolerated and immunogenic in

BCG-naïve and BCG-vaccinated healthy volunteers, with more potent immunogenicity in the latter group[193]. AERAS-402, based on Adenovirus Hu35, expresses three *M.tb* antigens: Ag85A, Ag85B and TB10.4[191, 197-199].

Chimpanzee adenoviral vectors were developed more recently to circumvent the potential problem of anti-vector immunity due to a high seroprevalence of neutralising antibodies in a target vaccine population. Reports of their use in clinical trials against malaria and influenza, alone and in heterologous prime-boost regimen, are published[200-202]. A first in man trial of a candidate TB vaccine with a chimpanzee adenoviral vector, ChAdOX1 85A, has just been completed in Oxford (ClinicalTrials.gov Identifier: NCT01829490). Another such vaccine, AdCh68Ag85A, is in preclinical stages of development[199].

Modified Vaccinia Ankara (MVA) used as a vector for boost vaccinations can enhance T cell responses as shown in clinical vaccine trials to a variety of pathogens[184, 196, 203, 204]. In a recent Ebola vaccine trial, a MVA boost vaccination was also able to enhance virus-specific humoral and cellular immune responses[196]. MVA85A administered intradermally was shown to boost pre-existing BCG-induced immune responses in adults. MVA85A is one of the most clinically advanced TB vaccine candidates. In a phase II trial in BCG-vaccinated infants, vaccine-induced cellular immune responses were much weaker than responses seen in adults; and no significant improvement in efficacy above BCG alone was demonstrated[130]. In a more recent phase II trial in HIV infected adults MVA85A also failed to demonstrate efficacy against *M.tb* infection or disease[205].

A phase I trial with AERAS-402, an adenoviral vector candidate TB vaccine mentioned earlier, followed by MVA85A has recently been reported. AERAS-402 was shown to

predominantly induce antigen-specific CD8+ T cell responses, whereas MVA85A is potent at inducing Ag85A-specific CD4+ T cell responses. The rationale for this clinical trial was that by combining the two a balance of CD4+ and CD8+ T cell responses could be achieved[206]. Adenoviral prime-MVA boost regimens proved highly immunogenic in pre-clinical and clinical trials for vaccine candidates other than TB[200, 207].The trial demonstrated increased durability of antigen-specific T cell responses and increased polyfunctionality of CD8+ T cells[208].

1.2.4 Other considerations for TB vaccine design

Concerns about potential immunopathology of TB vaccines and the so-called Koch phenomenon warranted a cautionary approach in TB vaccine development. The Koch phenomenon was a mycobacteria-specific immunopathology described after culture filtrate tuberculosis protein was given to patients with TB[1]. Pre-clinical evidence suggested the Koch phenomenon was a dose-dependent process as it was seen in immunotherapeutic but not in prophylactic mice models. In one murine study the Koch phenomenon was observed in the form of pulmonary necrosis following repeated DNA vaccination against TB but not after less frequent vaccination[209]. In another study BCG administered repeatedly to mice infected with *M.tb* led to abscess formation in the lung[210]. The Koch phenomenon was a concern in the early stages of vaccine development. However, serial testing in people with increasing mycobacterial burden over the last decade demonstrated no immunopathology with MVA85A or any other candidate TB vaccine tested to date[211].

Another concern has been the use of TB vaccines in HIV-infected individuals. This population is at increased risk of TB and therefore an important target population. The consensus is that clinical trials for live vaccines such as MTBVAC and VPM1002 had

to be performed in healthy unvaccinated adults first; but could then sequentially progress to BCG-vaccinated individuals and those exposed to TB and HIV[212]. Viral vector vaccines such as MVA85A were tested and demonstrated to be safe in people infected with HIV[213].

Candidate vaccines vary in their delivery systems and antigens. With the exception of MVA85A all candidate TB vaccines were tested in clinical trials by the systemic route of administration. There are concerns that any vaccine designed to boost BCG administered by the same route as BCG could still fail to protect from pulmonary TB. This has encouraged the TB vaccine community to consider and develop the mucosal route of vaccination, in order to match the route of vaccination to the route of natural infection, with the aim to establish local vaccine-induced immunity at the future first site of encounter with *M.tb*.[214]

1.3 Aerosol vaccination

1.3.1 Proof of concept

There are ongoing efforts to evaluate mucosal routes of vaccination for respiratory pathogens other than *M.tb*. The aerosol route of delivery has been explored for measles to improve coverage through easier administration[215, 216]. The licensed injectable vaccine is administered subcutaneously and target population coverage reported as 84% in 2013[217]. In all published comparative clinical trials, aerosolised measles vaccination was equally or more immunogenic than the subcutaneous vaccination in children aged 10 months or older[215]. Also, administering a booster dose by the aerosol route yielded a stronger and more durable antibody titre than when given by the subcutaneous route[218-220]. Mass campaigns in South African and Mexican

schoolchildren demonstrated that the aerosol route of vaccine delivery was not only safe and immunogenic but also an acceptable and feasible method[220, 221]. Aerosolised delivery of measles vaccine would require re-licensing. Post-licensing field trials would be required to demonstrate efficacy of this route. In a recent open-label non-inferiority trial aerosolised measles vaccine was not superior to the subcutaneous route at inducing seropositivity[222].

For seasonal influenza, an intranasally administered live attenuated influenza vaccine (LAIV, FluMist) has recently been licensed in addition to the injectable trivalent vaccine (TIV). LAIV demonstrated higher efficacy in children than TIV[223]. LAIV was also associated with more durable protection than TIV[224]. However, these features were all observed despite limited ability of LAIV to induce humoral and cellular effector responses[225, 226].

1.3.2 Aerosol vaccination for TB: rationale

The aerosol route of vaccination has potential practical advantages as it does not require highly trained personnel, thus facilitating deployment. It also avoids risks associated with use and disposal of needles and syringes[214]. The primary route of *M.tb* infection is *via* inhalation of aerosolised droplets containing *M.tb* resulting in a primary infection focus in the lung[28, 126]. Delivering a TB vaccine by aerosol directly to the respiratory mucosa might offer an immunological advantage.

Mucosal immunisation *via* the intranasal route has been considered. In the murine model, intranasal administration effectively enhanced immunogenicity and protective efficacy[141, 227]. In mice, the pharyngeal anatomy is such that a vaccine administered by the intranasal route is more likely to reach the lung. In humans intranasal

administration is more likely to end up in nasal passages, where it would induce local secretory IgA antibodies. This was demonstrated by intranasally administered LAIV shown to induce nasal associated lymphoid tissue and secretory IgA[228]. Antigen-specific IgA was detected in saliva; and antibody-secreting as well as memory-type B cell responses were found in tonsil tissue[228]. Therefore, in humans the aerosol route of delivery is likely to offer physiological advantages by delivering smaller particles to more distal airways which reach bronchus-associated lymphoid tissue. In the past the intranasal route of immunisation has raised safety concerns in vaccine development. An epidemiological association of facial nerve palsy following administration of an inactivated influenza virosome vaccine (NasalFlu) containing an *Escherichia coli* heat labile toxin adjuvant (ELT) was observed. This led to withdrawal of the vaccine[229]. Two further cases of transient facial nerve palsy were reported following administration of a nasal subunit vaccines against HIV and TB in two concurrent phase I clinical trials. In these trials, both vaccines were administered with the same ELT adjuvant[230]. The exact cause and pathogenesis of these facial nerve palsies were unclear. In the case of NasalFlu the investigators suggested "a strong association" and for the other two cases using the ELT adjuvant intranasally the investigators reported that “individual components for paralysis were not identified”[229, 230].

1.3.3 Aerosol vaccination for TB: animal models

Pre-clinical studies where BCG was administered to mice, guinea pigs or macaques by the intranasal or aerosol route, demonstrated greater protection by this route compared to the systemic route in *M.tb* challenge experiments[231-235]. Aerosolised BCG vaccination in non-human primates produced several features thought to be important in protective immunity to TB: antigen-specific Th1 and Th17 cells, polyfunctional T cells

in lung mucosal and systemic compartment, IFN-γ and serum IgG. Antigen-specific T cells expressed consistently high levels of lung homing integrin α4β1[236].

Two candidate TB vaccines, both recombinant viral vectors (AdHu5Ag85A and MVA85A), when administered directly to the respiratory mucosa, demonstrated efficacy in pre-clinical animal models. AdHu5Ag85A, delivered intranasally, conferred significant protection against aerosol *M.tb* challenge when administered either alone, or as a mucosal boost in BCG-vaccinated animals[227, 237, 238]. Single intranasal vaccination with AdHu5Ag85A also offered superior protection to cutaneous BCG vaccination alone[227]. There was no enhancement in protection above BCG alone when this vaccine was administered to mice intramuscularly[227]. In cattle, endobronchial boosting of BCG-vaccinated animals with AdHu5Ag85A induced local mucosal and systemic responses that were comparable in magnitude to those induced by intradermal boosting[194]. Mucosal delivery of AdHuAg85A has not yet been evaluated in humans. MVA85A, administered intranasally to BCG-vaccinated mice, also conferred significant protection in an aerosol *M.tb* challenge[141]. When MVA85A was administered by aerosol to BCG-vaccinated non-human primates, it induced potent mucosal and systemic immunity [239]. Interestingly, the strongest responses appeared to be in the compartment to which the vaccine had been administered, although the differences were not statistically significant. Anti-MVA IgG antibodies were detected in the serum of the animals vaccinated by the intradermal route, but not in those vaccinated by the aerosol route. In contrast to these findings with aerosolised AdHu5Ag85A and MVA85A in animal models, delivery of the viral vector vaccine AERAS-402 by the aerosol route to BCG-vaccinated or naïve non-human primates showed induction of antigen-specific cellular immune response in the lungs, but failed to confer additional protection against a high dose *M.tb* challenge[240].

Mucosal vaccination with an *M.tb* mutant in mice induced bronchus-associated lymphoid tissue, strong central memory T cell responses and reduced bacterial and disease burden[241].

The intranasal delivery of inert bacterial spores coated with TB antigens, MPT64 and a hybrid protein consisting of alpha- crystalline and antigen 85B, in mice was immunogenic and conferred significant protection against an intranasal *M.tb* challenge[242, 243].

This body of pre-clinical data suggests that the aerosol route of delivery may be a promising route of immunisation for TB vaccines, and in general potentially for recombinant viral vectors.

1.3.4 Aerosol vaccination for TB: humans

There is an early report of a study with aerosolised BCG in 1968[244]. Rosenthal *et al* reported aerosol nebulisation of BCG first in guinea pigs, then school children and medical students. TST conversion rates were used as an outcome measure in this study. Rates of TST conversion were 93% in guinea pigs, 85% in school children and 89% in medical students, at 2-6 months follow-up (for 100 tuberculin units)[244]. Although Rosenthal *et al* described technical limitations in this study, aerosolised BCG proved feasible to administer and was well tolerated in the populations studied. A decade later aerosolised BCG was trialled in two studies as an adjunct to standard treatment in patients with lung cancer[245, 246]. One study recruited patients with non-resectable primary bronchogenic carcinoma and the other one patients with metastatic lung cancer[245, 246]. All patients experienced flu-like symptoms, which resolved within 24-72 hours and gradually declined with continuation of treatment[245, 246]. Lung function

was unchanged from pre-treatment to post-treatment in the group with non-resectable primary lung cancer. In the group with metastatic lung cancer lung function declined[245, 246]. The rate of decline in lung function correlated with radiological evidence of cancer progression[245, 246]. Whilst there were no survival benefits reported in either studies, both demonstrated that delivery of aerosolised BCG in these patient groups was practicable. There was no evidence of BCG in the lungs of these patients on *post-mortem* examinations[245, 246]. A clinical trial delivering BCG by aerosol to healthy volunteers is currently underway in Oxford (ClinicalTrials.gov Identifier: NCT02709278).

The first clinical trial to administer a candidate TB vaccine directly to the respiratory mucosa in humans has been reported[247]. BCG-vaccinated healthy UK adults received MVA85A by the aerosol or intradermal route. The initial two volunteers received a dose of $1x10^8$ pfu of MVA85A but given that the proportion of Ag85A-specific T cell responses in the bronchoalveolar lavage fluid (BAL) of these volunteers were found to be very high and one volunteer developed shingles, a cautious approach was taken and the dose reduced to $1x10^7$ pfu MVA85A for remaining volunteers[247]. Safety was comparable between the two groups, and the aerosol route of immunisation was well tolerated. In BAL samples taken 7 days after immunisation, antigen-specific CD4+ T cells were detectable in both groups, but were significantly stronger in the subjects immunised by the aerosol route[247]. Systemic antigen specific CD4+ T cell responses were comparable between the two groups. Antigen-specific CD8+ T cell responses were higher in BAL than in blood after both routes of immunisation. In blood, antigen-specific CD8+ T cell responses were significantly higher after aerosol administration than after intradermal administration[247]. Serum antibodies to MVA were detected after intradermal immunisation but not after aerosol administration of the vaccine, which is

discussed later in this thesis[247]. The conclusions of this first-in-man trial of an aerosolised candidate TB vaccine were that the aerosol route is safe (based on the initial data), and more potent at inducing lung mucosal immune responses.

However, as this was only the first phase I trial, the aerosol route of MVA85A vaccination and any other vaccine candidates requires further evaluation. As mycobacterial burden is high in TB endemic countries, safety assessment in individuals with LTBI to assess the potential risk of the Koch phenomenon is important. A phase I clinical trial delivering aerosolised MVA85A to patients with LTBI was recently started in Oxford (ClinicalTrials.gov Identifier: NCT 02532036).

1.4 Anti-vector immunity

A limitation of use and re-use of all viral vector vaccines (discussed in section 1.2.3, p.43-45) is anti-vector immunity; i.e. immunity to the viral vector. Anti-vector immunity is either pre-existing to commonly naturally occurring human serotypes, or induced *de novo* after vaccination[197, 248-250]. Several strategies have been designed to circumvent the possible effects of anti-vector immunity. These include use of less prevalent serotypes, development of chimpanzee adenoviral vectors and mixed vector immunisation[192, 198, 251, 252].

However, the impact of the type or magnitude of anti-vector immunity on the efficacy of a viral vector vaccine is not known. Neither are the differential roles of pre-existing *versus* vaccine-induced or humoral *versus* cell-mediated anti-vector immunity well established. The literature regarding this is limited and somewhat contradictory. Anti-vector antibodies were shown to decrease expression of the insert antigen carried by the vector, diminishing the generation of antigen-specific CD8+ T cells and therefore

contribute to decreased insert immunogenicity[204, 249, 253-255] Conversely, a phase I trial with the AdHu585A vaccine demonstrated despite high prevalence and high levels of pre-existing AdHu585A-antibodies the impact on vaccine potency was minimal[193]. Furthermore, a phase I/IIa trial of an AdHu-5 based malaria vaccine demonstrated no effect of pre-existing or vaccine-induced AdHu-5 antibodies on humoral or cell mediated immunogenicity of the insert malaria antigen[195]. Thus, humoral anti-vector immunity does not always inversely correlate with the immunogenicity and potency of viral vector vaccines.

To add to the complexity, a clinical trial designed to test the ability of an AdHu5 vector HIV-1 vaccine to prevent HIV infection or reduce viral load in those later infected was halted early due to lack of efficacy. A *post-hoc* analysis revealed a non-significant higher incidence of HIV in vaccinated male subjects who had pre-existing AdHu5 antibodies and/or were circumcised[256-258].

The studies of aerosolised MVA85A delivered to non-human primates and humans demonstrated that systemic but not aerosol vaccination with MVA85A induced serum anti-vector antibodies. In non-human primates, intramuscular delivery of a recombinant poxvirus-based vector induced higher levels of serum anti-vector antibodies, whereas aerosolised delivery induced higher levels of anti-vector antibody in the vaginal mucosa. There was no evidence of insert mucosal humoral responses[259].

There is a need to understand better how pre-existing and *de novo* humoral and cellular anti-vector immune responses influence and predict the immunogenicity to the insert antigen of viral vector vaccines.

1.5Aim of this DPhil project

This DPhil was based on the hypothesis that combined administration routes of a candidate TB vaccine delivered to systemic and lung mucosal compartments would enhance responses to insert Ag85A in both body compartments of the vaccinated individual. The candidate TB vaccine used in this DPhil was MVA85A.

Previous studies demonstrated that a 3 week interval between homologous MVA85A–MVA85A boosting administered systemically did not enhance responses[197]. There are potential physiological and logistical advantages of delivering a TB vaccine by aerosol. The first-in-man trial of MVA85A delivered by aerosol demonstrated this route to be safe and immunogenic[247]. The aerosol and intradermal route of MVA85A delivery in humans were not previously combined.

The primary aim of this DPhil was to evaluate a homologous MVA85A-MVA85A prime-boost vaccine regimen delivered by heterologous routes (aerosol and intradermal), as a way to induce potent local and systemic cellular immunity to the insert Ag85A.

The secondary aims were to characterise lung mucosal and systemic insert (Ag85A) and vector (MVA) responses; evaluate the functional relevance of anti-vector immunity induced by aerosol or systemic prime immunisations on a subsequent MVA85A-boost vaccination by alternative routes, measuring insert Ag85A responses in systemic and lung mucosal compartments as a reflection of the effectiveness of this heterologous route approach.

2 Materials and methods

2.1 Study design

This was a phase I clinical trial using a candidate TB vaccine MVA85A. The aim of the study was to evaluate the effects of homologous boosting using MVA85A twice but by heterologous routes. The vaccination routes used were aerosol and intradermal. The boosting interval was 28 days. The effect was measured in terms of insert Ag85A responses in blood and BAL samples. For the clinical trial protocol please refer to Appendix 1.

The primary objective was

1. to evaluate the safety of aerosol administration and combined aerosol with intradermal administration of MVA85A.

The secondary objectives were

1. to measure lung mucosal and systemic insert antigen 85A (Ag85A) and vector Modified Vaccinia Ankara (MVA) responses and
2. to evaluate the functional relevance of any anti-vector immunity induced by aerosol or systemic prime immunisations on a subsequent MVA85A-boost vaccination by alternative routes, measuring insert Ag85A responses in systemic and lung mucosal compartments.

36 BCG-vaccinated healthy volunteers were randomised into 3 groups (Figure 2.1 Trial Schedule). All subjects had received a BCG vaccination at least 6 months prior to enrolment. Allocation was randomised by sequentially numbered sealed envelopes, which had been prepared by an independent statistician at the Centre for Statistics in Medicine, University of Oxford.

Each group received two doses of MVA85A vaccinations 4 weeks apart.

Group 1 received MVA85A administered by aerosol, and then boosted by a second dose of MVA85A administered intradermally.

Group 2 received MVA85A administered intradermally, and then boosted by a second dose of MVA85A administered by aerosol.

Group 3 received both doses of MVA85A intradermally (Figure 2.1 Trial Schedule).

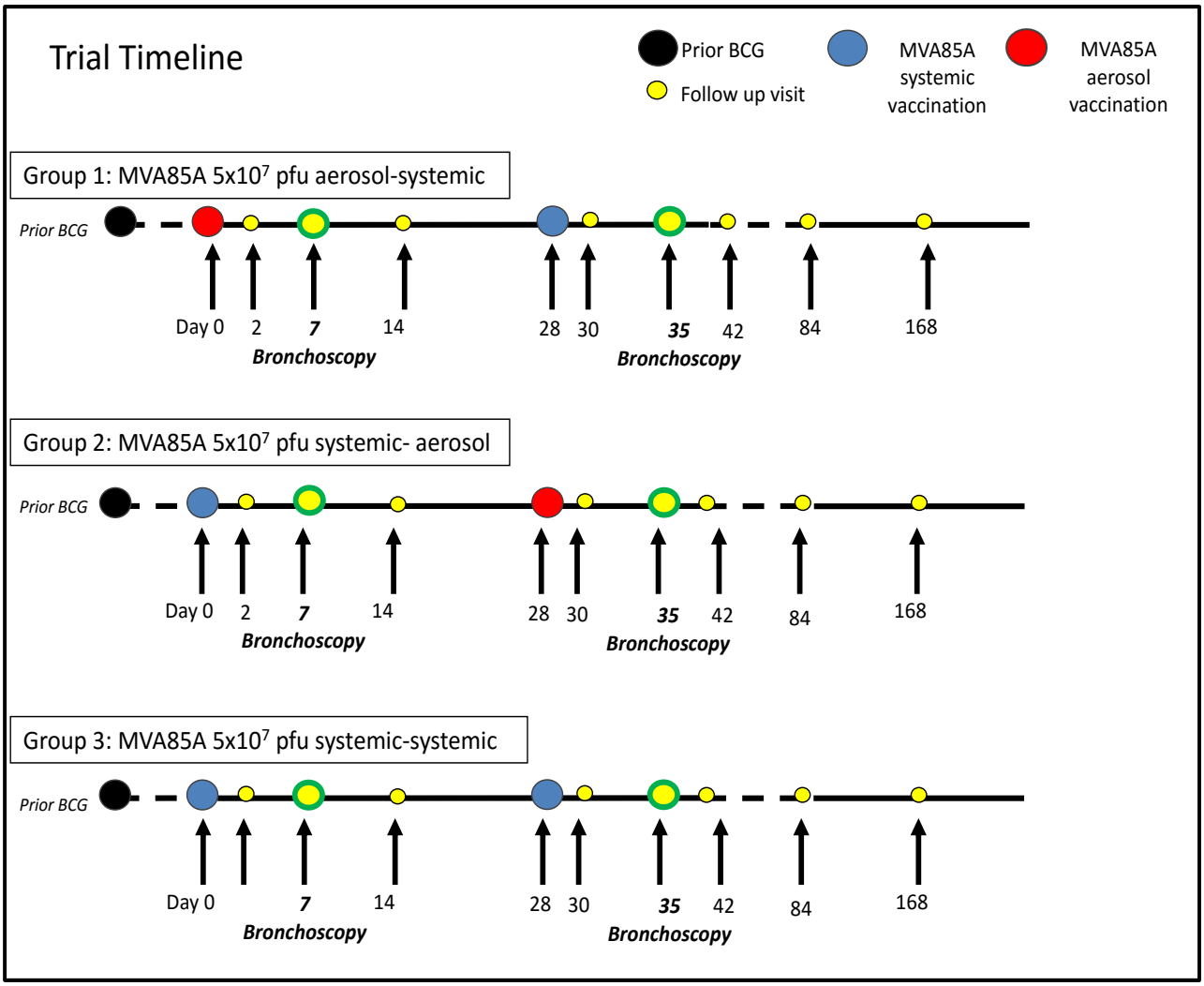
The dose of MVA85A vaccination was $5x10^7$ pfu. The aerosol was delivered using a handheld nebuliser device (Section 2.2.1, p.66-67). When MVA85A was administered by aerosol, a saline placebo was administered intradermally at the same time; conversely when MVA85A was administered by intradermal injection a saline placebo was administered by aerosol (paired placebo design).

Blood was taken at every scheduled trial visit (Figure 2.1 Trial Schedule).

Bronchoscopy and broncholaveolar lavage were performed 7 days after each vaccination in this study (i.e. on day 7 and day 35). The bronchoscopist was blinded to the route of vaccine administration.

The trial was registered with clinicaltrials.gov (NCT01954563).

Figure 2.1 ***Trial Timeline.*** *Groups 1-3 (top, middle bottom row). Black line is timeline with timepoints in days black circle is prior BCG; MVA85A Vaccinations on day 0 and day 28 by aerosol route (red circle) or intradermal (blue circle); yellow circles are follow up visits, yellow circles with green ring are bronchoscopy visit.*



During enrolment, it was noted that 2 out of 4 volunteers in Group 2 (but no volunteers in the other groups) exhibited mild transient respiratory symptoms following their day 28 aerosol boost vaccination. The protocol was amended to pause enrolment into this group until safety review. Envelopes with that group number were omitted whilst enrolment into the other two groups (Groups 1 and 3) continued in the randomised sequence. At that point, the only feature that distinguished the two affected Group 2 volunteers from the other volunteers in Group 2 was that they both had a clear history

of atopy. No other differences were noted. Following safety review of clinical and immunological data and consultation with the local safety monitor, enrolment into Group 2 was restarted with the implementation of more stringent exclusion criteria to exclude any volunteers with history of atopy. Also, only one volunteer at a time was to be enrolled into this group, with the subsequent volunteer only being enrolled after completion of both vaccinations and favourable safety review in the previous volunteer. Once the first two subjects were enrolled this way with a favourable safety review, subsequent volunteers could be enrolled together. When 3 more volunteers were affected by either respiratory adverse events or more severe systemic adverse events (compared to volunteers in the other groups) or both, an urgent safety measure was implemented to halt vaccinations in Group 2 (Appendix 1: Clinical Trial Protocol). The remaining 3 volunteers that had already been enrolled into Group 2 (074, 077 and 081) and had received the MVA85A intradermal prime vaccination, did not receive MVA85A aerosol boost on day 28.

2.1.1 Sample size

This was a phase I clinical trial with primarily descriptive endpoints. A twofold increase in the magnitude of the cellular immune response, as measured by IFN-γ ELISPOT response to Ag85A peptide stimulation (section 2.5.4, p.74-76) was considered immunologically meaningful. Extrapolating effect size and variability from the completed pilot aerosol vaccine safety and immunogenicity study, 8 volunteers were needed to have 80% power to detect an immunologically meaningful (twofold) difference by IFNγ ELISPOT in response to stimulating with a single peptide pool of all 66 peptides of Ag85A. This sample size of 12 volunteers per group was appropriate for a proof-of-concept phase I safety study.

2.1.2 Study conduct

Ethical (NRES South Central- Oxford A, 13 SC 0329), MHRA, NHS Research and Development and Oxford GMO approvals for the study were obtained. The University of Oxford was the Sponsor for the trial. The trial was performed in accordance with Good Clinical Practice. Independent monitoring was undertaken by Clinical Trials and Research Governance, University of Oxford. Healthy BCG-vaccinated volunteers were recruited by advertisement as outlined in the approved study protocol (Appendix 1).

2.1.3 Volunteer visits

Volunteers who expressed interest were sent the volunteer information sheet (VIS) and offered a screening visit. Volunteers were asked to read the VIS at least 24 hours prior to attending for screening. At the screening visit, the clinical trial was explained in detail and questions about the screening process and the trial were answered. Written informed consent was obtained for screening evaluation which consisted of medical history and examination. Also, blood samples were obtained for full blood count, biochemistry and serological studies for blood borne viruses (Table 2.1). Blood was also taken for an *ex-vivo* IFN-γ ELISPOT assay for baseline antigen-specific responses to PPD, antigen 85A and two *M.tb* specific antigens, ESAT-6 CFP-10. Spirometry, pulse oximetry, urine analysis (including pregnancy tests in females) and chest radiographs were also performed at this visit. At enrolment and follow up visits the following schedule of trial procedures was observed (Table 2.1).

***Table 2.1 Schedule of trial procedures**. S= Screening visit. X marks events scheduled to occur and if in brackets only when indicated. Grey highlights vaccination days (days 0 and 28), Blue highlights bronchoscopy days (days 7& 35)*

Timeline (days)	*S*	*0*	*2*	*7*	*14*	*28*	*30*	*35*	*42*	*84*	*168*
Inclusion/ exclusion criteria	*X*										
Review contra-indication	*X*	*X*		*X*		*X*		*X*			
Informed consent	*X*										
Medical history	*X*	*(X)*	*(X)*	*(X)*	*(X)*	*(X)*	*(X)*	*(X)*	*(X)*	*(X)*	*(X)*
Physical examination	*X*			*X*				*X*			
Vital signs	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*
Urinalysis	*X*										
Chest x ray	*X*										
Pulmonary function tests	*X*	*X*	*X*	*X*		*X*	*X*	*X*			
Beta-HCG urine test	*X*	*X*		*X*		*X*		*X*			
Vaccination		*X*				*X*					
Bronchoscopy				*X*				*X*			
Local & systemic events		*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*
Diary cards provided		*X*				*X*					
Diary cards collected					*X*				*X*		

Table 2.1 continued: Trial Schedule Blood Tests. *Safety blood tests at screening, day 7 and day 35. HBV=Hepatitis B virus, HCV= Hepatitis C virus, HIV=Human immunodeficiency virus, HLA=Human leukocyte antigen.*

Timeline (days)	*S*	*0*	*2*	*7*	*14*	*28*	*30*	*35*	*42*	*84*	*168*
Biochemistry (4mL)	*X*			*X*				*X*			
Haematology (2mL)	*X*			*X*				*X*			
Coagulation (4mL)	*X*										
HBV, HCV, HIV (10mL)	*X*										
HLA typing (4mL)		*X*									
Exploratory Immunology(20-60mL)	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*
Blood volume (mL)	*50*	*64*	*20*	*66*	*60*	*60*	*20*	*66*	*60*	*60*	*60*

2.1.4 Eligibility

Inclusion and exclusion criteria were defined in the approved clinical trial protocol (Appendix 1) to identify individuals suitable for vaccination and minimise risks at bronchoscopy. To participate, volunteers were required to meet all of the inclusion criteria and none of the exclusion criteria.

Inclusion criteria were:

- Healthy adult aged 18-55 years
- Resident in or near Oxford for the duration of the trial period
- No relevant findings in medical history or on physical examination
- Confirmation of prior vaccination with BCG not less than 6 months prior to projected trial vaccination date (by visible BCG scar on examination or written documentation)
- Allow the Investigators to discuss the individual's medical history with their GP
- Use effective contraception 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 subject details with a confidential database to prevent concurrent entry into clinical trials
- Able and willing (in the Investigator's opinion) to comply with all the trial requirements

Exclusion criteria were:

- Any respiratory disease, including asthma
- Current smoker
- Clinically significant abnormality on screening chest radiograph
- Clinically significant abnormality of pulmonary function tests
- Any nasal, pharyngeal, or laryngeal findings which preclude bronchoscopy
- Current use of any medication taken through the nasal or inhaled route including cocaine or other recreational drugs
- Laboratory evidence at screening of latent *M.tb* infection (LTBI) as indicated by a positive IFN-γ ELISPOT response to ESAT-6 or CFP-10 antigens
- Clinical, radiological, or laboratory evidence of current active TB disease
- Previous vaccination with candidate vaccine MVA85A or candidate vaccine FP85A or any other recombinant MVA vaccine
- 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
- History of serious psychiatric condition
- 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 vaccine, sedative drugs, or any local or general anaesthetic agents
- Any abnormality of screening blood or urine tests that is deemed to be clinically significant or that may compromise the safety of the subject in the trial
- Positive HBsAg, HCV or HIV antibodies
- Female currently lactating, confirmed pregnancy or intention to become pregnant during trial period
- Use of an investigational medicinal product or non-registered drug, live vaccine, or medical device other than the trial vaccine for 30 days prior to dosing with the trial vaccine, or planned use during the trial period
- Administration of immunoglobulins or any blood products within the three months preceding the planned trial vaccination date
- Any other significant disease, disorder, or finding, which, in the opinion of the Investigator, may either put the subject at risk or may influence the result of the trial or may affect the subject's ability to participate in the trial

Atopy was incorporated as an exclusion criterion after the first Group 2 safety review, suggesting more atopy in the two Group 2 subjects who suffered respiratory adverse events following day 28 boost aerosol vaccination compared to all other volunteers.

Subjects, who were excluded from the trial because a previously undiagnosed condition had been identified during screening procedures, were referred to either their GP or to NHS specialist services for further investigation and treatment. Subjects

discovered to have evidence of latent *M.tb* infection as defined by positive IFN-γ ELISPOT responses to ESAT-6 and CFP-10, were screened for evidence of active TB disease either on clinical or radiological grounds and referred to the TB specialist service for consideration of chemoprophylaxis.

2.2Vaccines

MVA85A was manufactured under Good Manufacturing Practice conditions by IDT Biologika GmbH, Germany. MVA85A lot 0050811 was supplied as a sterile liquid in glass vials. Each vial contained 400 µL of vaccine at a concentration of 8.4×10^8pfu/mL in 10mM Tris buffer 140mM NaCl, pH 7.7. Vaccine was stored between 70°C and –90°C, in a locked freezer at the University of Oxford.

2.2.1 Vaccinations

Vaccine was allowed to thaw to room temperature; and diluted to the correct concentration using 0.9% saline. Diluted vaccine was then administered by intradermal injection or aerosol. Intradermal injection was performed over the deltoid region of the upper arm using a syringe pre-loaded with a 29G diameter needle. Aerosol administration was performed using an approved battery operated electromedical ultrasonic mesh nebuliser device MicroAIR NE-U22 (Omron® Healthcare UK, Ltd., Milton Keynes, UK, CE0197, EAN code 40 15672 10142 1). The vaccine was diluted in normal saline made up to a total volume of 1ml (Appendix 1: Clinical trial protocol). The nebuliser containing the vaccine was connected to a mouthpiece. The aerosol was delivered to volunteers through the mouthpiece. Volunteers were sitting upright on the edge of the clinical examination couch. This device is designed for the delivery of bronchodilators and antibiotics[260] The nebuliser uses ultrasound to push liquid

through a fine metal mesh with 6000 tapered holes generating an aerosol mist with consistently sized particles of about 3-5 µm in diameter. The devices were cleaned and the mesh replaced between vaccinations. Aerosol delivery ranged between 3 and 7 minutes with a median of 5 minutes. The nebuliser was removed from the volunteer after confirming that no residual vaccine remained in the reservoir. Subjects were observed for 60 minutes after vaccination to detect any immediate adverse events and followed up as per study protocol (Appendix 1).

2.3 Safety assessment

Information about local and systemic adverse events were collected actively at each visit by medical history of solicited local, respiratory and systemic as well as any unsolicited adverse events, examination and measurement of the injection site, spirometry and vital signs. Passive data collection on adverse events was obtained from diary cards for two weeks following each vaccination (days 0 to 14 and days 28 to 42). Adverse events from visits and diary cards were collated and chronologically ordered for analysis by group. Bronchoscopic examination of the lungs 7 days after each vaccination provided further safety data. Safety blood tests (Full blood count and biochemistry profile) were collected at screening and 7 days after each vaccination (day 7 and day 35). When Group 2 was restarted following the safety pause, an amendment was made to also measure C-reactive protein of volunteers following vaccination in order to quantify inflammation and correlate this with the clinical picture.

2.3.1 Causality assignment

Causality of adverse events was determined by the lead clinician after completion of the trial with the premise that all solicited adverse events were considered definitely causally related to intradermal vaccination (i.e. relationship grading 4). The greatest degree of causal relationship (definite > probable > possible > unlikely related > not related) was determined for all unsolicited adverse events and then the assignment subjected to independent peer review with discussion for discordant causality assignments. The following guidelines were used to assess the relationship of all adverse events to the administration of the vaccine (Table 2.2).

Table 2.2 ***Guidelines for assessing relationship of adverse events to administration of vaccine.*** *Solicited adverse events following vaccination were assumed to be causally related. Greatest degree of causal relationship of unsolicited adverse events was assigned.*

Relationship grading	*Guidelines*
0= no relationship	*No temporal relationship to vaccine; and alternate aetiology (clinical state, environmental or other interventions); and does not follow known pattern of response to the vaccine*
1=unlikely relationship	*Unlikely temporal relationship to vaccine; and alternate aetiology likely (clinical state, environmental or other interventions); and does not follow known typical or plausible pattern of response to the vaccine*
2=possible relationship	*Reasonable temporal relationship to vaccine; or event not readily produced by clinical state, environmental or other interventions; or similar pattern of response to that seen with the vaccine*
3 = Probable relationship	*Reasonable temporal relationship to vaccine; and event not readily produced by clinical state, environment, or other interventions or known pattern of response seen with the vaccine*
4 = Definite relationship	*Reasonable temporal relationship to vaccine; and event not readily produced by clinical state, environment, or other interventions; and known pattern of response seen with the vaccine*

2.3.2 Severity assessment

Adverse events were graded by severity by volunteers themselves in diary card and at clinic review. The following guidelines were provided for severity grading on a scale of 1-4 where 0= absent, 1 =mild, 2=moderate and 3= severe (Table 2.3).

Table 2.3 ***Severity grading for solicited and unsolicited adverse events.*** *This guidance was given to volunteers at enrolment. Severity was graded by volunteers themselves in diary cards or as reported by them in clinic.*

Adverse event	*Grade*	*Measurement*
Tenderness or pain at intradermal injection site	*0*	*No pain at all*
	1	*Painful on touch; easily tolerated*
	2	*Painful when limb is moved; interferes with daily activity*
	3	*Severe pain at rest; prevents daily activity*
Redness at intradermal injection site	*0*	*0 mm*
	1	*1 – 50 mm*
	2	*51 – 100 mm*
	3	*> 100 mm*
Swelling at intradermal injection site	*0*	*0 mm*
	1	*1 – 20 mm*
	2	*21 – 50 mm*
	3	*> 50 mm*
Fever (oral)	*0*	*≤37.5°C*
	1	*37.6 – 38.0 °C*
	2	*38.1 – 39.0 °C*
	3	*>39.0 °C*
Other AEs	*0*	*Absence of symptom*
	1	*Awareness of symptom but tolerated; transient or mild discomfort; little or no medical intervention required*
	2	*Discomfort enough to cause limitation of usual activity; some medical intervention or therapy required*
	3	*Incapacitating, absent from work, or bed rest required; hospitalisation possible*

2.4 Bronchoscopies

The bronchoscopies were carried out in a dedicated NHS bronchoscopy suite at the Oxford Centre for Respiratory Medicine by consultant respiratory physician Dr. Bettinson according to local policy and guidelines[261]. Intravenous sedation (Midazolam up to 3mg and Fentanyl up to 100µg) and local anaesthesia spray delivered by metered dose valve above the cords (Xylocaine delivering ~0.02mg in 0.1ml/spray) and Lidocaine 2% (2ml) above and below the cords were administered to reduce discomfort and facilitate the bronchoscopy. Trained, experienced clinical staff, facilities for resuscitation and drugs for reversal of sedation were available in accordance with guidelines[261]. Macroscopic appearances were examined and photographs taken before bronchoalveolar lavages (BAL) were obtained from the right middle lobe (medial segment) using 100 ml of normal saline. No Biopsies were taken.

2.5Immunological assays

2.5.1 Reagents

Table 2.4 ***Reagents used for immunological assays***

Reagent	***Supplier***
Anti CD49d and Anti CD28 antibodies	*Becton Dickinson UK Ltd*
85A peptides: Single pool of 66 15mer peptides, overlapping by 10 amino acids, spanning the length of antigen 85A	*Synthesized by Peptide Synthetics, UK*
Blocker Casein in PBS	*Pierce*
Blood collection tubes	*Becton Dickinson UK Ltd*
Bovine Serum Albumin	*Sigma Aldrich, UK*
Brefeldin	*Sigma Aldrich, UK*
Casyton	*Sedna Scientific, UK*
Cytofix/Cytoperm Fixation and permeabilisation kit™	*Becton Dickinson UK Ltd*
EDTA (Ethylenediaminetetraacetic acid)	*Gibco, Thermo Fisher Scientific, UK*
ELISPOT coating buffers	*Mabtech, UK*
ELISPOT substrate (5-Bromo-4-chloro-3-indoxyl phosphate/ Nitro blue tetrazolium solution)	*Europa Bioproducts, UK*
FACS lysing Solution	*Becton Dickinson UK Ltd*
Flow cytometry antibodies	*Various, see below Table 2.6*
Goat anti-human γ-chain whole IgG alkaline phosphatase conjugate	*Sigma Aldrich*
Goat anti-human α-chain whole IgA alkaline phosphatase conjugate	*Sigma Aldrich*
Goat anti-human u-chain whole IgM alkaline phosphatase conjugate	*Sigma Aldrich*

Reagent	***Supplier***
Goat anti-human ε-chain whole IgE alkaline phosphatase conjugate	*Sigma Aldrich*
Leukosep	*Greiner Bio-One, UK*
Lymphoprep	*Axis Shield, UK*
Modified Vaccinia Ankara virus, batch 1377	*Vector Core group,* *Jenner Institute, UK*
MVA peptides: Single pool of CD4 (27 14-21mer peptides) published epitopes[248]; and single pool of CD8 (36 9mer peptides) published epitopes [248]	*Synthesized by Peptide synthetics, UK according to published literature[248]*
Paraformaldehyde (PFA)	*Sigma Aldrich, UK*
Phosphate Buffered Saline (PBS)	*Sigma Aldrich, UK*
PPD	*Statens Serum Institut, Denmark*
Recombinant Ag 85A	*Lionex Diagnostics and Therapeutics GmbH, Germany*
RPMI cell media	*Sigma Aldrich, UK*
Sodium Azide	*Sigma Aldrich, UK*
5x Diethanolamine (DEA)	*Fisher*
4- nitrophenylphosphate tablet (4-npp)	*Sigma Aldrich*

The following reagents were prepared according to recipes below: "R10 media" was prepared by mixing RPMI, Foetal Calf Serum 10%, Penicillin/Streptomycin 1%, Glutamine 1% and Sodium Pyruvate1%. "FACS buffer" referred to in this chapter was made up from 0.1% BSA (Sigma Aldrich), 0.01% Sodium azide (Sigma Aldrich) in 1l of PBS. "Perm/wash buffer" referred to in this chapter was prepared by diluting BD Perm/Wash™ 1 in 10 in dH_2O to give a 1x working concentration. "FACS Lysing Solution" was used diluted 1 in 10 in dH_2O to give a 1x working concentration. "1% paraformaldehyde" was prepared from 6.25mL 16% stock solution of PFA diluted in 100mL PBS.

2.5.2 Peripheral blood mononuclear cells isolation

For Peripheral Blood Mononuclear cell (PBMC) isolation, blood samples were drawn into lithium heparin BD vacutainers™ (Becton Dickinson) and processed within 4 hours. PBMC were separated using Leucosep tubes (Greiner); up to 30ml of whole blood was added to each Leucosep tube which was pre-filled with 15 ml Lymhoprep (Axis Shield); samples were then centrifuged at 1000xg for 13 minutes at room temperature without brake. PBMC were collected from the interface with a Pasteur Pipette, transferred to a new 50ml Falcon tube and washed twice with R0 media (RPMI, Penicillin/Streptomycin1%, L-glutamine 1%). Cells were counted using a Casy counter (Sedna Scientific) with cell size set for human PBMCs (5.75-15µm). Concentration of cells was adjusted for *ex-vivo* IFNγ ELISPOT and the remaining cells were cryopreserved for later immunological analysis.

2.5.3 Serum harvesting

Coagulated blood obtained in red BD vacutainers™ (Becton Dickinson) was centrifuged at 1800rpm at room temperature with brake for 5 minutes. Serum was collected and frozen directly at -20˚C (long term storage at -80˚C).

2.5.4 *Ex-vivo* IFN-γ Enzyme-Linked ImmunoSpot

Enzyme-Linked ImmunoSpot (ELISPOT) plates (Millipore) were coated with anti-IFNγ capture antibody at 15µg/ml in 0.05M carbonate-bicarbonate buffer and incubated for 1-3 days at 4˚C. On the day of the assay, plates were washed with Phosphate Buffered Saline (Sigma Aldrich) and blocked with R10 media for 2-5 hours at 37˚C. Cell suspensions were prepared in R10 media. PBMCs were added in a cell suspension of $0.1x10^6$ and $0.3x10^6$ PBMC/80 µl/well in triplicates. On the day of the expected peak

effector response (day 7) further triplicates of a cell suspension concentration at 0.05x10^6 PBMC/80 µl/well were also included. The following antigens and R10 (as negative control for the assay) were added (20 µl/well):

Table 2.5 ***Antigens used for IFNγ ELISPOT Assay.*** *PBMC were stimulated with these antigens (triplicate wells per antigen) for 18-20 hours.*

Antigen	*Detail*	*Concentration*
Ag85A	*Single pool of 66 15mer peptides, overlapping by 10 amino acids, spanning the length of antigen 85A*	*2 µg per ml*
PPD	*Tuberculin purified protein derivative*	*20 µg per ml*
MVA CD4	*Single pool of CD4 (27 14-21mer peptides) published epitopes*[248]	*2 µg per ml*
MVA CD8	*Single pool of CD8 (36 9mer peptides) published epitopes* [248]	*2 µg per ml*
ESAT-6	*Single pool of 17 15mer peptides overlapping by 10 amino acids*	*10 µg per ml*
CFP-10	*Single pool of 18 15mer peptides overlapping by 10 amino acids*	*10 µg per ml*
SEB	*Staphylococcal Enterotoxin B, positive control*	*2 µg per ml*
R10 media	*R10 media, negative control*	*As detailed above*

The prepared plate with PBMC and stimulating antigens was incubated for 18-20 hours at 37˚C. Plates were then washed with 0.05% PBS Tween 20 (Sigma Aldrich). Secondary antibody (1mg/ml biotin anti-IFNγ antibody, Mabtech) diluted 1:1000 in PBS was added and washed off following 2 hour incubation at room temperature. Next, Streptavidin conjugated to Alkaline Phosphatase (Mabtech) diluted 1:1000 in PBS was added and incubated for 1 hour at room temperature. Plates were washed prior to the addition of Developing Buffer containing substrate (5-Bromo-4-chloro-3-indoxyl

phosphate/Nitro blue tetrazolium solution, Europa Bioproducts). The substrate reaction was terminated after 5 minutes by washing with tap water. Plates were dried overnight, read using AID ELISpot reader (Germany), and then stored protected from light and humidity.

2.5.5 Analysis of IFN-γ ELISPOT

Each Elispot plate was calibrated on the ELISPOT reader (Autoimmun Diagnostika) prior to reading. Quality control was performed on each well and variability between triplicates had to be less than 50% in a total of 90% of all wells. Each well was examined for artefacts. Results were expressed in spot forming cells (SFC) per million PBMC with background responses in negative control wells subtracted. Less than 20 spots were accepted in negative control wells. Positive control wells (SEB) were considered positive and passed the quality control criteria if there were at least 200 spots per well. The spots in the positive control wells were estimated at 500 spots per million as there were consistently too many spots to count (blackout). More than 20 spots per million PBMC in ESAT-6 and/or CFP-10 pools were considered to be indicative of LTBI.

2.5.6 Whole blood stimulation

Whole blood (WB) was added to vials (500 µl) prepared with co-stimulant cocktail of Anti-CD49d and Anti-CD28 (Becton Dickinson) at 0.5µg/ml each and antigen at 2µg/ml (85A peptide pool, MVA CD4 and MVA CD8 peptides[248]). An unstimulated negative control and Staphylococcal Enterotoxin B (SEB) at 5µg/ml (Sigma Aldrich Aldrich) as a positive control were also included. Samples were incubated at 37◦C for 5-6 hours. Then Brefeldin A (Sigma Aldrich) was added at 3 µg/ml, and samples incubated in a

timed waterbath at 37◦C for 5 hours. Next, 2mM EDTA (Gibco) was added. Cells were lysed with FACS lysing solution (BD Biosciences), washed and cryopreserved for later batched surface and intracellular staining and analysis.

2.5.7 WB: surface and intracellular cytokine staining

Stimulated whole blood was thawed in batches to process samples from all timepoints of one volunteer in one run. Samples were centrifuged in a microfuge at 6000 rpm for 5 minutes, supernatant discarded, and samples transferred to plates for staining. WB samples were stained using two flow cytometry antibody panels (Table 2.6). The first panel was aimed at detecting Th1 cells and cytokines[247], while the second one was developed to investigate a possible role of granulocytes or inflammatory monocytes in the adverse reactions detected in Group 2 (Exploratory Group 2 Panel).

For the Th1 panel, samples were permeabilised with BD Cytofix/Cytoperm™ fixation/permeabilisation solution and washed again with BD Perm/Wash™ Buffer before addition of ICS antibody mix for 30 minutes at 4 ◦C. Samples were washed with BD Perm/Wash™ Buffer before being resuspended in 100 μL FACS buffer 1% paraformaldehyde and transferred to cluster tubes for acquisition. For the Exploratory Group 2 panel, unstimulated WB samples were surface stained with the antibody mix for 30 minutes at 4 ◦C before washing twice with FACS buffer and then resuspended in 100 μL FACS buffer 1% paraformaldehyde for acquisition. Fluorescence-minus-one (FMO) gating controls were included in this panel as deemed necessary from optimisation experiments during panel development. Compensation controls were prepared separately for each antibody using compensation beads according to manufacturer`s instruction. All samples were acquired on BD LSRII flow cytometer

using BD FACSDiva software. Compensation corrections for spectral overlap were calculated using BD FACSDiva software.

Table 2.6 ***Th1 (Top) and Exploratory Group 2 (bottom) flow cytometry panels.*** *From left to right: markers and fluorochromes, clone, source and volume of antibody used per test.*

Marker	***Clone***	***Source***	***µL / test***
Th1 panel antibodies and fluorochromes			
CD14 ECD	*RMO52*	*Beckman Coulter*	*5*
CD19 ECD	*J3-119*	*Beckman Coulter*	*5*
CD4 Pacific Blue	*RPA-T4*	*Biolegend*	*1*
CD3 AF-700	*UCHT1*	*EBioscience*	*0.5*
CD8 APC-H7	*SK1*	*Becton Dickinson*	*2*
IFNγ PE-Cy7	*4S.B3*	*EBioscience*	*0.25*
IL-2 PE	*N7.48A*	*Beckman Coulter*	*2*
TNFα AF-647	*MAb11*	*Biolegend*	*1*
IL-17 AF-488	*BL168*	*Biolegend*	*4*
Exploratory Group 2 panel antibodies and fluorochromes			
CD3 BV510	*UCHT1*	*Becton Dickinson*	*1*
CD56 AF700	*HCD56*	*Biolegend*	*2*
CD14 efluor450	*61D3*	*Biolegend*	*2*
CD11b PE-Cy7	*ICRF44*	*Biolegend*	*0.25*
CD16 APC-Cy7	*B73.1*	*Biolegend*	*2*
CD66b Percpcy5.5	*G10FS*	*Biolegend*	*2*
CD15 FITC	*HI98*	*Biolegend*	*3*
CCR3 (CD193) AF647	*5E8*	*Biolegend*	*5*
Siglec-8 PE	*7C9*	*Miltenyi Biotech*	*4*

2.5.8 Bronchoalveolar lavage processing

Bronchoalveolar lavage (BAL) samples were transported to the laboratory at ambient temperature and processed within 2 hours of harvest. BAL samples received ranged between 28ml and 61ml. Samples were centrifuged at 400g for 10 minutes. Supernatant was cryopreserved. Cell pellets were pooled and resuspended in R10 media and counted. Stimulation of BAL cells was performed in 5ml round bottom polystyrene tubes (Falcon), with $1x10^6$ BAL cells per condition: Ag85A (2 µg/ml), PPD (20 µg/ml), MVA CD4 and MVA CD8 peptides (2 µg/ml), SEB (positive control, 5 µg/ml) or left unstimulated as negative control for the assay. Co-stimulatory antibodies, anti-CD28 and anti-CD49d (BD) at 1µg/ml each were added to all samples. After a 2 hour incubation in 5%CO2 at 37 ^oC, 3µg/ml of Brefeldin A (Sigma Aldrich) was added, the samples incubated overnight and stained the following morning.

2.5.9 BAL: cell surface and intracellular cytokine staining

BAL cells were first surface stained for viability (Live/Dead Stain, Invitrogen) for 10 minutes at 4 ^0C, then, samples were incubated with surface staining antibody mix of antibodies to CD4, CD14 and CD19 for 30 minutes at 4 ◦C. Next, samples were washed twice with FACS buffer, permeabilised with BD Cytofix/Cytoperm™ fixation/permeabilisation solution and washed again with BD Perm/Wash™ Buffer before addition of intracellular cytokine staining (ICS) antibody mix containing antibodies to CD3, CD8, IFNγ, TNFα, IL-2 and IL-17. Cells were fixed with 1% paraformaldehyde for immediate acquisition on the BD LSRII flow cytometer using BD FACSDiva software.

2.5.10 Analysis of flow cytometry data (WB and BAL)

All flow cytometry data was analysed on FlowJo (Treestar, Inc., USA) V8 for BAL data or V9 for all other data.

For Th1 flow cytometry panel in whole blood and BAL the following gating strategy was applied (Figure 2.2). First, lymphocytes were gated on a forward scatter area (FSC-A) *vs.* side scatter (SSC) plot. Next, duplets were excluded on a forward scatter height (FSC-H) *vs.* FSC-A. Also, CD14+ and CD19+ cells were excluded. For BAL samples dead cells were excluded as well. Then within CD3+ lymphocytes, CD4+ and CD8+ subsets were determined. Presented are percentages of cytokine positive CD4+ and CD8+ T cells (for IFNγ, TNFα, IL-2 and IL-17) with background (i.e. responses in unstimulated cells) subtracted.

For the Exploratory Group 2 flow cytometry panel in whole blood the following gating strategy was applied (Figure 2.3). First white blood cells were gated on a forward scatter area FSC-A *vs.* SSC plot. Next, duplets were excluded on a FSC-H *vs.* FSC-A; then CD3+ and CD56+ positive cells were excluded. Within the remaining population, CD16+ and CD16- were gated against FSC-H[262, 263]. Subsets of CCR3+ or CD11b+ populations were gated within CD16+ and CD16-[262-264]. CD16+ CD66b+ and CD16-Siglec8+ populations were gated (gates not shown)[262-264]. Quadrants were gated for CD16/CD14 to determine CD16+CD14+ inflammatory monocytes[265]. Gates for CD16, CD56, CCR3 and Siglec-8 gates were ascertained using FMO controls that excluded these markers. Other markers were found not to require FMO controls during panel development. Presented are percentage of the respective cell subsets.

Figure 2.2 ***Gating Strategy for WB ICS Th1 Panel****. 1st row from left to right: Gating of Lymphocytes on FSC-A vs. SSC; Singlets FSC-A vs. FSC-H, and CD3+.T cells. 2nd row: CD4+ and CD8+ T cells; Cytokines: TNFα (T) vs. IFN-γ (G), IL-17 and IL-2 in CD4+(3rd row) & CD8+ (4th row) T cells.*

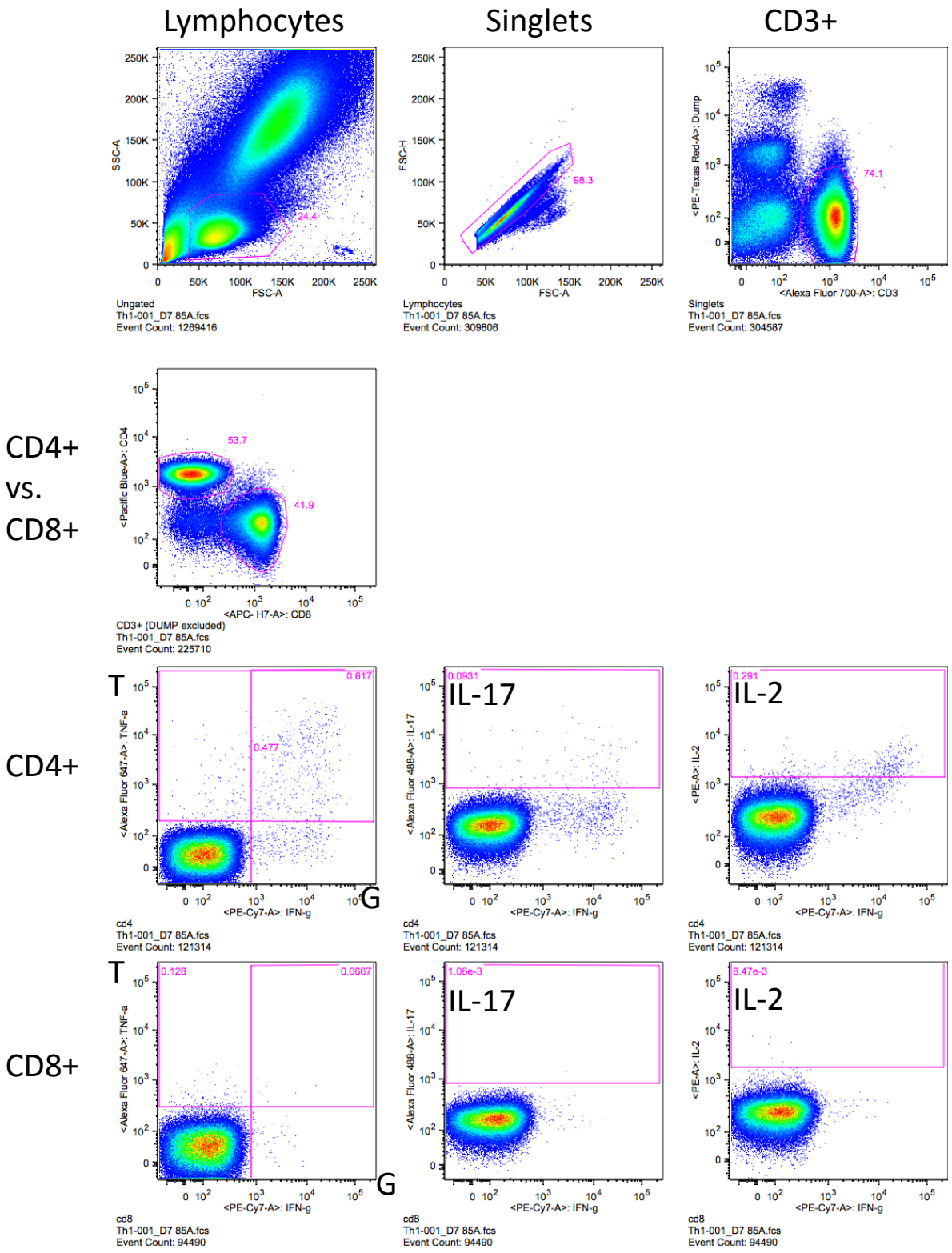
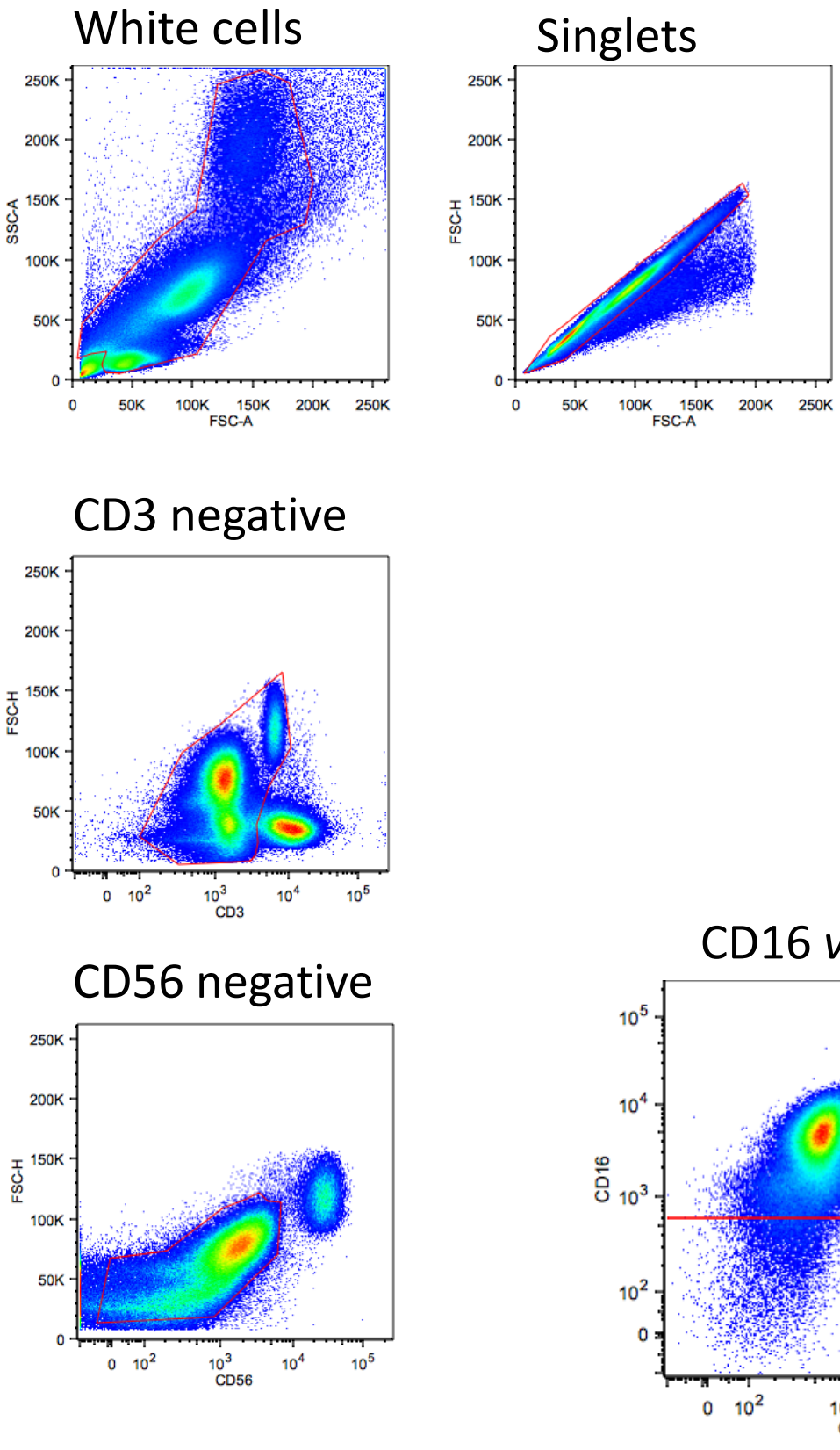


Figure 2.3 Abbreviated ***Gating Strategy for WB ICS Exploratory panel****. 1st row from left to right: Gating of White cells on FSC-A vs. SSC; Singlets FSC-A vs. FSC-H,2nd row: CD3 negative cells, 4th row from left to right: CD56 negative cells, CD16 vs. CD14.*



2.5.11 Enzyme-Linked Immunosorbent Assay

Insert- and vector-specific humoral antibody (IgG, IgA and IgM) responses were assessed by Enzyme-Linked-Immunosorbent-Assay (ELISA) in serum and BAL fluid samples in triplicates. Nunc Immunoplates were coated with 50 µl per well of r85A (2ug/ml, Lionex) or non-recombinant MVA ($5x10^5$ pfu/well, Virus Vector Core Group, Jenner Institute, Oxford) and incubated overnight for adsorption. The next morning samples were prepared by diluting test serum 1:10 in Casein blocking buffer (Sigma Aldrich). Coating solution was discarded, plates washed 6 times with PBS/Tween and blocked with 200ul of Casein per well for 1 hour at room temperature. Blocking solution was discarded. Samples were added in triplicates at 50µl per well and incubated for 2 hours at room temperature. Serum samples of all 7 timepoints from 4 volunteers were processed on the same plate. Blank wells, positive and negative control samples were included. Contents were discarded and plates washed 6 times with PBS/Tween. 50µl of 1/100 diluted goat anti-human secondary antibody conjugated to alkaline phosphatase (Sigma Aldrich) was added and incubated for 1 hour. Plates were washed with PBS/Tween and substrate: 4-npp in DEA buffer was added (Sigma Aldrich). Optical Density (OD) was read at 405 nm absorbance. Data presented are OD values with background subtracted. Acceptable threshold for background was an OD value of <0.15 at 405nm absorbance in blank wells.

2.6 Statistical analysis

All data management and analysis was performed on Excel 2013 and GraphPad Prism Software 6. Box-and-Whisker-Plots were used to summarise the data; where boxes indicate the interquartile range between 25th to 75th percentiles, the line within the box the median and the whiskers the minimum and maximum values. Non-parametric tests were applied as the data was not normally distributed on normality testing. Within each group paired data was analysed using Wilcoxon matched pairs test for comparison of 2 sets of paired data or Friedman`s test for comparison of 3 or more paired data sets. To compare unpaired datasets between groups, a non-parametric Mann Whitney test was used for comparison of 2 data sets and Kruskal Wallis test was used for comparison of 3 or more data sets. Dunn`s multiple comparison test was used for *post hoc* corrections where applicable. Differences between medians between timepoints or groups were presented along with their p-value. Associations were tested using Spearman's non-parametric rank correlation and expressed indicating correlation efficient r. The significance level was assumed to be $p<0.05$ for all of the above tests with a 95% confidence interval. An area-under-curve analysis of deviation from baseline was used to compare the overall spirometry values between the three study groups in the time period following vaccination (negative peak area only) and the Ag85A-specific ELISPOT responses. These analysis made use of data collected at all time points.

3 Results: Clinical

3.1Introduction

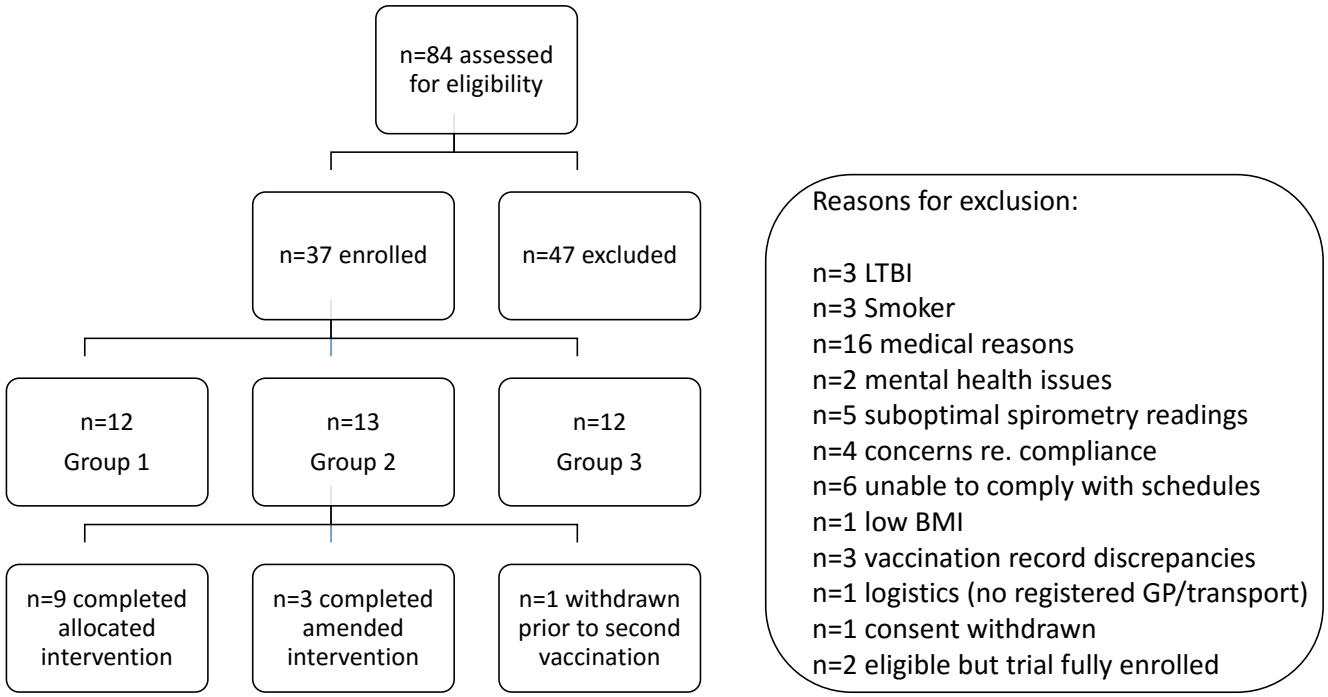
This trial was designed to deliver a candidate TB vaccine MVA85A to two body compartments, systemic (intradermal) and lung mucosal (aerosol), using alternating routes of vaccine delivery, in succession. The aim was to induce and potentially synergise a vaccine response to insert Ag85A in both compartments. Details of the trial design and conduct are outlined in Chapter 2: Materials and Methods (p.56-62) and Appendix 1: Clinical Trial Protocol. A dose of $5x10^7$pfu MVA85A, was delivered by either inhaled aerosol or intradermal route as per group allocation: Group 1 received aerosol MVA85A followed by intradermal MVA85A one month later, Group 2 received intradermal MVA85A followed by aerosol MVA85A one month later, and Group 3 received MVA85A twice by the same (intradermal) route one month apart. In order to ensure volunteers were blinded to their route of vaccination, saline as paired placebo was administered concurrently by the alternative route at the vaccination visits. This paired placebo design also enabled differentiation between symptoms after inhalation *per se* compared to inhalation of MVA85A vaccine. All volunteers underwent a bronchoscopy with bronchoalveolar lavage (BAL) 7 days following each vaccination, i.e. day 7 and day 35; and blood was sampled at all visits (days 0, 2, 7, 14, 28, 30, 35, 42, 84 and 168).

3.2Volunteers

Enrolment commenced on the 4th December 2013 and was completed on the 26th August 2015. Eighty-four healthy volunteers were assessed for eligibility and 37 healthy previously BCG-vaccinated volunteers were enrolled into the trial. One

volunteer was withdrawn by the clinical team prior to the second vaccination due to concerns relating to a change in the volunteer`s circumstances and subsequent ability to comply with study requirements. This volunteer was replaced. Of those screened, 47 volunteers were excluded for reasons detailed in the CONSORT diagram below (Figure 3.1). Volunteers excluded with suspected latent *M.tuberculosis* infection (LTBI) were referred and followed up in the local NHS TB clinic for consideration of chemoprophylaxis. Volunteers with incidental medical findings were referred to the GP for further investigation and management, if necessary.

*Figure 3.1**CONSORT diagram***



3.3Demographics

Demographic details are summarised in Table 3.1. In total, 28 females and 9 males were enrolled with a median age of 25 years. 33 subjects were Caucasian and 4 were Asian. There was no significant difference in any of the demographic characteristics between the groups (p=0.7, corrected for multiple comparisons) and no difference in baseline lung function, as measured by percentage of predicted Forced Expiratory Volume in the first second (FEV1), Forced Vital Capacity (FVC) and SpO_2 (data not shown) between the 3 groups (p=0.3, corrected for multiple comparisons).

Table 3.1 ***Baseline demographic and respiratory health of volunteers.*** *Demographic information and baseline spirometry at screening.*

Characteristic	**Group 1 (n=12)**	**Group 2 (n=13)**	**Group 3 (n=12)**
Sex, n=female (%)	11 (92%)	11 (85%)	6 (50%)
Median age in years (range)	25 (22-42)	25 (20-37)	25 (21-30)
Ethnicity	10 Caucasian 2 Asian	12 Caucasian 1 Asian	11 Caucasian 1 Asian
Median time interval since BCG vaccination in years (range)	12(1-37)	13 (1-28)	12(1-29)
Median % of predicted FEV1	102	91.5	98
Median % of predicted FVC	101	97	96.5
Median baseline % SpO_2	97	98	98

3.4 Safety

3.4.1 Group 2 protocol amendment

Allocation of volunteers into the 3 trial groups followed the randomisation sequence until a safety concern in subjects enrolled into Group 2 arose in October 2014. Enrolment into Group 2 was temporarily paused, but enrolment into Groups 1 and 3 continued. At that point, 13 volunteers had been enrolled in total, of which 4 volunteers had been enrolled into Group 2. Of the 4 volunteers in Group 2, two volunteers (009 and 026) had experienced transient (24-48 hours) mild to moderate respiratory symptoms, severe (grade 3) systemic reactions and a transient reduction in pulmonary function following their aerosol boost vaccination on day 28 (Table 3.2). Both volunteers made a full recovery within 48-72 hours following vaccination and proceeded with their scheduled bronchoscopy on day 35 (7 days following their aerosol boost vaccination). Macroscopic appearances at bronchoscopy in these individuals were unremarkable.

Table 3.2 ***Summary of lung function in the first two affected Group 2 volunteers from day 28 (day of second vaccination) to day 35 (one week after second vaccination and day of bronchoscopy).*** *FEV1 and FVC in litres (L) for 009 (left) and 026 (right).*

Volunteer 009	**FEV1 in L**	**FVC in L**	**Volunteer 026**	**FEV1 in L**	**FVC in L**
D28	3.8	4.86	D28	3.47	3.9
D28 Evening	3.17	4.09	D28 Evening	3.42	4.03
D29 Morning	2.28	2.87	D29 Morning	3.11	3.73
D29 Evening	2.13	3.19	D29 Evening	3.22	3.8
D30 Morning	2.58	3.47	D30 Morning	3.05	3.51
D30	2.92	3.61	D30	3.48	4.02
D30 evening	3.46	4.27	D30 evening	3.23	3.79
D31 Evening	3.13	3.88	D31 Evening	3.19	3.73
D32 Evening	3.98	4.05	D32 Evening	3.26	3.8
D33 Evening	3.46	4.15	D33 Evening	3.3	3.79
D34 Evening	3.53	4.19	D34 Evening	3.33	3.76
D35	3.53	4.16	D35	3.39	3.99

Review of the clinical data for the two affected volunteers in Group 2 was compared with the data for the two unaffected volunteers in Group 2 and those enrolled in the other two groups. A more marked clinical history of atopy was noted in the two affected subjects (009 and 026). Although volunteers with a history of asthma, egg allergy and previous vaccine reactions were excluded by pre-defined criteria, a history of allergic rhinitis, eczema or in this context not relevant allergies, had not been an exclusion criterion to date (Appendix 1: Clinical Trial Protocol). Review of the available immunology data did not reveal anything to predict this pattern of clinical response. The safety and immunology data was discussed with the local safety monitor and a consensus was reached to exclude all individuals with a history of atopy going forward. In January 2015 the decision was made to cautiously restart enrolment into Group 2. The decision to restart enrolment into Group 2 was made on the basis that continued

enrolment into the other two groups had shown no unexpected adverse events and the eligibility criteria had been made more stringent excluding any significant clinical history of atopy. The first 2 volunteers in Group 2 after the pause were enrolled one at a time, with the second volunteer only enrolling after completion of both vaccinations in the preceding Group 2 volunteer, and subsequent favourable safety review. Once the safety review was completed following the second vaccination on the second volunteer, with a favourable outcome, progress was then made to complete enrolment of Group 2.

The first vaccination (day 0) of the first volunteer enrolled into Group 2 following the pause (062) was uneventful. Following the day 28 aerosol boost vaccination, she reported mild systemic adverse events in the first two days following vaccination but she did not record any cough, wheeze or shortness of breath in her diary card during that period. At her day 30 review visit (2 days following aerosol boost vaccination) she reported some degree of discomfort in her throat with possible sensation of restricted breathing. In her diary card she documented a transient dip in self-recorded spirometry on the morning following her vaccination (day 29 a.m.) which recovered by the evening (Table 3.3). Her formal lung function, done at the Oxford Centre for Respiratory Medicine on day 30, confirmed baseline lung function parameters. Chest radiograph was unremarkable and CRP was 64.3mg/L (normal range 0-5 mg/L).

Table 3.3 ***Summary of spirometry for first volunteer enrolled into Group 2 after the pause.*** *FEV1 and FVC in litres (L) for volunteer 062*

Volunteer 062	**FEV1 in L**	**FVC in L**	**Oxford Centre for Respiratory Medicine**		
D28	3.61	3.71			
D28 Evening	3.44	3.49			
D29 Morning	2.76	2.76			
D29 Evening	3.32	3.33			
D30 Morning	3.46	3.67	FEV1 in L	FVC in L	
D30	3.44	3.47	3.55	3.85	
D30 evening	3.32	3.47			
D31 Evening	3.44	3.49			
D32 Evening	3.62	3.7			
D33 Evening	3.66	3.69			
D34 Evening	3.52	3.79			
D35	3.45	3.63			

As adverse events in this volunteer were within the range of the expected profile following MVA85A vaccination, and systemic symptoms were the predominant clinical feature, the decision was made to enrol the next Group 2 volunteer. All subsequent volunteers underwent formal pre-vaccination pulmonary function tests at the Oxford Centre for Respiratory Medicine in order to verify spirometry readings from the handheld spirometer and provide optimal baseline readings. Volunteer (073) was the next volunteer enrolled and the first vaccination in this volunteer proceeded uneventfully. At the day 30 visit (2 days following aerosol boost vaccination), the volunteer reported having experienced moderate (grade 2) systemic symptoms (myalgia, arthralgia and headache), a sore throat and chest tightness (grade 2) in the 48 hours following vaccination but denied cough, shortness of breath or wheeze at any point. The volunteer also reported reduced exercise tolerance on cycling to clinic limited by constitutional symptoms and chest discomfort. On assessment in clinic on

day 30, the volunteer described a full collection of systemic adverse events at grade 1 and also grade 1 chest discomfort. Physical examination including examination of the respiratory system was unremarkable. Spirometry and carbon monoxide diffusing capacity obtained at the Oxford Centre for Respiratory Medicine were normal (no change from baseline) and in keeping with readings from the handheld device (data not shown). CRP was 8.3mg/L (normal range 0-5 mg/L). As the symptoms following aerosol boost vaccination remained within the range of expected adverse events and were predominantly systemic in nature, the decision was made to continue enrolment into Group 2. Subsequently, three further volunteers were enrolled into Group 2 on the same day (063, 070 and 072), as per amended protocol; and remaining volunteers within the following two weeks (074, 077, 081). One of those volunteers enrolled into Group 2 (072) reported chest tightness associated with shortness of breath on the day of aerosol vaccination which had improved by the following morning. On review in clinic (day 29, 1 day following boost aerosol vaccination) she reported grade 3 fatigue, grade 1 shortness of breath and some grade 1 systemic symptoms. Her spirometry values are summarised in Table 3.4. She denied a cough and had no evidence of fever recorded. Chest radiograph was normal and CRP 57.1mg/L (normal range 0-5 mg/L). The other two Group 2 volunteers (063 and 070) were reviewed at their day 30 scheduled visits the following day. They reported predominantly systemic symptoms the preceding day (day 29 of their trial schedule), and were both noted to have recorded a transient dip in their self-recorded spirometry (Table 3.4).

Table 3.4 ***Summary of spirometry of the last 3 volunteers in Group 2.*** *FEV1 and FVC in litres (L)*

	Volunteer 063		Volunteer 070		Volunteer 072	
	FEV1 in L	FVC in L	FEV1 in L	FVC in L	FEV1 in L	FVC in L
D28	3.23	3.68	3.4	3.53	2.58	3.11
D28 Evening	3.13	3.6	3.09	3.3	2.25	2.92
D29 Morning	2.7	3.32	2.7	2.84	2.08	2.79
D29 Evening	2.73	3.4	2.54	2.78	2.21	2.85
D30 Morning	3.14	3.5	2.7	3.26	2.31	3.05
D30	3.16	3.74	2.74	3.51	2.25	2.88
D30 evening	3.2	3.56	2.67	3.29	2.29	2.8
D31 Evening	3.16	3.62	2.97	3.56	2.41	3.02
D32 Evening	3.14	3.64	3.11	3.55	2.42	3.1
D33 Evening	3.14	3.56	2.98	3.53	2.32	2.8
D34 Evening	3.28	3.7	3.13	3.71	2.34	2.9
D35	3.28	3.79	3.24	3.73	2.52	3.04

At this stage, enrolment into Group 2 was completed. The last 3 volunteers had received their day 0 intradermal prime vaccination and were scheduled to receive their day 28 aerosol boost. Due to the ongoing respiratory symptoms observed in Group 2, a decision was made not to proceed with aerosol boost vaccinations in these remaining three volunteers, but instead to administer a double placebo (saline by intradermal and aerosol routes) on day 28 in *lieu* of vaccination, and proceed with bronchoscopy on day 35 with the prospect of gaining data on macroscopic appearance and level of lung mucosal immunity measured in BAL 35 days after intradermal prime vaccination. Volunteers who had not completed their vaccinations at that stage were

individually consented for this amendment but remained blinded to their individual group allocation.

3.4.2 Vaccination adverse events

There were no serious adverse events in this trial. Adverse events were collected as outlined in Chapter 2: Materials and Methods (p.67-71). Briefly, adverse events were collected actively at scheduled trial visits and passively from diary cards for 2 weeks following each vaccination. Data collected included solicited (i.e. part of a uniform set of questions asked in diary card or at clinic visits) or unsolicited (i.e. information of any symptom not solicited) adverse events. Adverse events experienced after prime and boost vaccinations (day 0 and day 28 respectively) were analysed in three categories: local, respiratory and systemic.

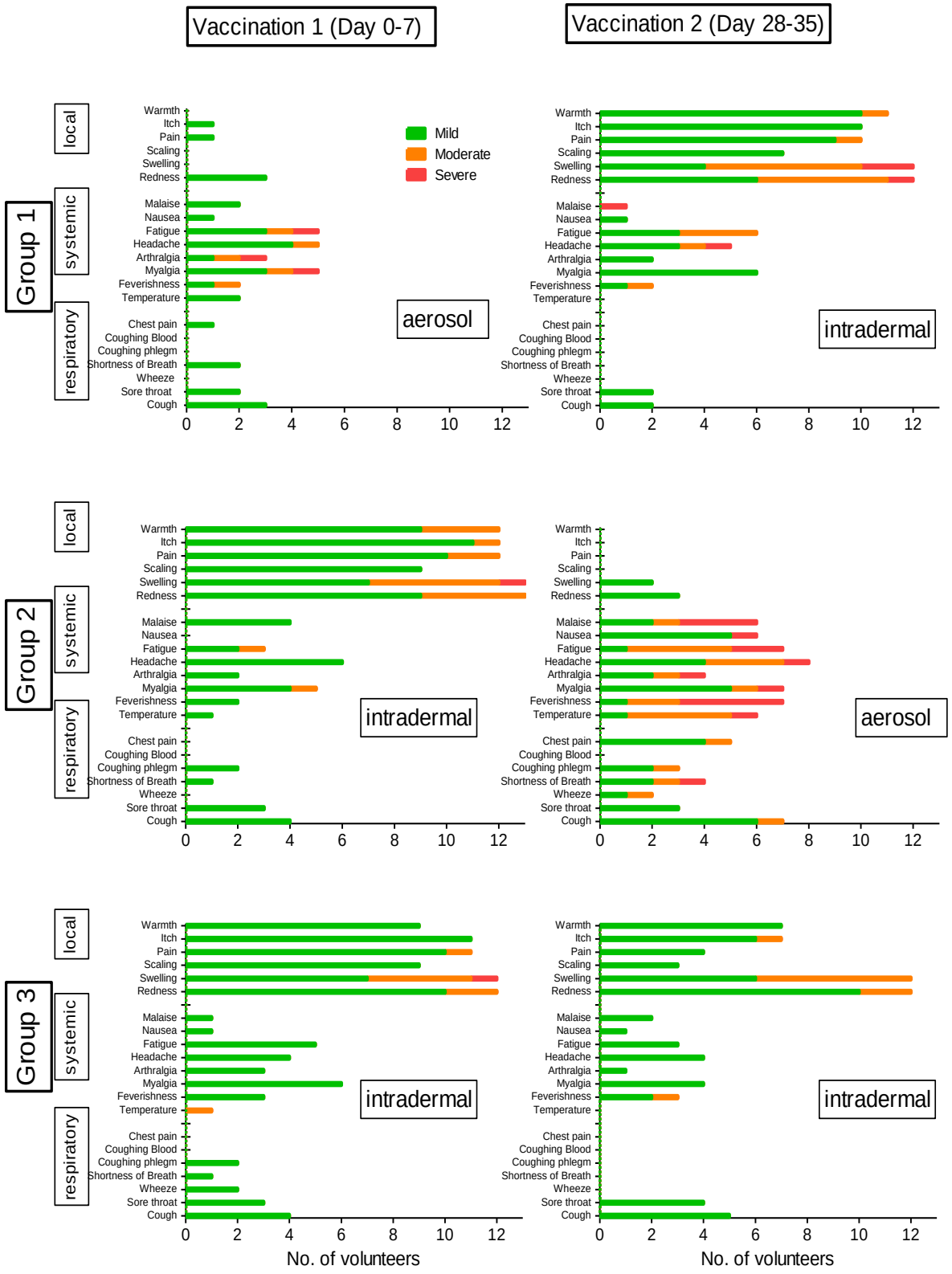
The 4 volunteers who did not complete the original trial regimen (n=1 withdrawn, n=3 urgent safety measure) were included in the safety analysis (intention-to-treat).

Adverse events were calculated as the number of volunteers recorded to have experienced a solicited adverse event once or more at the peak severity grading of the event in the 7 day period following vaccination (i.e. day 0 to day 7 following prime vaccination and day 28 to day 35 following boost vaccination, Figure 3.2). The most frequently occurring were local reactions at the intradermal MVA85A injection site occurring mostly in Group 1 following intradermal day 28 boost, in Group 2 following intradermal day 0 prime and in Group 3 following both day 0 and day 28 intradermal prime and boost vaccinations. These local adverse events made up 58% of all adverse events in the 7 day period following the first vaccination and 50% following the second vaccination. The majority of these were mild with 79% and 78% of all local adverse

events classed as grade 1 for first and second vaccination, respectively. Respiratory adverse events made up 12% and 16% of all adverse events following first and second vaccination, respectively. There was no significant difference in the frequency of adverse events between any of the groups at any timepoint (p=0.37, corrected for multiple comparisons). However, 65% of all recorded respiratory adverse events in the day 28 to day 35 period were reported in Group 2 volunteers. All respiratory adverse events following the day 0 prime vaccination were mild (grade 1 severity). However, following the aerosol boost vaccination 21% of all respiratory adverse events were graded as moderate and 4% severe. These were exclusive to Group 2 volunteers following their day 28 vaccination.

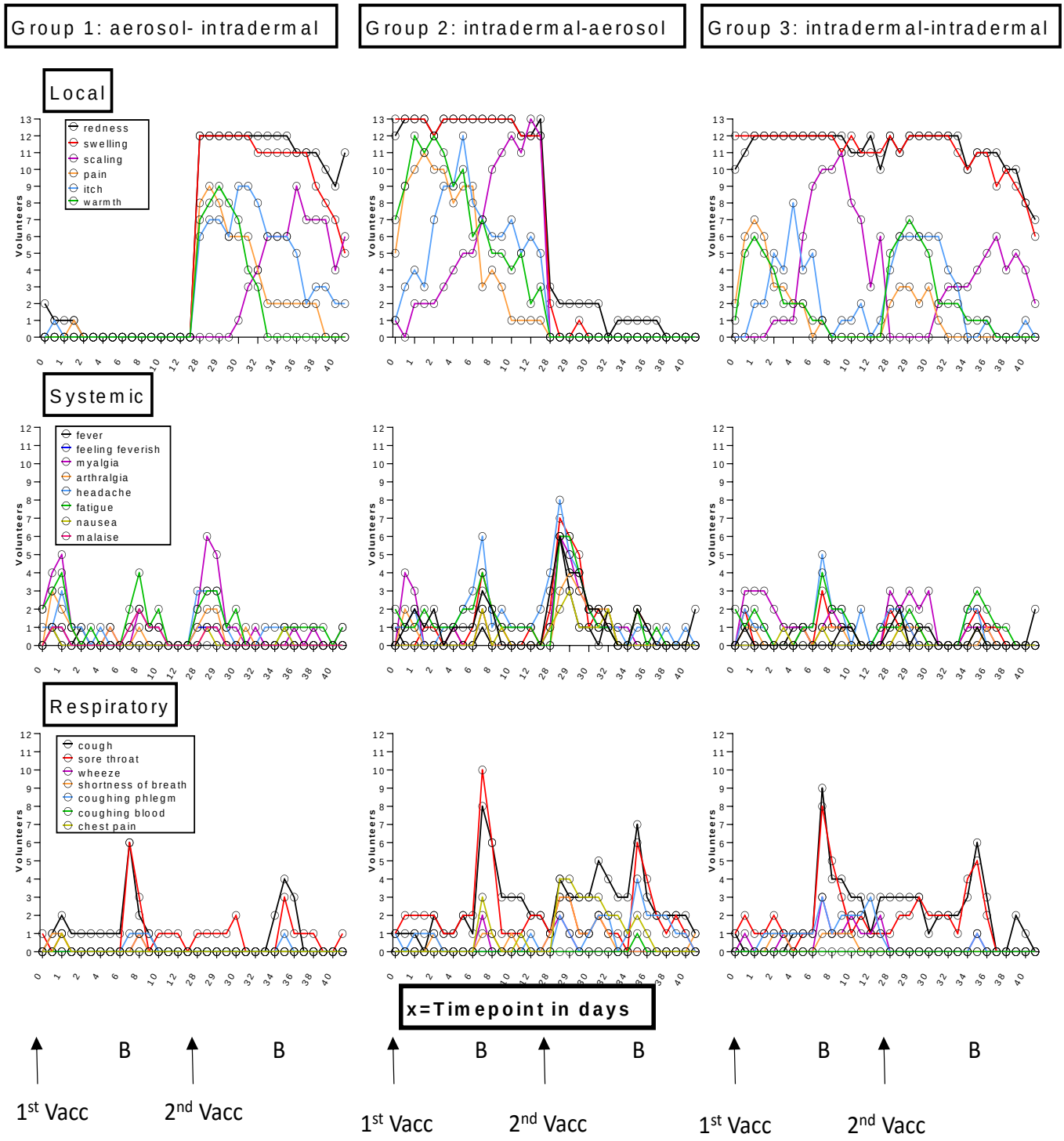
Systemic adverse events were comparable between the groups with no significant differences between the groups at any timepoint (p=0.37, corrected for multiple comparisons). However systemic adverse events were most frequent following aerosol boost vaccination in Group 2, with 67% of all systemic adverse events in the period between day 28 to day 35 attributable to Group 2 volunteers. Severity grading of systemic adverse events overall was higher following their day 28 aerosol boost vaccination compared to the day 0 prime vaccination with 50% and 15% of all systemic adverse events being graded as moderate (grade 2) or severe (grade 3). The majority of moderate and severe systemic adverse events occurred in Group 2 following their day 28 aerosol boost vaccination, with 39% of all systemic adverse events being grade 2 or 3 in Group 2 in that period. Overall, respiratory adverse events were more frequent and more severe, and systemic adverse events more severe in Group 2 following day 28 aerosol boost compared to the other two groups, although these differences did not reach statistical significance.

Figure 3.2 ***Frequency of solicited adverse events experienced at least once; recorded at peak severity grading in the 7 days following each vaccination.*** *Red= severe, yellow=moderate, green=mild. X=no. of volunteers, y=symptoms*



Next, all three categories of adverse events were analysed by timeline from day 0 through to day 42. Shown in Figure 3.3 are the total frequency of solicited adverse events in this period. Occurrence of local adverse events coincided with local intradermal injection (Group 1 day 28 boost, Group 2 day 0 prime, Group 3 day 0 prime and day 28 boost). Respiratory adverse events predominantly occurred on the day of bronchoscopy or the day after; and also in Group 2 in the 4 day period following their aerosol boost vaccination (day 28 to day 32). Systemic adverse events occurred throughout the trial period following vaccination by either route.

Figure 3.3 ***Local, systemic and respiratory adverse events experienced by volunteers between day 0 and day 42 of the trial period by group*** *(x= timeline in days, B= bronchoscopy days, arrows indicating vaccination days; y=Number of volunteers experiencing the adverse events per timepoint)*



Adverse events were also analysed by individuals in the 7 days following vaccinations (i.e. day 0 to day 7 and day 28 to day 35; Figure 3.4). This analysis demonstrates the overall pattern of adverse events in the different individuals and groups; and highlights the affected Group 2 individuals. The figure illustrates the mild local adverse events following intradermal vaccination. With the exception of Group 2 aerosol boost vaccination, the pattern of respiratory and systemic adverse events was comparable.

In all the above analyses, data elicited at scheduled visits and from diary cards were combined. Severity grading was performed by volunteers themselves as per guidance provided to them at enrolment (Chapter 2: Materials and Methods, p.69-70 and Appendix 2: Diary card). All severity gradings were reviewed and confirmed by clinical staff at the scheduled trial visits.

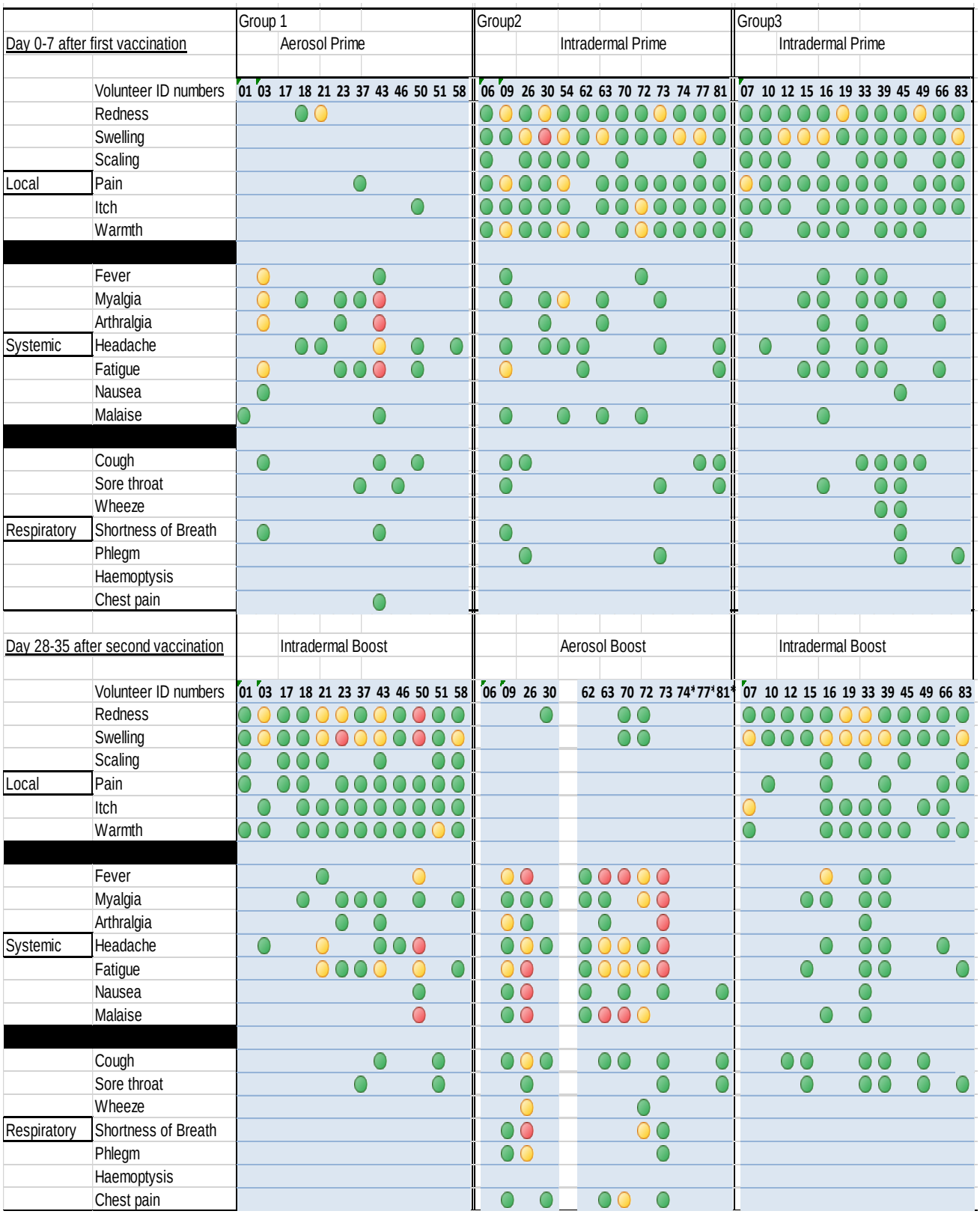
Detailed data summarising diameter of swelling and erythema from intradermal injection is not presented here as this was as expected and in keeping with previous published studies on MVA85A administered intradermally. The median diameter of the highest recorded erythema in the 28 days after placebo intradermal injection of saline was 0mm in Group 1 (range 0-12mm) and 1mm in Group 2 (range 0-2mm). For intradermal vaccination with MVA85A this was 12mm in Group 1(range 9-12mm), 13mm in Group 2(range 3-13mm) and 12mm and 11mm in Group 3 for first and second vaccination respectively (ranges 10-12mm after first and 7-12mm after second vaccination). In terms of erythema, the median diameter recorded after placebo intradermal injection of saline was 0mm (range 0-12mm) in Group 1 and 0mm (range 0-1mm) in Group 2. For intradermal vaccination with MVA85A this was 11mm in Group 1(range 5-12mm), 13mm in Group 2 (2-13mm), and 12mm and 11m in Group 3 after

first and second vaccination respectively (ranges 11-12mm after first and 6-12mm after second vaccination).

There was no difference between the groups following intradermal MVA85A given as prime (Group 2), boost (Group 1) or both (Group 3; data not shown).

Local and systemic adverse events following intradermal MVA85A as a prime or boost vaccination were in keeping with data published from previous clinical trials with intradermal MVA85A[130, 146, 197, 205, 247, 248, 266].

Figure 3.4. ***Adverse events by Individuals in Groups.*** *Local, systemic and respiratory adverse events 7 days after each vaccination. Severity: green=mild, yellow=moderate, red=severe*



3.4.3 Lung function

Pulmonary function was monitored throughout the 14-day period following each vaccination using a handheld spirometer (Chapter 2: Materials and Methods, p.61). Volunteers were shown how to use the handheld spirometer at the screening visit with formal training at the enrolment visit prior to vaccination. The technique was then monitored at all subsequent scheduled visits.

The forced expiratory volume in the first second (FEV1) is the maximal volume of air that can be exhaled in the first second of forced expiration after maximal inspiration. The forced vital capacity (FVC) is the total volume of air that can be forcibly exhaled following maximal inspiration. FEV1 and FVC were interpreted as percentage of predicted, adjusted for age, height and sex, at baseline and throughout the trial. Shown here are FEV1 and FVC analysed as negative or positive percentage change from that volunteer`s baseline to allow comparison between volunteers. Baseline was considered to be the pre-vaccination values on day 0 (Figures 3.5- 3.7). FEV1 and FVC at baseline (day 0) was comparable between groups (p=0.3147, corrected for multiple comparisons). This summary includes measurements recorded by volunteers themselves at home in their diary cards and recordings obtained at clinic visits. The only marked deviation from baseline is noted in Group 2 volunteers in the 7 day period following their aerosol boost vaccination (day 28- day 35). All volunteers made a full recovery in lung function within 24-72 hours following their day 28 aerosol boost vaccination, and all were eligible to proceed with bronchoscopy on day 35.

Figure 3.5 **Group 1:** ***Percentage change from baseline Forced Expiratory Volume in the 1st second (y=FEV1% change, top row) and Forced Vital Capacity (y=FVC % change, bottom row) over the 14 day period following each vaccination*** *(left column: x=days 0 to 14; right column: x=days 28 to 42). Each circle represents percentage change from baseline of an individual volunteer`s FEV1 or FVC. Recordings at scheduled visits and recordings from diary cards are included.*

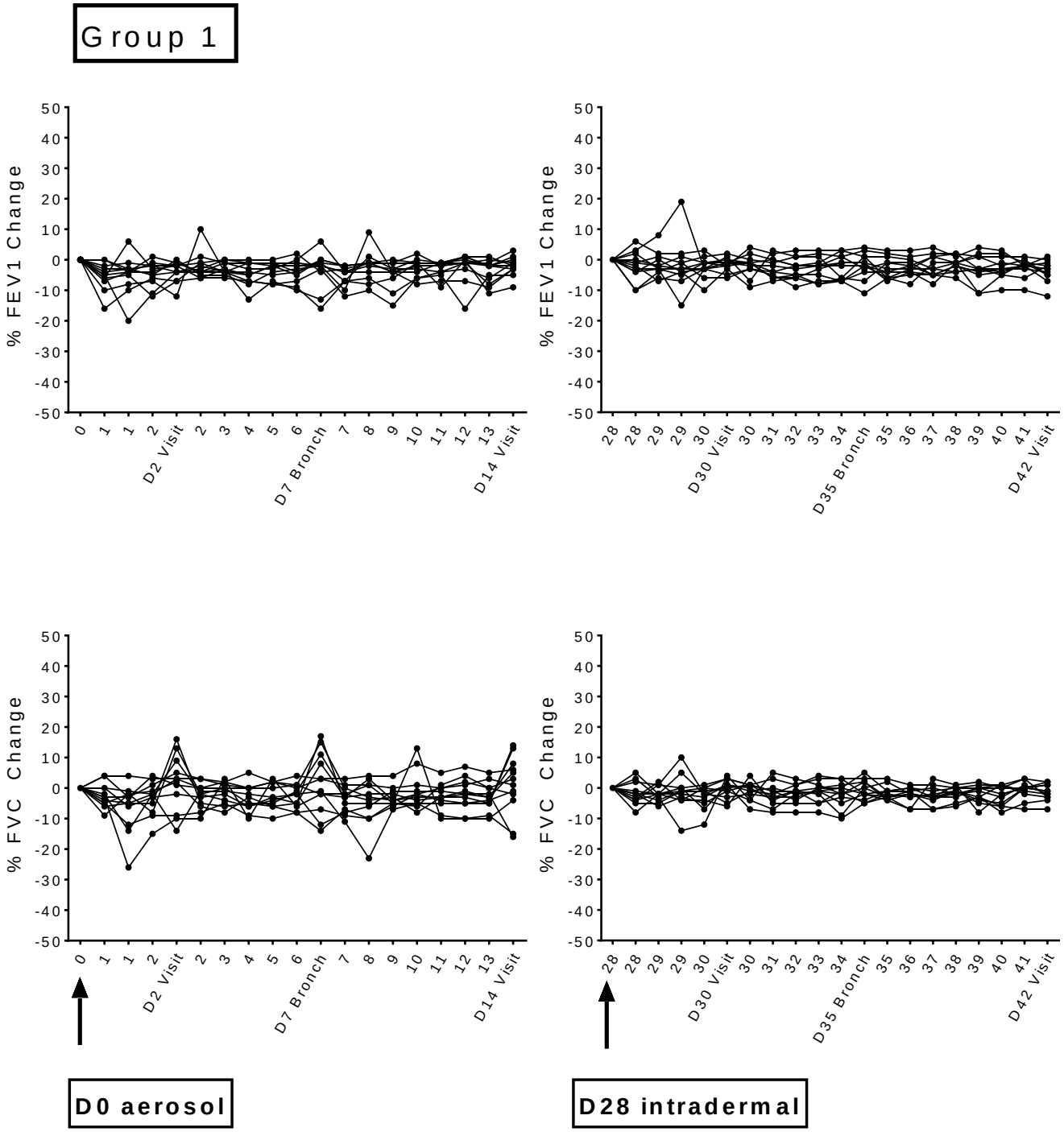


Figure 3.6 ***Group 2: Percentage change from baseline Forced Expiratory Volume in the 1st second (y=FEV1% change, top row) and Forced Vital Capacity (y=FVC % change, bottom row) over the 14 day period following each vaccination*** *(left column: x=days 0 to 14; right column: x=days 28 to 42). Each circle represents percentage change from baseline of an individual volunteer`s FEV1 or FVC. Recordings at scheduled visits and recordings from diary cards are included. Two outliers (volunteers 054- green and 063- pink) highlighted in colour)*

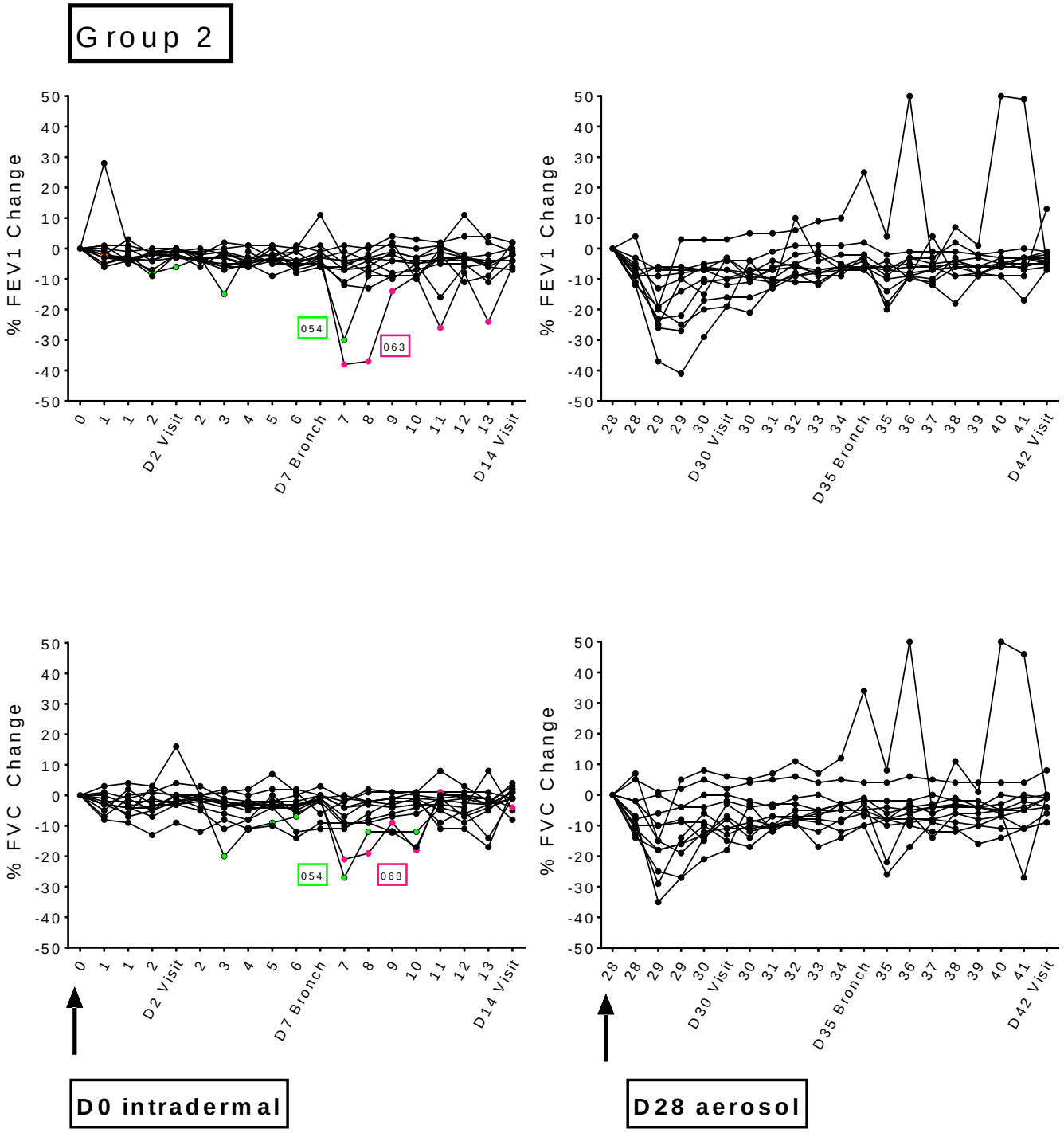
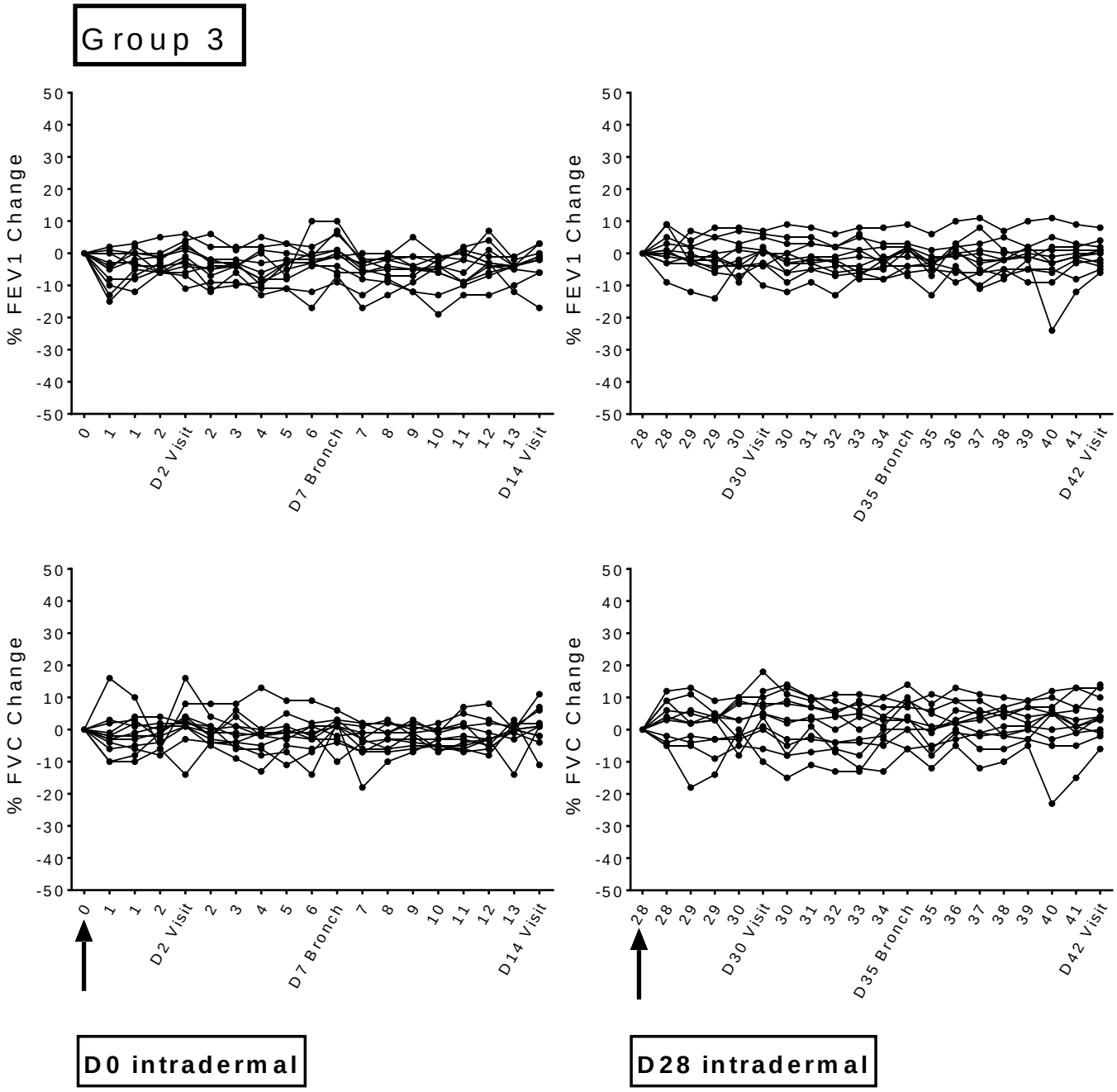


Figure 3.7 ***Group 3: Percentage change from baseline Forced Expiratory Volume in the 1st second (y=FEV1% change, top row) and Forced Vital Capacity (y=FVC % change, bottom row) over the 14 day period following each vaccination*** *(left column: x=days 0 to 14; right column: x=days 28 to 42). Each circle represent percentage change from baseline of an individual volunteer`s FEV1 or FVC. Recordings at scheduled visits and recordings from diary cards are included.*



Differences in spirometry values between the groups were compared as negative deviation from day 0 baseline, (i.e. any values below 100% baseline; Figures 3.5- 3.7). Median area-under-the-curve (AUC) values (negative peak area only) for FEV1 and FVC for all 3 groups from day 0 to day 7 (following the first vaccination) and from day 28 to day 35 (following the second vaccination) are summarised in Table 3.5.

Table 3.5 ***Median area-under-the-curve (AUC) values of negative peak areas of FEV1 and FVC*** *from day 0 baseline following first and second vaccination*

1st Vaccination	Group 1	Group 2	Group 3
FEV1	27.5	22.4	24.5
FVC	21.6	21.1	8.4
2nd Vaccination	Group 1	Group 2	Group 3
FEV1	15.8	54.9	21.9
FVC	13.9	58.9	0.4

Comparison between the groups demonstrated significant differences in Group 2 following the second vaccination compared to Groups 1 and 3 (day 28 to day 35 median AUC of negative peaks from baseline, Group 1 *vs*. Group 2: FEV1 p=0.0024 and FVC p=0.0179; Group 2 *vs*. 3 FEV1 p=0.0035 and FVC p=0.0122, Table 3.5 and Figures 3.5-3.7). A trend towards marginally increased volumes can be observed at clinic visits compared to self-recorded diary card readings.

Peripheral oxygen saturation was also obtained at all scheduled visits and was always within the normal range. Deviation from normal ranges was only observed during the bronchoscopy procedure when volunteers were sedated and the scope was passed (data not shown). Supplemental oxygen via nasal cannula was administered routinely during the procedure to manage this expected transient drop in peripheral oxygen saturation (data not shown).

3.4.4 Temperature

Temperatures were recorded orally at clinic visits and by the volunteer at home. A temperature greater than 37.6 ^{O} C was considered abnormal and severity graded as defined in Chapter 2: Materials and Methods (p.70). Summarised below are fevers documented in the period following vaccinations and bronchoscopies (Table 3.6).

Table 3.6 ***Frequency of fever occurring in volunteers*** *following vaccination and bronchoscopy by group, graded by severity*

Post- Vaccination Fevers

Day 0-7	mild	moderate	severe
Group 1	2	0	0
Group 2	1	0	0
Group 3	1	0	0

Day 28-35	mild	moderate	severe
Group 1	0	0	0
Group 2	1	4	1
Group 3	0	0	0

Post Bronchoscopy Fevers

Day 7-14	mild	moderate	severe
Group 1	0	0	0
Group 2	1	0	0
Group 3	0	0	0

Day 35-42	mild	moderate	severe
Group 1	0	0	0
Group 2	0	0	0
Group 3	0	0	0

3.4.5 Causality assessment

Solicited local, respiratory and systemic adverse events recorded within the diary card period (day 0 to day 14 and day 28 to day 42 for prime and boost vaccination respectively) were not assessed for causality but analysed and presented in full above. Solicited adverse events occurring beyond the diary card periods (day 14 to day 28 and day 42 to day 168) as well as unsolicited adverse events were collected and listed including details on whether it was a pre-existing condition. At every scheduled visit these adverse event listings were reviewed to ascertain when the symptoms had stopped, or if ongoing to review severity. For all adverse event listings, causality was

assigned following completion of the trial with subsequent independent peer review. A total of 258 unsolicited adverse events were listed in this trial (Group 1=54, Group 2= 99, and Group 3=105). The distribution of causally related adverse events was found to be as outlined in the following table.

Table 3.7 ***Causal relationship of listed adverse events by group****, including unsolicited adverse events occurring at any time in the trial period and solicited adverse events occurring outside the diary card period.*

Causal Relationship	No relationship	Unlikely	Possible	Probable	Definite	Total
Group 1	13	7	2	0	32	54
Group 2	30	16	3	4	46	99
Group 3	15	30	2	2	56	105

3.4.6 Bronchoscopies

All 36 enrolled volunteers who completed the trial underwent two bronchoscopies with no procedural complications. The volunteer who was withdrawn from the study (054) prior to her second vaccination underwent only one bronchoscopy on day 7. Seven volunteers opted not to have sedation at either one or both of the bronchoscopies. BAL with 100ml normal saline was obtained from the right middle lobe, medial segment. Return volume of lavages ranged between 28ml and 61ml (median= 47ml). Of the 73 total bronchoscopies conducted in this trial, 68 were reported as having normal macroscopic appearances. Two volunteers were described to have mild erythema throughout at bronchoscopy (001, Group 1 at day 35 bronchoscopy following intradermal boost vaccination and 006, Group 2 at day 7 bronchoscopy following intradermal prime vaccination). One volunteer (010, Group 3: intradermal-intradermal) was found to have petechiae in her left main bronchus of unknown aetiology at her second bronchoscopy on day 35. One volunteer (026, Group 2: intradermal-aerosol)

was noted to have some subtle mucosal irregularities in her left upper lobe which was considered to be normal variant.

Bronchoscopic images for the volunteers with these abnormal findings (001, 006, 010 and 026) can be found in Appendix 3. An example of bronchoscopic images of a volunteer reported to have normal macroscopic appearances after aerosol vaccination is also included for reference (003).

One volunteer (050, Group1: aerosol-intradermal) had abnormal secretions indicative of a chest infection at bronchoscopy on day 7. She was asymptomatic; and had been deemed fit for bronchoscopy. A chest radiograph following bronchoscopy revealed no evidence of consolidation. Her CRP on day of bronchoscopy was 2.2 mg/L (normal range 0-5 mg/L) and her white cell count was 7.32 mg/L (normal range 4-11 mg/L) with a normal differential white cell count. Empirical antibiotic therapy with co-amoxiclav and clarithromycin was commenced. BAL microscopy, culture and sensitivity testing revealed respiratory tract flora only. Her recovery following bronchoscopy was uneventful.

Post-bronchoscopy adverse events are summarised in Table 3.8. Two volunteers experienced mild flu-like symptoms and a transient dip in lung function on the evening of their first bronchoscopy (volunteers 063 and 054; Figure 3.6)

Table 3.8 ***Respiratory adverse events following bronchoscopy****. Self-recorded respiratory symptoms in diary card between day 7 (evening following bronchoscopy) and day 14.*

1st Bronchoscopy	**Group 1**	**Group 2**	**Group 3**
number of volunteers	*n=12*	*n=13*	*n=12*
Cough (productive)	6 (1)	8 (2)	10 (4)
Sore Throat	6	11	11
Wheeze	1	2	5
Shortness of breath	1	2	3
2nd Bronchoscopy	**Group 1**	**Group 2**	**Group 3**
number of volunteers	*n=12*	*n=12*	*n=12*
Cough (productive)	4(1)	7(4)	8(1)
Sore Throat	3	6	5
Wheeze	0	0	1
Shortness of breath	0	0	0

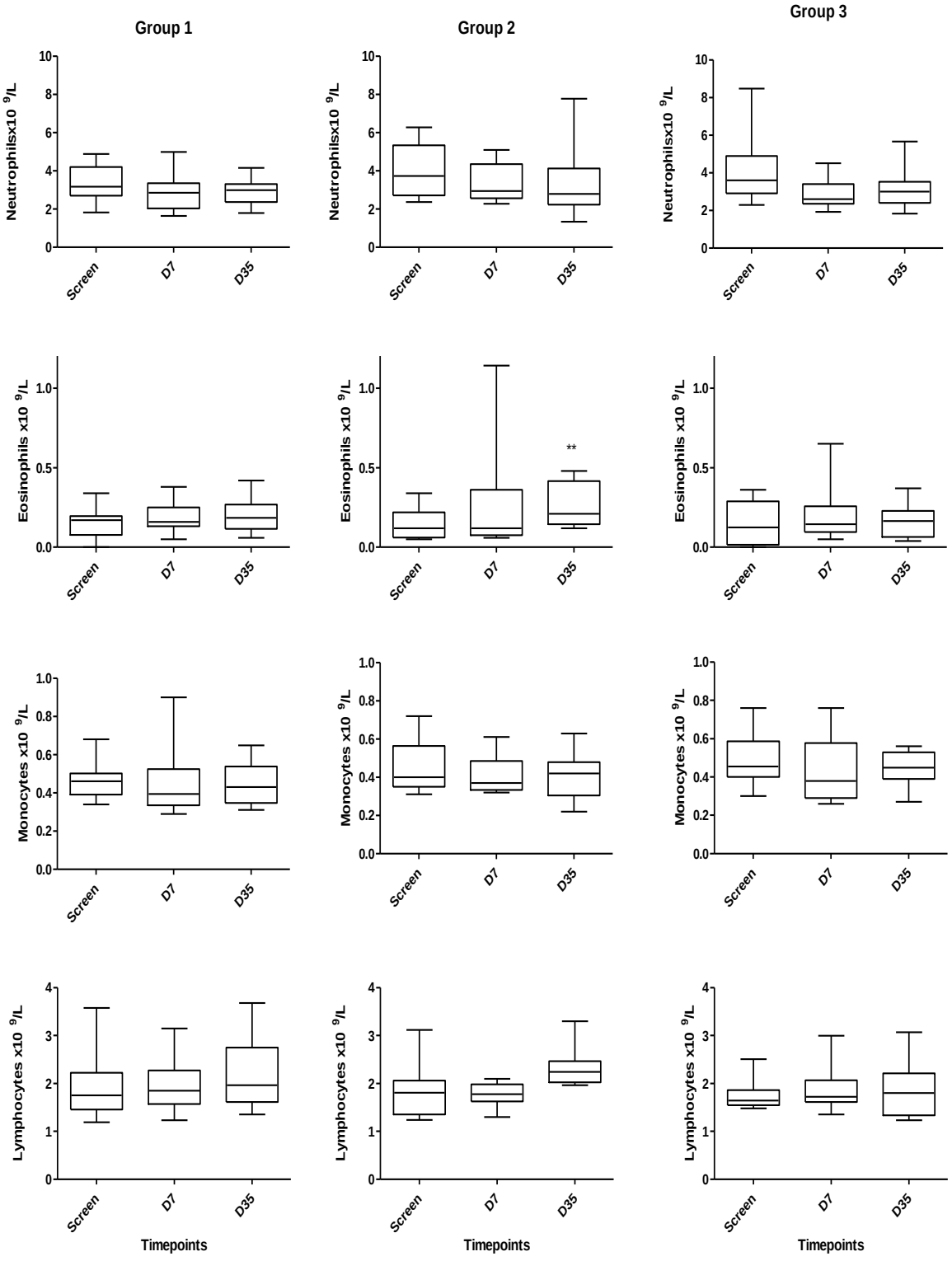
3.4.7 Laboratory adverse events

Laboratory adverse events detected at screening were repeated and confirmed. Volunteers were usually excluded and referred to their GP if necessary. Severity grading was standardised as described in Chapter 2: Materials and Methods (p.70) and Appendix 1: Clinical Trial Protocol. Laboratory adverse events noted during the trial period in enrolled volunteers are summarised below:

One volunteer (073, Group 2) experienced a grade 1 neutropenia on day 35 ($1.34x10^9$/L; normal range 2-7 $x10^9$/L) that had resolved by day 42. The same female volunteer had a haemoglobin value classed as being grade 1 severity (11g/dL; normal range 12-15g/dL) on day 7 which remained stable over repeated measurements throughout the trial (range 11-12g/dL). Her mean cell volume remained in the normal

range throughout. No other haematological parameter abnormalities were noted. Figure 3.8 illustrates the differential white cell count between the groups. Comparisons between timepoints in each group and comparisons between the groups were made (corrected for multiple comparisons). The only statistically significant difference was the eosinophil count in Group 2 between screening and day 35 (p= 0.0078, corrected for multiple comparisons). However, there was no difference in eosinophil count between the groups on day 35 (p=0.4052, corrected for multiple comparisons).

Figure 3.8 ***Differential white cell count at screening, day 7 and day 35****: Neutrophils (top row), Eosinophils (2nd row), Monocytes (3rd row) for Group 1 (left column), Group 2 (middle column), Group 3 (right column). ** refers to p value of 0.0078 in Group 2 eosinophil count. Non-significant differences (p>0.05) omitted for clarity.*



One volunteer experienced a grade 1 reduction in serum potassium on day 7 of the trial (074, Group 2), another volunteer a grade 2 reduction in serum sodium on day 7 (023, Group 1) and the same volunteer a repeat reduction in serum sodium on day 35 (grade 1). One volunteer experienced an isolated grade 1 elevated bilirubin on day 7 (045, Group 3) with liver enzymes within the normal range; which resolved spontaneously on repeat testing prior to the day 28 boost vaccination (data not shown). Following restart of group 2 enrolment, CRP was measured as an additional biochemical parameter of acute inflammation in these individuals. In Group 2 the CRP values on day 30 ranged between 55.7 and 156 (normal range 0-8 mg/L), whilst the ranges in Groups 1 and 3 on day 30 were 2.1 to 26mg/L and 0.1 to 25.5 mg/L, respectively

3.5 Exploratory analysis of adverse reactions in Group 2

In view of the clinical and laboratory observations in Group 2 volunteers, I designed a retrospective exploratory experiment, to try to determine the cause of this adverse reaction. The hypothesis behind this experiment was that the reactions were due to innate immune responses given the timeframe they occurred in. The aetiology was not clear but given the differential diagnoses considered (see section 3.7 p.120-122) detection of markers for eosinophils, neutrophils or inflammatory monocytes were included in this exploratory experiment.

Using multi-parameter flow cytometry, I developed a panel for surface markers to characterise neutrophils, eosinophils and inflammatory monocytes, in order to determine if these cells were implicated in the underlying immunological mechanism

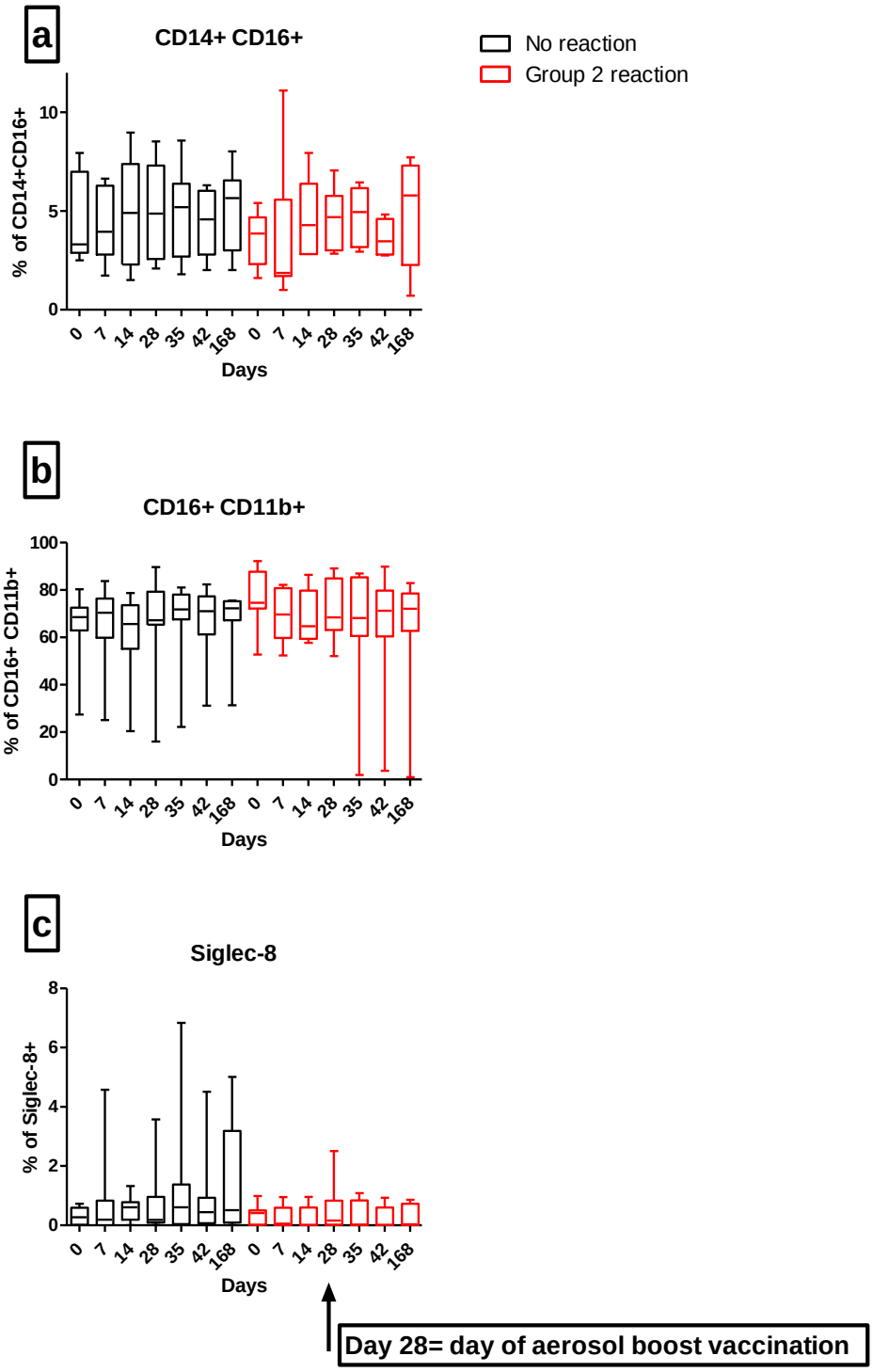
behind the reactions seen. This pilot experiment was performed on unstimulated whole blood samples which were already being collected for study of the antigen-specific Th1 biased immune responses. This additional exploratory study was performed on a total of 18 samples, including all volunteers in Group 2; and 9 additional volunteers from the other groups as controls (selected at random). Details of the flow cytometry panel are described in Chapter 2: Materials and Methods (p.80, 82). There were no differences detected in any of the markers examined at any timepoint between affected Group 2 volunteers and unaffected volunteers ($p>0.05$, Figure 3.9). Summarised below are the proportions of three of the studied populations: inflammatory monocytes (CD14+ CD16+), activated neutrophils (CD16+CD11b+) and the eosinophils expressing Siglec-8 (Figure 3.9).

Other markers that were included in the panel were CD15+, a marker of phagocytosis and chemotaxis in granulocytes; CD66b another marker of neutrophil activation; and CCR3 an eosinophil chemokine receptor involved in eosinophil migration and function (data not shown). These markers reflect the findings of populations demonstrated in Figure 1.9 and were therefore not included. There was no significant differences between affected Group 2 volunteers and unaffected volunteers in any of these markers (data not shown).

Comparison was made between the 7 affected Group 2 volunteers and the two volunteers in Group 2, who did not display these features after boosting by the aerosol route. Comparison was also made between the 7 affected Group 2 volunteers and the 9 volunteers from the other groups. Lastly, comparison was made between 7 affected Group 2 volunteers and the other 11 unaffected volunteers. There were no differences at any timepoint between the groups in any of the studied markers between the groups

(all p>0.05, corrected for multiple comparisons). In the affected volunteers, there was also no difference at day 28 or day 35 from baseline (day 0) and no difference from day 28 to day 35 for any of the studied marker (all p>0.05, data paired and corrected for multiple comparisons).

Figure 3.9 ***Pilot exploratory analysis on unstimulated whole blood samples of affected Group 2 volunteers with described reaction following aerosol boost (n=7) and unaffected volunteers (n=11).*** *Selected cell populations shown below are: (a) inflammatory monocytes: CD14+CD16+, (b) activated neutrophils CD16+CD11b+and (c) an eosinophil expressing Siglec-8. Comparisons between groups and timepoints not statistically significant (p>0.05)*



3.6Volunteer feedback

A retrospectively designed feedback questionnaire was sent out to all volunteers by email. Participation was voluntary. Twenty out of 37 enrolled volunteers responded. All 20 volunteers reported they would be happy to receive another aerosol vaccination. Only 17 of the respondents would agree to undergo a bronchoscopy again, with two of the volunteers describing the experience of the bronchoscopy as causing some degree of discomfort; and 8 volunteers considering the bronchoscopy the worst aspect of the trial. When asked if they would recommend the trial to a friend 16 volunteers answered “yes”, one answered “no”, two were undecided and one did not respond. In qualitative statements added as free text by volunteers, 8 volunteers commented positively on their encounter with trial staff, 4 commented that they were interested in the experience of taking part in a clinical trial, three commented positively on trial conduct and one volunteer emphasized a preference for aerosol compared to intradermal vaccination. In terms of negative comments, three volunteers disliked the waiting periods for clinic visits or bronchoscopy and 4 found the daily completion of home diary cards and spirometry following vaccinations tedious.

With regards to sedation for bronchoscopy 5 of the respondents had opted not to have sedation for both bronchoscopies and two had opted not to have sedation for one of the two bronchoscopies. When asked for reasons for choosing to opt out of sedation, the 7 volunteers who opted for no sedation (for one or both bronchoscopies) did so due to work or personal commitments (including travelling) which required them not to be under the effects of sedation for the remainder of the day. Of the two respondents who experienced one bronchoscopy with and one bronchoscopy without sedation, one

would be willing to have the procedure done without sedation again but the other one would not.

3.7 Discussion

The primary objective was to evaluate safety of the aerosol route as a prime and as a boost vaccination. The safety profile of the aerosol route as a prime vaccination was comparable to that of the intradermal route and acceptable. Respiratory symptoms in volunteers receiving aerosol prime vaccinations (Group 1) *vs.* those receiving intradermal prime vaccinations with aerosol paired placebos (Group 2 and 3) were comparable and likely attributable to aerosol administration or a placebo effect rather than aerosol vaccination. Bronchoscopy appearances revealed no differences in macroscopic mucosal inflammation between the groups, suggesting that aerosol prime vaccination did not lead to macroscopically detectable airway inflammation. FEV1 is a measure of airway obstruction, and would likely be affected by airway inflammation. The fact that oscillation of FEV1 and FVC was minimal following aerosol prime vaccination (Group 1) and comparable to that of volunteers receiving intradermal prime vaccination and aerosol placebo (Groups 2 and 3) is supporting evidence that aerosol prime vaccination with MVA85A does not induce airway inflammation. This is in keeping with lung function parameters in the first clinical trial of aerosolised MVA85A[247]. There was a general trend towards improved spirometry readings at visits in all groups, which probably reflects increased effort in the presence of a healthcare professional. This was also observed in the first trial of aerosolised MVA85A[247]. Collectively, the evidence from this trial and the first trial of aerosolised MVA85A suggests that aerosol prime vaccination is safe and comparable to intradermal vaccination.

However, when MVA85A aerosol was administered as a boost vaccination (Group 2) some volunteers transiently experienced more frequent and more severe transient respiratory and systemic adverse events than the volunteers in the other groups; with full resolution in 48-72 hours. During the first pause in Group 2 enrolment, clinical and immunological data available was closely reviewed and the preponderance of atopy in the affected volunteers was the only noticeable difference that distinguished those two affected volunteers from all the other enrolled volunteers. At the final analysis the only deviation in the laboratory blood profile of Group 2 was the change in eosinophil counts observed from baseline (screening) to day 35 (7 days after second aerosol boost vaccination). These observations raised the question as to whether any history of atopy was a predisposing factor. Volunteers with any history of wheeze as well as an established diagnosis of asthma had been excluded from enrolment. However, initially volunteers who had a history of allergic rhinitis, eczema or certain allergies as part of the atopy syndrome were not excluded, because this had not been considered clinically relevant for suitability in this clinical trial. An exploratory analysis of some different innate cell types in blood (eosinophils, neutrophils and inflammatory monocytes) to determine the aetiology of these adverse events did not suggest an association. The two key questions proposed were: Firstly, was there any signal at day 0 (baseline) or day 28 (baseline for second vaccination) that distinguished affected Group 2 volunteers from their peers, and could this have aided prediction of the adverse event profile seen? Secondly, was there any signal detected after the second vaccination which would have pointed towards the immunological mechanism to explain this reaction? Analysis of the studied populations and markers revealed no apparent differences. However, this experiment was retrospectively designed, exploratory and limited for reasons mentioned. One reason for failure to detect

differences could have been the fact that samples were collected when volunteers were asymptomatic, i.e. day 28 and day 35, but not in the time window in between, which was when volunteers reported peak severity of the described adverse event profile (day 29 to 32) and would coincide with the timeline of innate immune responses. Another reason for failure to detect a signal could have been that some markers could not be included for technical reasons mentioned. For example, CD49d, a marker of leucocyte trafficking (positive on eosinophils and negative on neutrophils) could not be included in the panel as it was used as a co-stimulatory antibody in these whole blood samples at initial processing (Chapter 2: Materials and Methods, p.76). The panel was not comprehensive for all markers of those populations, only selected key markers. The final reason why this experiment did not reveal any differences could be due to the fact that whole blood might not have represented what was happening on the lung mucosa of these volunteers. BAL or induced sputum rather than whole blood might have shown differences if these cells contributed to aetiology. Atopy could have played a role, at least in the first two affected Group 2 volunteers. However, the occurrence of the same symptom profile in further volunteers following stringent exclusion of atopic volunteers suggested that this was probably not the sole predisposing factor.

Another possibility that was considered was the possibility of hypersensitivity pneumonitis (HP)[267, 268]. One of the causes of HP described has been exposure to non-tuberculous mycobacteria (NTM) from hot tubs (so called "hot tub lung"), although there is some controversy whether it is truly HP or infection with NTM[268, 269]. The clinical presentation in the affected Group 2 volunteers was in keeping with acute HP[268, 270, 271]. Whilst there are several agreed relevant clinical factors for a diagnosis of HP, consensus of diagnostic criteria are still being reached[270, 271]. Some characteristics in the affected Group 2 volunteers were consistent with HP: the history

of exposure (through intradermal prime vaccination) and the clinical features following the aerosol boost vaccination which were well documented by the volunteers in their diary cards and reviewed by the clinical team at their clinic visits. In terms of lung function, there was a transient relatively proportional reduction in both FEV1 and FVC with normal oxygen saturations. Carbon monoxide diffusing capacity was normal in the volunteers who had this performed. The magnitude of increase in CRP following the aerosol boost vaccination in Group 2 volunteers, (although not specific) indicated an inflammatory process[268, 270]. The BAL immunogenicity data is discussed in detail in the next chapter (Chapter 4: Cell Mediated Immunity, p.157-173). Briefly, in the affected Group 2 volunteers there was no change in percentage of CD3+ lymphocytes detected in BAL from the first to the second bronchoscopy (as detected by flow cytometry). A relative lymphocytosis would have been typical of the lymphocyte rich alveolitis indicative of HP[268, 270]. There is some evidence for the evaluation of CD4:CD8 ratio in HP but it can be influenced by a variety of factors[268, 270]. Group 2 volunteers had no change in CD4:CD8 ratio from day 7 to day 35 as measured in BAL. Radiological examination was limited but chest radiographs in affected volunteers were all unremarkable. However, a normal chest radiograph would not contradict the diagnosis[268, 270]. The assessment was not sufficiently comprehensive to be conclusive but HP was a possible consideration.

A third possibility considered was a potent vector-specific or insert-specific response to the aerosol boost but at the initial safety review no signal supporting this was apparent (discussed in more detail in Chapter 4: Cell Mediated Immunity, p.178).

There were some mild and expected respiratory adverse events following the bronchoscopy. Flu-like symptoms for 12 to 24 hours following BAL are well recognised

in up to one third of patients undergoing BAL, and the symptoms reported by two volunteers in this clinical trial were in keeping with the expected range and timeframe[272].

In terms of limitations of the clinical trial, the blinding was imperfect. Saline injected intradermally did not mirror the local intradermal reaction seen with MVA85A. Therefore, volunteers could potentially deduce their group allocation, particularly following boost vaccination. This could have introduced reporting bias. Whilst partial blinding of study investigators was also a limitation of the study methodology, full blinding would have been difficult due to the issue of concealment. Given that this was a proof-of-concept phase 1 trial and endpoints were safety and immunogenicity, partial blinding of study investigators was considered acceptable and was unlikely to influence interpretation of safety and immunogenicity readouts. In fact, with events in Group 2 unblinded clinical staff aided urgent and comprehensive safety review. Breaking of the randomisation code during assessment of observations in Group 2 was another limitation but given the challenging logistics of the trial and the unexpected respiratory adverse events, this was the best possible option for continuing the trial safely at the time.

There were also many positive points in this study. Full enrolment with no drop-outs and no loss to follow up was achieved despite the logistical challenges and complex schedules including two bronchoscopies. This has provided an extensive and detailed safety data set. The overall positive feedback of the volunteers reflected the acceptability and tolerability of aerosol vaccination and bronchoscopy in the research setting. Furthermore, the fact that there were no bronchoscopy procedural complications and no problems with sedation further supported the feasibility and

safety of research bronchoscopies. Another positive point was the paired placebo design which, despite the issue of masking, was a way of evaluating respiratory adverse events attributable to nebulisation independent of vaccination.

There are further considerations in the wider context of developing an aerosol TB vaccination that were highlighted by this study.

Firstly, nebulisation requires some degree of cooperation and therefore might not be suitable for younger age ranges or those unable to cooperate. However, aerosol administration has been successfully performed using modified paediatric masks in anesthetised non-human primates, indicating that this could be overcome by device adaptation[236, 239]. Another device-related consideration is the requirement for a reliable portable device, as used in this clinical trial. This produces respirable particles large enough to contain the candidate vaccine and small enough to reach the distal airways. These devices are increasingly refined, user friendlier, widely available and more affordable. Their availability is an unlikely barrier to implementation of aerosol vaccination[273-275].

To make the aerosol route of vaccination more deployable in the field, modification of the vaccine formulation would also be beneficial. Powdered BCG vaccine for inhalation has been generated by spray drying [276] and aerosol delivery of BCG nanomicroparticles has been shown to protect guinea pigs from *M.tb* challenge better than parenteral BCG [233].

The questions of bioavailability, the dose of aerosolised MVA85A which actually reaches and acts on the lung mucosa, how much undergoes lung first-pass metabolism and how much is absorbed remain unanswered. MVA85A is the first viral vector vaccine and the first candidate TB vaccine administered to humans by the

aerosol route[247]. The device used, rate of production of aerosol, and individual inspiratory flow tidal volume during the aerosol inhalation were likely to have influenced how much MVA 85A vaccine reached the target site[273]. The action of any inhaled drug and also aerosolised MVA85A will be a combination of local and systemic action from absorption through lung and oral mucosa[277]. Any adverse events can therefore also be attributable to local and systemic effects.

Inhalation for drug delivery is widely used in respiratory medicine. Inhaled insulin as a needle-free alternative has been an area of interest and debate. Cough was a frequent side effect of inhaled insulin and alveolar toxicity of insulin and the potential for growth stimulatory and hence carcinogenic effects have been hypothetical concerns[278]. In one study, long term follow up of patients receiving inhaled insulin as an adjunct to oral therapy for glycaemic control demonstrated a decrease in FEV1 and carbon monoxide diffusion capacity after 6 months which plateaued over the remaining 1.5 years of the study[279, 280]. Whilst lessons can be learned from delivery of all drugs by the inhaled route and the effect on different patient groups, an inhaled vaccine would not be given daily nor life-long.

In contrast to inhaled drugs, where airway selectivity and high topical potency is desirable and systemic absorption undesirable[273, 277], inhaled vaccines require systemic absorption to induce systemic immunity. Equally, inadvertent oral absorption might hinder an effective vaccine response by inducing oral tolerance[281]. A pre-clinical study used *in vivo* real time scintigraphy to monitor the fate of aerosolised radiolabelled poxvirus related vectors delivered to anaesthetised non-human primates. This demonstrated consistent delivery to the respiratory tract with approximately 4% reaching the lungs[259]. This study also demonstrated that there was no evidence of

brain or lung pathology in *post-mortem* examinations. Similar studies using labelled MVA85A vaccine *in vivo* would help to answer questions about pharmacodynamics and pharmacokinetics of aerosolised vaccines. Further studies examining the effect of aerosolised MVA85A in other mucosal compartments will be important to consider.

There is also a need for reliable and reproducible immunomonitoring of the lung mucosal compartment following aerosol vaccination. Bronchoscopy and BAL are likely to represent a robust and representative sample of mucosal immunity but remain an invasive method. One method that could be explored for immunomonitoring of respiratory mucosal immunity is induced sputum. Induced sputum has been used to assess airway inflammation in asthma, cystic fibrosis and healthy volunteers and also as an immunoassay in patients with tuberculosis[282-286]. Furthermore, robust surrogate markers in the blood that correlate with established immunity in the respiratory mucosa, would be a useful non-invasive tool for immunosurveillance especially in those considered high risk. However, these would need to be validated for the indication. In patients with eosinophilic asthma, sputum eosinophilia has been used as a non-invasive marker of eosinophilic lung inflammation and to guide treatment[287]. However, in a study to identify patients suitable for treatment with mepolizumab, a monoclonal anti-IL-5 antibody recently licensed for eosinophilic asthma, sputum eosinophils did not predict treatment response and therefore blood eosinophils are used as a biomarker to identify patients suitable for treatment with mepolizumab and monitor their progress[288]. This emphasises that a robust biomarker needs to be relevant to the specified indication, i.e. a biomarker for LTBI or TB might not necessarily be useful for predicting treatment outcome or protective vaccine responses. Lastly, the population enrolled in this study was a selected population of

healthy non-smokers without LTBI resident in Oxford, who might not be representative of the target population.

4 Results: Cell mediated immunity

4.1 Introduction

As outlined in the Introduction, cell mediated immunity is a critical component of anti-mycobacterial immunity.

The aim of the clinical trial conducted as part of this DPhil was to identify whether insert Ag85A responses could be enhanced through homologous boosting by heterologous routes. One of the secondary objectives in this clinical trial was to characterise lung mucosal and systemic insert (Ag85A) and vector (MVA) responses. This chapter summarises cellular immune responses to Ag85A and MVA in the lung mucosal compartment as assessed by BAL ICS and in the systemic compartment as evaluated by *ex-vivo* IFN-γ ELISPOT and whole blood ICS.

IFN-γ ELISPOT and BAL ICS were performed in real time on fresh cells. Whole blood was stimulated and frozen; and then thawed in batches for ICS.

To ensure stringent analysis and avoid false estimation of effect of the trial regimen, the three Group 2 volunteers who received a prime intradermal vaccination but then no boost vaccinations (volunteers 74, 77, 81) were excluded from the immunogenicity analysis beyond day 28, but their BAL data is presented separately.

Supplementary table 2 in appendix 4 provides a brief summary of cell mediated immune responses.

4.2Interferon-γ ELISPOTs

Ex-vivo IFN-γ ELISPOTs were performed as described in Chapter 2: Materials and Methods (p.74-76) on fresh PBMC for Ag85A (as a pool of 66 overlapping 15mer peptides), MVA CD4+ (27 14-21mer peptides) and MVA CD8+ (36 9-mer peptides) epitopes and PPD (data not shown). Antigen-specific responses to the insert, Ag85A, and MVA CD4+ and MVA CD8+ T cell epitopes are presented here as spots per million PBMC for all timepoints with background in wells with medium only subtracted (Chapter 2: Materials and Methods, p.76).

4.2.1 Antigen specific IFN-γ ELISPOT responses

Peripheral blood IFN-γ ELISPOT responses are summarised in Figure 4.1 for Ag85A (top row), MVA CD4 peptides (middle row) and MVA CD8 peptides (bottom row). There were no significant differences at baseline between the groups for any antigen (p= 0.4093 for Ag85A; p=0.1904 for MVA CD4; p=0.1316 for MVA CD8). IFN-γ ELISPOT responses were considered in terms of peak, boosting and durability.

In all three groups, Ag85A-specific IFN-γ ELISPOT responses peaked on day 7 following the first vaccination (Day 0 *vs.* day 7: Group 1 p= 0.0025, Group 2 p= 0.0039, Group 3 p= 0.0005). Comparison of day 28 (4 weeks after prime and day of second vaccination) and day 35 (1 week after second vaccination) revealed boosting of Ag85A-specific levels beyond that induced by the prime vaccination in Group 2 only (intradermal- aerosol; day 28 *vs*. day 35: p=0.0273) but not in Groups 1 or Group 3 (Group 1: aerosol-intradermal, day 28 > day 35 p=0.0454, Group 3: intradermal-intradermal, day 28 *vs*. day 35: p=0.0771). Ag85A-specific responses remained significantly elevated compared to day 0 baseline in all groups until day 84, and in

Groups 1 and 3 until day 168 (Day 0 vs. day 84: Group 1 *p*=0.0025, Group 2 *p*=0.0039, Group 3 *p*=0.0005 and Day 0 vs. day 168: *p*=0.0025 for Group 1 and *p*=0.0005 for Group 3). Ag85A-specifc responses in Group 2 on day 168 were not above baseline (Day 0 *vs*. day 168: *p*= 0.0969).

Median Area-Under-the-Curve (AUC) values of Ag85A-specific ELISPOT responses were also compared (data not shown). AUC was calculated for frequency of antigen-specific IFN- γ secreting cells as measured by IFN- γ ELISPOT responses to Ag85A for each group over time. No significant difference in median AUC values was detected between groups (p=0.9273)

MVA CD4-specific T cell responses in Group 1 peaked on day 7 post-aerosol immunisation (day 0 *vs*. day 7: *p*=0.0025). There was boosting of MVA CD4-specific responses on day 42 in Group 1 following the intradermal boost vaccination (day 28 *vs*. day 42 *p*=0.0076). MVA CD4-specific responses in Group 1 remained significantly elevated above baseline until day 168 (day 0 *vs.* day 168: *p*=0.0053).

MVA CD4-specific responses in Group 2 also increased on day 7 post-intradermal immunisation but were not significantly above baseline until day 35, i.e. 7 days after aerosol boost vaccination (day 0 vs. day 35 *p*=0.0319). Similar to Group 1, there was boosting of MVA-CD4 specific responses on day 42 in Group 2 (day 28 *vs.* day 42: *p*=0.0391). The MVA CD4-specific response then contracted and on day 168 were no longer significantly elevated above baseline (day 0 *vs.* day 168 *p*=0.3135).

In Group 3, MVA CD4-specific responses in Group 3 also increased on day 7 following intradermal prime vaccination (day 0 *vs.* day 7: *p*=0.0038) and peaked on day 14 (day 0 *vs*. day 14: *p*=0.0005). These responses were not boosted by a second intradermal boost vaccination on day 28 (day 28 *vs.* day 42: *p*=0.5828). However, MVA CD4-

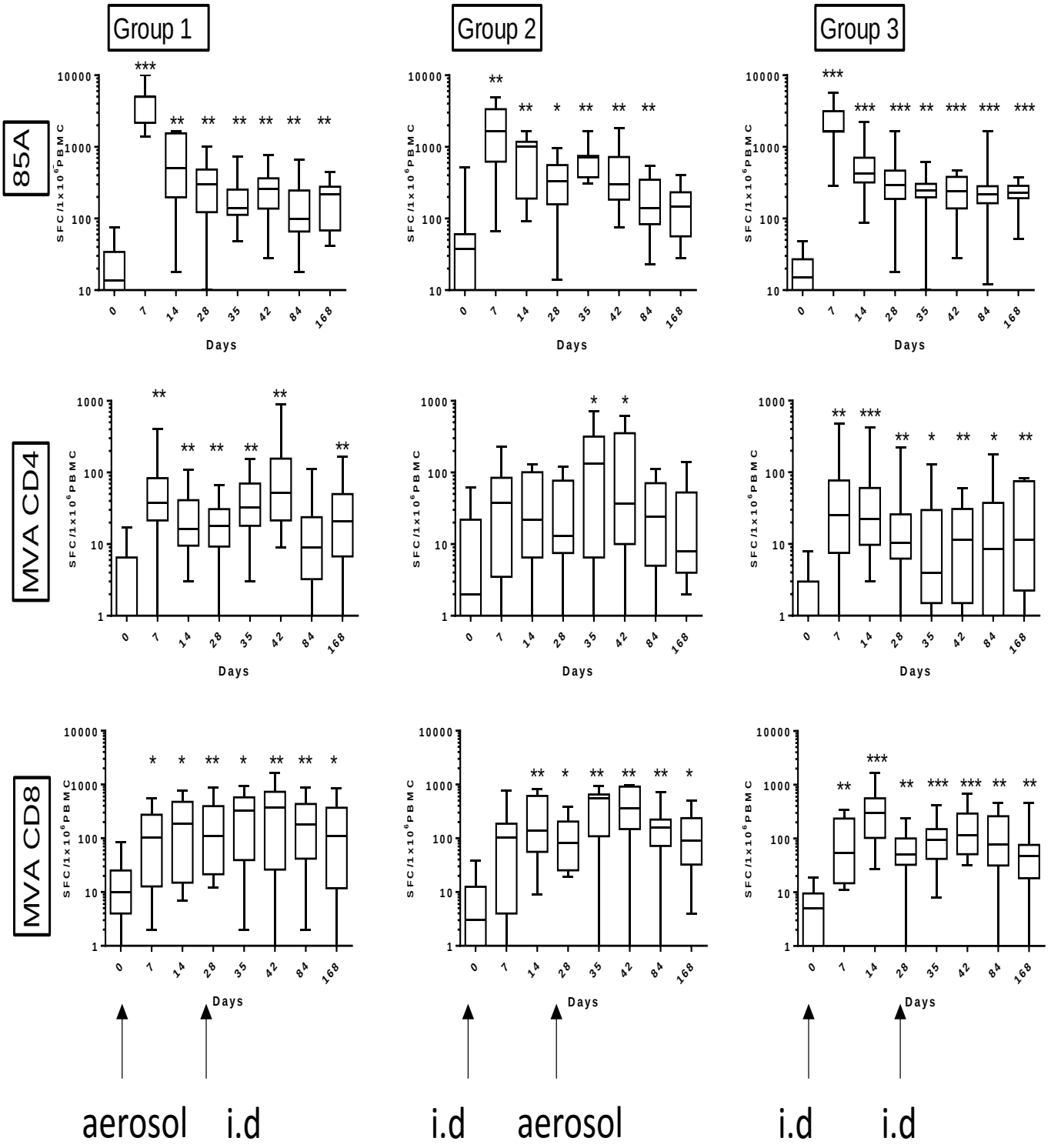
specific responses in Group 3 were still higher than day 0 baseline by day 168 (day 0 *vs.* day 168 p=0.0076).

MVA CD8-specific responses in Group 1 increased after the aerosol prime vaccination (day 0 *vs*. day 7: p=0.0206) and peaked on day 28 (p=0.0025). MVA CD8-specific responses peaked again on day 42 (day 0 *vs*. day 42: p=0.0086) but were not boosted compared to day 28, day of aerosol boost vaccination (day 28 *vs.* day 42: p=0.0652). MVA CD8-specific responses in Group 1 remained significantly higher than baseline until day 168 (day 0 *vs.* day 168 p=0.0108).

MVA CD8-specific T cell responses in Group 2 peaked on day 14 following intradermal prime vaccination (day 0 *vs.* day 14; p= 0.0039) and (in contrast to Group 1) were boosted following the day 28 aerosol boost vaccination (day 28 *vs.* day 42 p=0.0078). This MVA CD8-specific response in Group 2 then contracted but remained significantly elevated above baseline at day 168 (day 0 *vs.* day 168: p=0.0117).

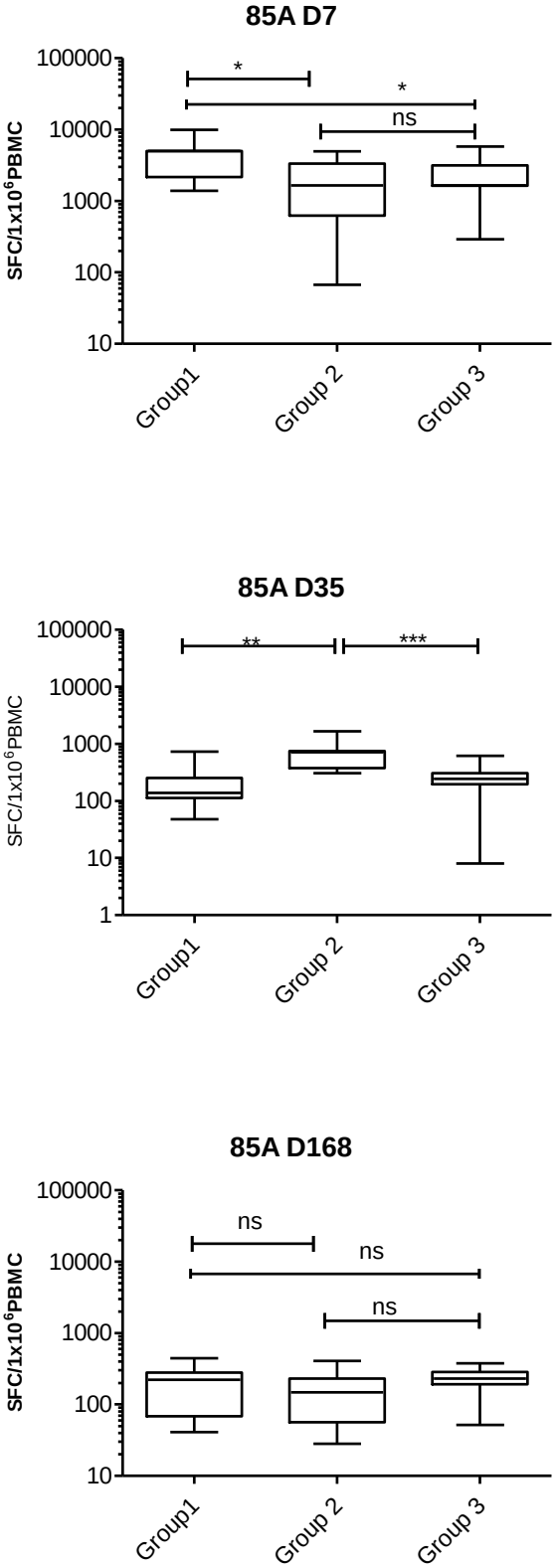
In Group 3, MVA CD8-specific responses peaked on day 14 post intradermal prime vaccination (day 0 *vs.* day 14: p=0.0005) and were boosted following a second intradermal vaccination (day 28 *vs*. day 42: p=0.0093). MVA CD8- specific responses in Group 3 remained significantly higher than baseline on day 168 (day 0 *vs.* day 168 p=0.0024).

Figure 4.1 ***Frequency of antigen-specific IFN- γ secreting cells as measured by IFN- γ ELISPOT responses to Ag85A (top row), MVA CD4 (middle row), MVA CD8 (bottom row)*** *in Group 1(left column), Group 2 (middle column) and Group 3 (right column), x axis: timepoints in days, y axis: Spots per 1x10^{6}PBMC. Arrows indicate timings of aerosol/intradermal (i.d) vaccines. Shown are significant differences from baseline (day 0). Non-significant differences (p>0.05) omitted for clarity. * for p ≤ 0.05, ** for p≤0.01 and *** for p≤0.001*



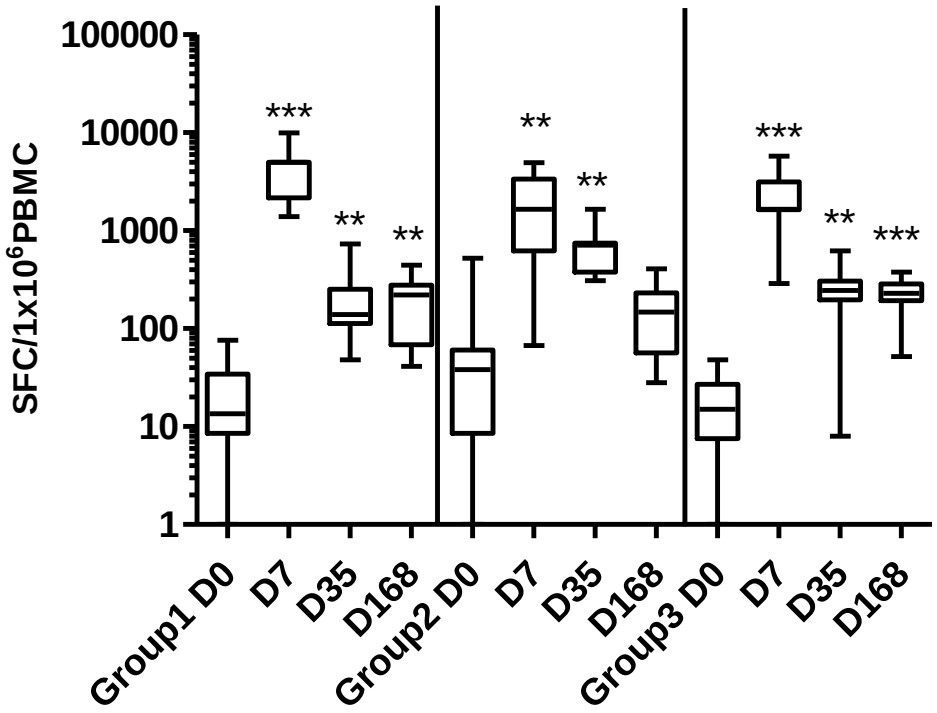
In Figure 4.2, Ag85A-specific responses across the three groups at peak timepoints (i.e. 7 days following each vaccination) and plateau (day 168) are compared. On day 7, Ag85A-specific IFN-γ ELISPOT responses in Group 1 after aerosol prime were significantly higher than in Groups 2 and 3 after intradermal prime (Group 1 *vs.* Group 2: p=0.0205, Group 1 *vs.* Group 3: p=0.0240). When Ag85A- specific IFN-γ ELISPOT responses from Groups 2 and 3 on day 7 were combined (as both had received an intradermal day 0 prime vaccination), the comparison to Group 1 on day 7 was also highly significant (Group 1 *vs*. Groups 2&3: p=0.007, data not shown). On day 35, which was 7 days following boost vaccination, Ag85A-specific IFN-γ ELISPOT responses in Group 2 after aerosol boost were significantly higher than those in Groups 1 and 3 after intradermal boost (p=0.0016 Group 2 *vs.* Group 1 and p=0.0007 Group 2 *vs.* Group 3, corrected for multiple comparisons). Therefore, the maximum peak Ag85A-specific responses were always observed following an aerosol vaccination. There was no significant difference between the groups by day 168 (all p>0.05).

Figure 4.2 ***Comparison of Ag85A IFN- γ ELISPOT responses on days 7(top), 35 (middle) and D168 (bottom) between groups****. x axis: groups, y axis: Spots per 1x10^6 PBMC). Significant differences between groups shown. ns= non-significant differences (p>0.05). * for p ≤ 0.05, ** for p≤0.01 and *** for p≤0.001*



In Figure 4.3, the effects of priming and boosting across the three groups are summarised. Ag85A-specific IFN- γ ELISPOT responses increased in all three groups following priming on day 7 compared to baseline. These responses were boosted after the second vaccination in Group 2 only (day 28 *vs.* day 35 Group 2 p=0.0273), but not in Groups 1 and 3 (day 28 *vs.* day 35: Group 3 p=0.0771, day 28> day 35 in Group 1 p=0.0454). On day 168, there was no difference between groups (Figure 4.2).

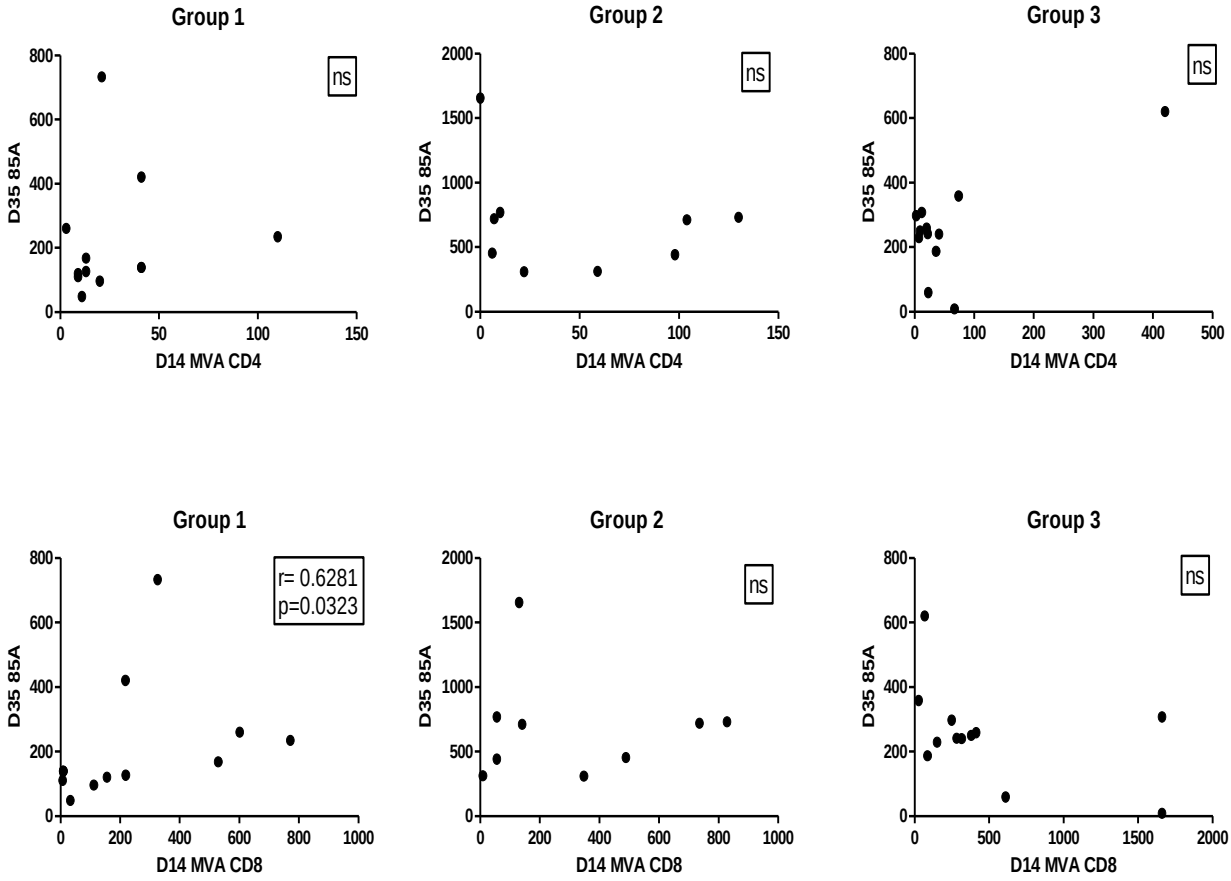
Figure 4.3 ***IFN- γ ELISPOT responses to Ag85A at baseline (day 0), peak time points following first and second vaccination (day 7 and day 35 respectively) and last follow up (day 168).*** *x axis: timepoints in days for each group, y axis: Spots per 1x10^{6}PBMC). Shown are significant differences from baseline (day 0). Non-significant differences (p>0.05) omitted for clarity. ** for p≤0.01 and *** for p≤0.001*



4.2.2 IFN-γ ELISPOT vector-insert correlations

The peak response to insert Ag85A as measured by IFN-γ ELISPOT was 7 days after each vaccination (i.e. day 7 and day 35 in this clinical trial). The peak of the MVA CD4-specific responses measured by IFN-γ ELISPOT occurred between day 7 and day 14 and for MVA CD8-specific responses between day 14 and day 28. Correlating MVA CD4 and MVA CD8 vector- specific IFN-γ ELISPOT responses at peak (day 14) following prime vaccination, with insert Ag85A-specific responses following boost vaccination at the peak timepoint (day 35) was only statistically significant for MVA CD8 in Group 1 (Figure 4.4, r=0.6281, *p*=0.0323). When MVA CD4-specific and MVA CD8-specific IFN-γ ELISPOT responses on day 14 were combined, the correlation with Ag85A-specifc IFN-γ ELISPOT responses on day 35 was only statistically significant in Group 1 (r=0.7474, *p*=0.0070; data not shown). Neither MVA vector responses on day 7, nor MVA vector responses on day 28 (baseline boost vaccination) significantly correlated with Ag85A responses on day 35, either (as measured by IFN-γ ELISPOT, data not shown).

Figure 4.4 ***Correlation of D14 MVA CD4-specific (top row) and MVA CD8-specific (bottom row) IFN- γ ELISPOT responses with insert Ag85A IFN- γ ELISPOT responses on day 35*** *(7 days/peak following boost vaccination) for Group 1 (left column), Group 2 (middle column), Group 3 (right column); ns=not statistically significant (p>0.05)*



4.3Whole blood ICS responses

Whole blood (WB) was stimulated (Ag85A and MVA CD4/CD8 peptide pool) and cryopreserved and then thawed and processed in batches, as described in Chapter 2: Materials and Methods (p.76-78). Shown below are Ag85A-specific and MVA-specific CD4+ and CD8+ T cell responses positive for cytokines IFN-γ, TNF-α, IL-2 and IL-17. Baseline responses on day 0 for Ag85A-specifc and MVA-specific CD4+ and CD8+ T cell response as measured by WB ICS were not significantly different between groups, i.e. groups comparable at baseline (all $p>0.05$)

4.3.1 Ag85A-specific WB ICS

For Ag85A-specific CD4+ T cells responses in all three groups, IFN-γ and TNF-α positive responses increased from baseline on day 7 (day 0 *vs.* day 7: Group 1 $p=0.0005$, Group 2 $p=0.0039$, Group 3 $p=0.0010$, Figure 4.5). Comparison between the groups showed that day 7 Ag85A-specific IFN-γ and TNF-α CD4+ responses were significantly higher in Group 1 than Groups 2 and 3 (for IFN-γ: p= 0.0116 Group 1 *vs.* Group 2 and $p=0.0102$ Group 1 *vs.* Group 3 and for TNFα: $p=0.0275$ Group 1 *vs.* Group 2 and $p=0.0117$ Group 1 *vs.* Group 3, corrected for multiple comparisons; Figure 4.5). There was no boosting of Ag85A-specific IFN-γ and TNF-α CD4+ responses in any of the groups from day 28 to day 35 ($p>0.05$ in all 3 groups, Figure 4.5). There were also no significant differences in Ag85A-specific IFN-γ and TNF-α CD4+ responses between the three groups on day 35 ($p=0.1913$, Figure 4.5).The responses contracted by day 168, but remained above day 0 baseline, although this only reached statistical significance for Group 3 for IFN-γ ($p=0.0039$) and for Groups 1 and 3 for TNF-α ($p=0.0024$ and $p=0.0098$ respectively, Figure 4.5).

Ag85A-specific CD4+ T cell responses positive for IL-2 and IL-17 were much lower in magnitude than IFN-γ and TNF-α positive responses and were not significantly different from baseline in any of the groups with the exception of IL-17 positive responses on day 7 compared to baseline in Group 1 (day 0 *vs.* day 7, p=0.0137, Figure 4.5). The IL-17 positive responses were significantly higher in Group 1 compared with Group 2 on day 7 (p=0.0214, Figure 4.5). The IL-2 positive responses were significantly higher in Group 3 compared with Group 1 on day 168 (p=0.0057, Figure 4.5).

There was no significant increase (compared to day 0 baseline) in Ag85A-specific CD8+ responses positive for IFN-γ in any of the groups at any time point although the day 7 responses in Group 1 were trending to be higher (Figure 4.6).

Ag85A-specific CD8+ T cell responses positive for TNF-α in Group 1 increased from day 7 following aerosol prime vaccination (p=0.0303, day 0 *vs.* day 7, Figure 4.6). In Group 1 following the second vaccination (day 28, intradermal), there was no boosting of CD8+ T cell responses positive for TNF-α evident (day 28 *vs.* day 35: p=0.3750); but responses were still higher than baseline (day 0 *vs.* day 35: p=0.002, Figure 4.6). By day 168, Ag85A-specific CD8+ T cell responses positive for TNF-α in Group 1 remained elevated above baseline (day 0 *vs.* day 168: p=0.0029, Figure 4.6).

In Group 2, Ag85A specific CD8+ T cell responses positive for TNF-α were not significantly different from day 0 baseline at any timepoint; there was no boosting on day 35 compared to day 28 (day 28 *vs.* day 35 p=0.8438), and there were no differences when compared to the other two groups (all p>0.05, Figure 4.6).

In Group 3, Ag85A-specific CD8+ T cell responses positive for TNF-α increased on day 7 but were only significantly higher than day 0 baseline on day 28 (day 0 *vs.* day

28: p=0.0273). There was no boosting of Ag85A-specific CD8+ T cell responses positive for TNF-α in Group 3, either (day 28. vs day 35 p=0.6250). Responses on day 168 were not above baseline (day 0 *vs.* day 168 p=0.1563).

Ag85A-specific IL-2 and IL-17 positive CD8+ responses were undetectable.

Figure 4.5 ***Whole blood ICS Ag85A specific CD4+ T cells positive for cytokines IFN-γ, TNF-α, IL-2 and IL-17.*** *X-axis= timepoints in days, y-axis= %CD4+T cells positive for that cytokine. Statistically significant differences from baseline and between groups shown. Non-significant differences (p>0.05) omitted for clarity. * for p ≤ 0.05, ** for p≤0.01 and *** for p≤0.001*

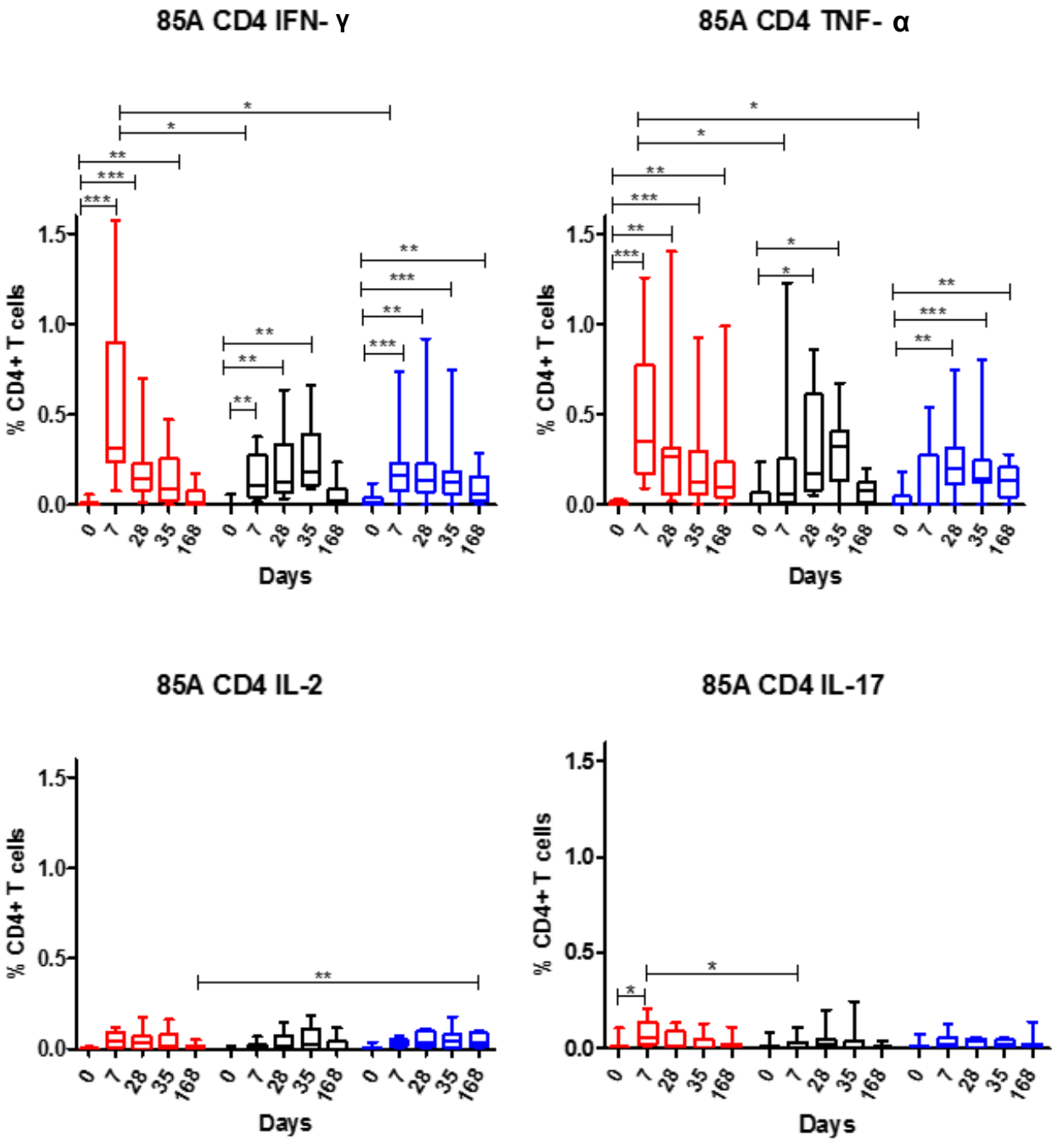
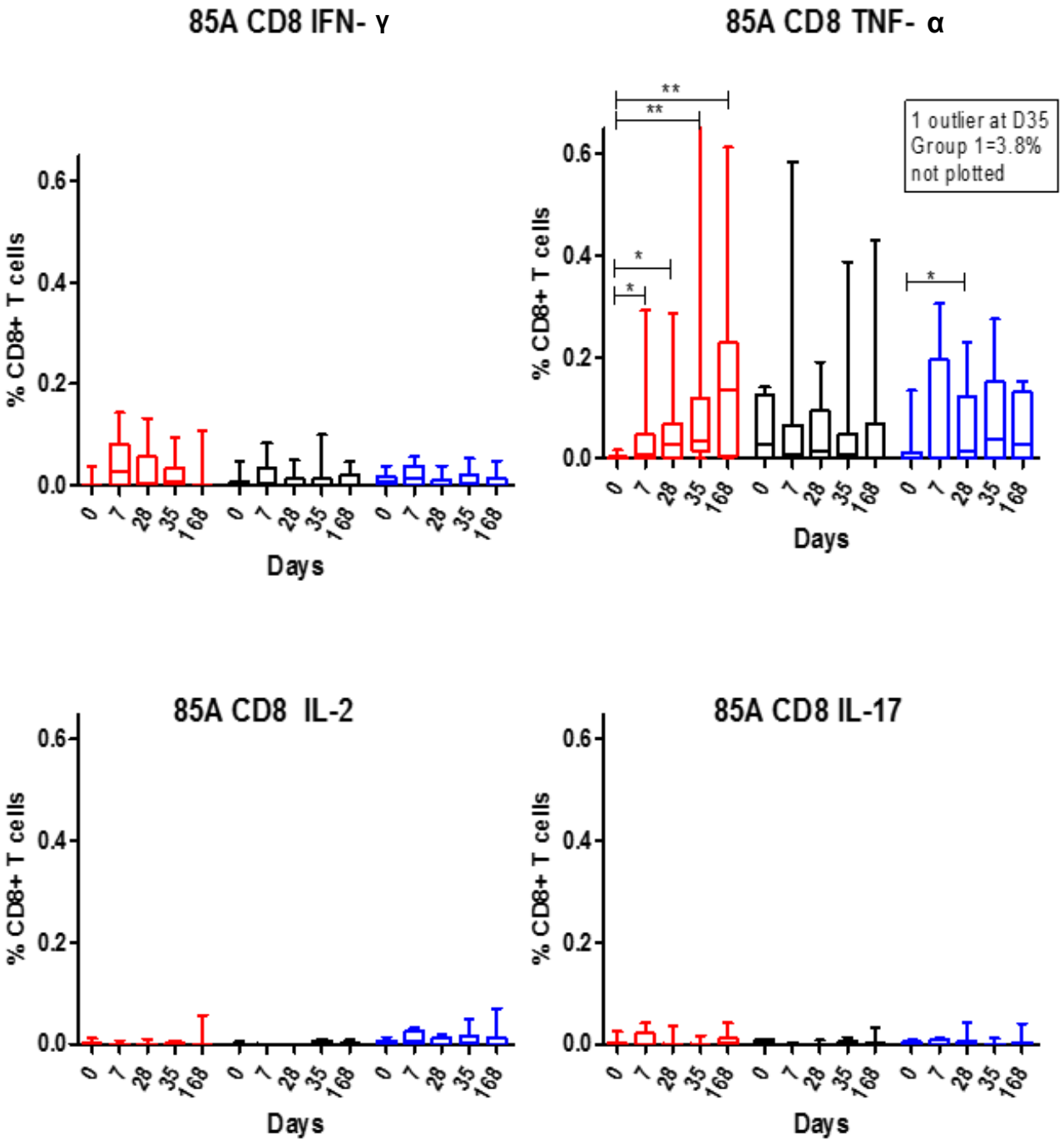


Figure 4.6 ***Whole blood ICS Ag85A specific CD8+ T cells positive for cytokines IFN-γ, TNF-a, IL-2 and IL-17.*** *X-axis= timepoints in days, y-axis= %CD8+T cells positive for that cytokine. Statistically significant differences from baseline and between groups shown. Non-significant differences (p>0.05) omitted for clarity. * for p ≤ 0.05, ** for p≤0.01 and *** for p≤0.001*



4.3.2 MVA-specific WB ICS

In Group 1, MVA-specific CD4+ T cell responses positive for IFN-γ, were not significantly different from baseline at any timepoint (all p>0.05, Figure 4.7).

In Group 2, only day 42 responses of MVA-specific CD4+ T cells positive for IFN-γ were significantly increased above baseline (day 0 *vs.* day 42: *p*=0.0313, Figure 4.7).

In Group 3, MVA-specific CD4+ T cell responses positive for IFN-γ were only significantly increased above baseline on day 14 (day 0 *vs.* day 14: *p*=0.0371, Figure 4.7).

There was no boosting of MVA-specific CD4+ responses positive for IFN-γ following the second vaccination in any of the groups (day 28 *vs.* day 42 *p*>0.05 for all groups, Figure 4.7). Comparison of day 42 MVA-specific CD4+ responses positive for IFN-γ showed those in Groups 2 and 3 were significantly higher than in Group 1 (*p*=0.0253 and *p*= 0.0308 respectively). On day 168, MVA-specific CD4+ responses positive for IFN-γ were not above baseline in any of the groups (all *p*>0.05, Figure 4.7).

MVA-specific CD4+ responses positive for TNF-α in Group 1 were significantly higher than baseline on day 28 (day 0 *vs*. day 28: *p*=0.0195) and day 42 (day 0 *vs.* day 42: *p*=0.0371) but this was not maintained until day 168 (day 0 *vs.* day 168: *p*=0.0537). In Groups 2 and 3 MVA-specific CD4+ T cell responses positive for TNF-α were never above baseline. There was no boosting of MVA-specific CD4+ T cell responses positive for TNF-α following the second vaccination in any group (day 28 *vs.* day 42 all p>0.05 Figure 4.7).

For MVA–specific CD4+ T cell responses positive for IL-2 and IL-17, none of these were above baseline and there were no significant differences between the groups (all p>0.05, Figure 4.7).

MVA-specific CD8+ T cell responses positive for IFN-γ were significantly above baseline in Group 1 on day 14 (day 0 *vs.* day 14: *p*=0.0248), day 28 (day 0 *vs.* day 28: *p*=0.0078) and day 42 (day 0 *vs.* day 42: *p*=0.0156) but this response contracted and was no longer significant above baseline by day 168 (day 0 *vs.* day 168, *p*=0.1094, Figure 4.8). There was no boosting of MVA-specific CD8+ T cell responses positive for IFNγ in Group 1 following the intradermal boost vaccination.

In Group 2, MVA-specific CD8+ IFN-γ+ responses were significantly above baseline on day 14 (day 0 *vs.* day 14: *p*=0.0156) but no longer above baseline on day 28 (day 0 *vs.* day 28: *p*=0.0625, Figure 4.8). Group 2 was the only group in which MVA-specific CD8+ responses positive for IFN-γ were boosted following the second (aerosol) vaccination (day 28 *vs.* day 42 *p*=0.0156). Comparison of day 42 MVA-specific CD8+IFN-γ+ T cell responses in Group 2 compared to the other two groups demonstrated these to be significantly higher than those in Group 3 (*p*=0.0363, corrected for multiple comparisons). Group 2 was also the only group to still have MVA-specific CD8+IFN-γ+ T cell responses above baseline by day 168 (day 0 *vs*. day 168: *p*=0.0156).

In Group 3, MVA specific CD8+ T cell responses positive for IFN-γ were not significantly above baseline at any timepoint.

Figure 4.7 ***Whole blood ICS MVA specific CD4+ T cells positive for cytokines IFN-γ, TNF-α, IL-2 and IL-17.*** *X-axis= timepoints in days; y-axis= %CD4+T cells positive for that cytokine. Statistically significant differences from baseline and between groups shown. Non-significant differences (p>0.05) omitted for clarity. * for p ≤ 0.05, ** for p≤0.01 and *** for p≤0.001*

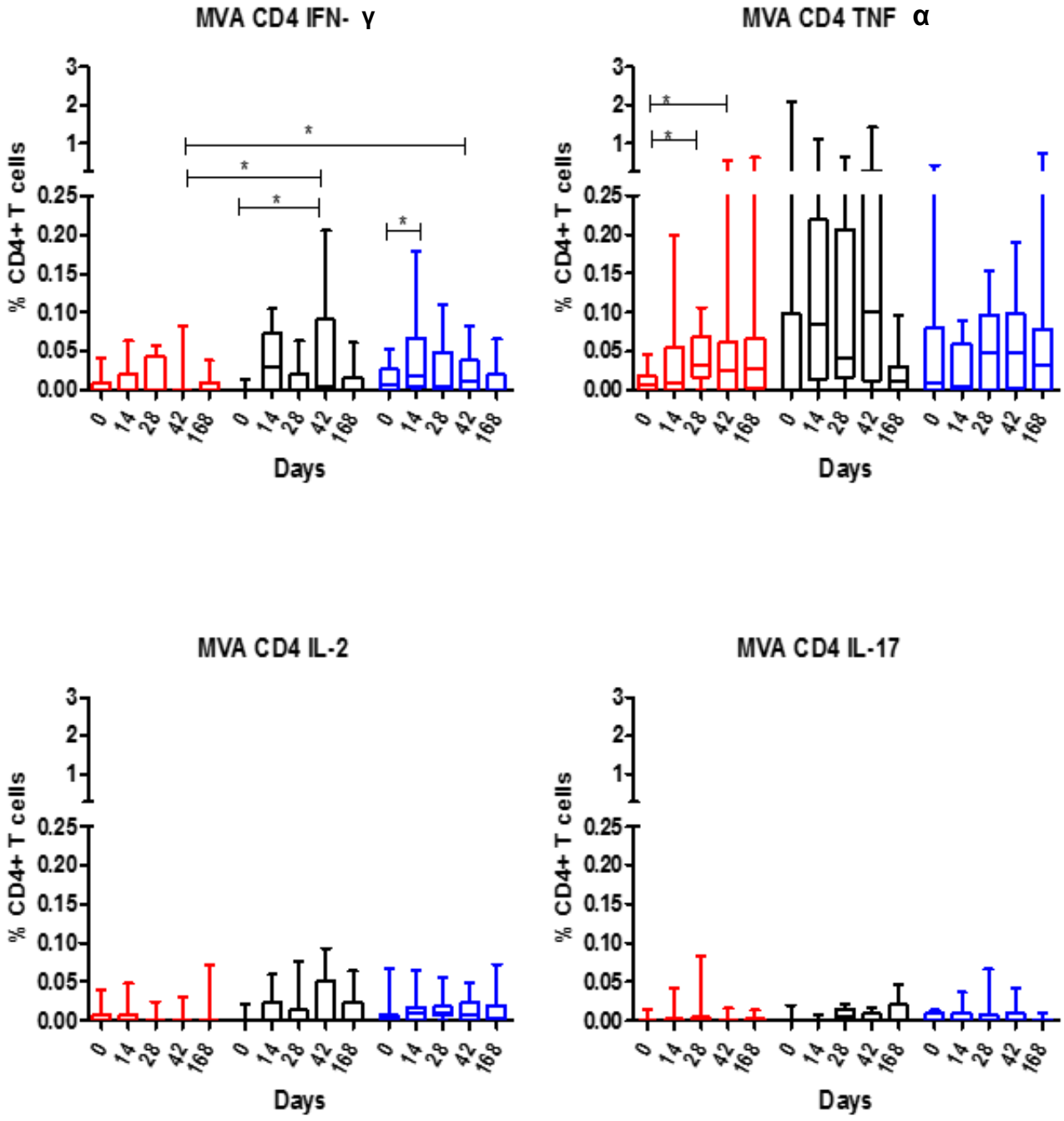
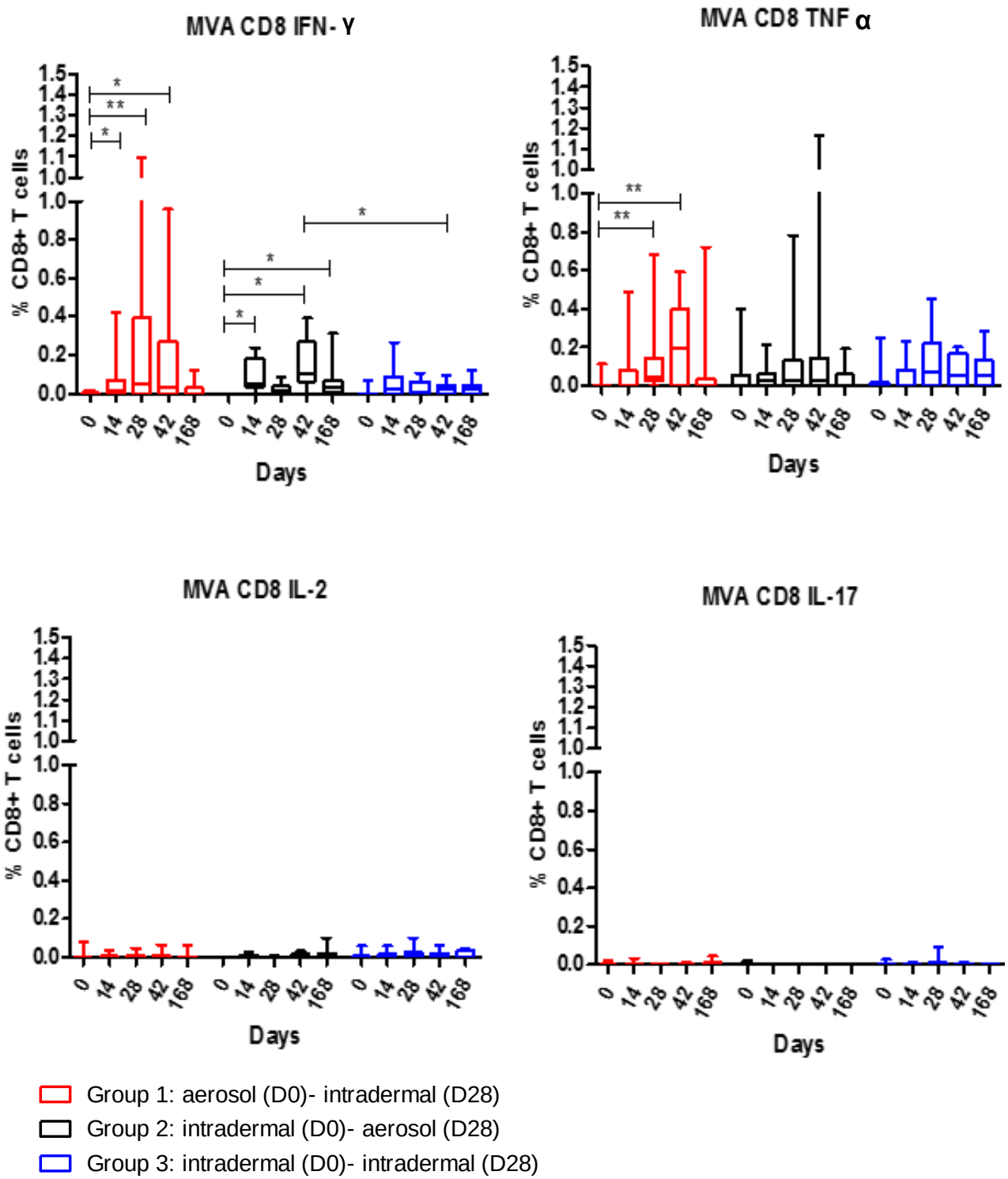


Figure 4.8 ***Whole blood ICS MVA specific CD8+ T cells positive for cytokines IFN-γ, TNF-a, IL-2 and IL-17****. X-axis= timepoints in days, y-axis= %CD8+T cells positive for that cytokine. Statistically significant differences from baseline and between groups shown. Non-significant differences (p>0.05) omitted for clarity. * for p ≤ 0.05, ** for p≤0.01 and *** for p≤0.001*



4.3.3 Polyfunctional WB ICS

Next, polyfunctionality in T cells detected in WB ICS samples was examined, given the significance of polyfunctional T cells in mycobacterial immunity as described in Chapter 1: Introduction (p.36). Only detectable functional phenotypes are summarised here. Cytokines are abbreviated as "g" for IFN-γ, "t" for TNF-α, "2" for IL-2 and "17" for IL-17 for clarity.

4.3.3.1 Ag85A-specific Polyfunctional CD4+T cells

Ag85A-specific polyfunctional CD4+ T cells are summarised in Figure 4.9. Overall the highest frequency of Ag85A-specific polyfunctional CD4+T cells was detected in Group 1 at day 7. At this day 7 time point, for all depicted phenotypes, the percentages in Group 1 were significantly higher than at day 0 baseline, with the exception of phenotype t+2+, where the increase was significant only by day 28 (day 0 *vs.* day 28: p=0.002). There was no detectable boosting of any functional phenotypes at day 35 in any of the groups (day 28 *vs*. day 35 p>0.05 for all three groups in all phenotypes).

For the g+t+2+17+ phenotype, the percentage of Ag85A-specific CD4+ T cells in Group 1 increased on day 7 (day 0 *vs.* day7: p=0.002) peaked on day 35 (day 0 *vs.* day 35: p=0.0078) and remained significantly above baseline by day 168 (day 0 *vs.* day 168: p=0.0156, Figure 4.9). In Group 2, the g+t+2+17+ phenotype of Ag85A-specific CD4+ T cells followed a similar pattern to Group 1, with an increase on day 7 (day 0 *vs.* day 7: p=0.0156), which then contracted but remained significantly above baseline by day 168 (day 0 *vs.* day 168: p=0.0469, Figure 4.9). In Group 3, the g+t+2+17+ phenotype of Ag85A-specific CD4+ T cells increased on day 7 (day 0 *vs.* day 7 p=0.0313), and then contracted, still remaining above baseline on day 168 (day 0 *vs.* day 168 p=0.0156, Figure 4.9). The magnitude of Group 3 responses for this

g+t+2+17+ phenotype throughout was slightly lower than in the other two groups although the difference was only significant compared to Group 2 on day 35 (Group 2 *vs.* Group 3 on day 35: *p*=0.0386, corrected for multiple comparisons, Figure 4.9).

The t+17+ phenotype of Ag85A-specific CD4+ T cell responses, in Group 1 increased significantly on day 7 then declined, in Group 2 did not increase above day 0 baseline, and in Group 3 increased on day 7, peaked on day 28 and then declined (Group 1 day 0 *vs.* day 7: p 0.0273; Group 2 day 0 *vs.* day 28: *p*=0.0195; Figure 4.9).

For the g+t+17+ phenotype of Ag85A-specific CD4+ T cell responses, in Group 1 levels increased on day 7 before declining (day 0 *vs.* day 7: *p*=0.0137; Figure 4.9). In Group 2, the pattern was similar and levels of Ag85A-specific g+t+17+ CD4+ T cell responses were never above baseline. In Group 3 levels were significantly above baseline from day 7 until day 35 (day 0 *vs.* day 7: *p*=0.002; day 0 *vs.* day 28: *p*=0.0059; day 0 *vs.* day 35: *p*=0.0068, Figure 4.9).

For the g+2+ phenotype, Ag85A-specific CD4+ T cells peaked on day 7 in all 3 groups (day 0 *vs.* day 7 Group 1: *p*=0.002, Group 2: *p*=0.0391, Group 3: *p*=0.0156, Figure 4.9). In Group 1, g+2+ Ag85A-specific CD4+ T cell levels remained significantly above baseline on day 168 (day 0 *vs.* day 168 *p*=0.0078). In Group 2 levels were still significant above baseline on day 35, and also significantly higher than responses in Groups 1 and 3 at that timepoint (Group 2 day 0 *vs.* day 35 *p*=0.0156; Group 2 *vs.* Group 1: *p*=0.0324; Group 2 *vs.* Group 3: 0.0017, adjusted for multiple comparison). By day 168, responses were no longer significantly above baseline (Group 2 day 0. *vs*. day 168: *p*=0.0625, Figure 4.9)

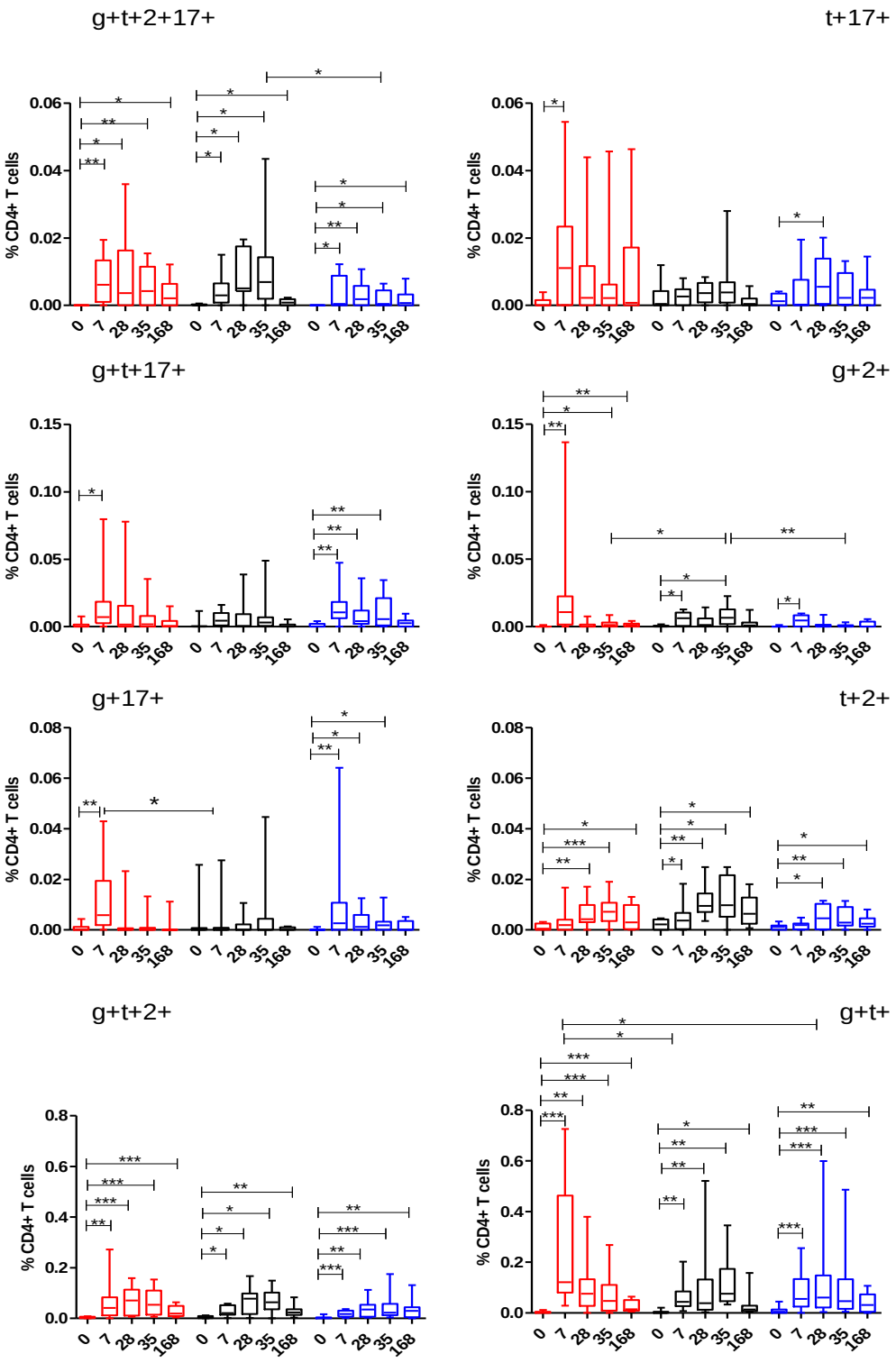
For the g+17+ phenotype, Ag85A-specifc CD4+ T cell levels in Groups 1 and 3 were higher than baseline on day 7 (day 0 *vs.* day 7: Group 1 *p*=0.0059, Group 3 *p*=0.0078).

This increase was sustained until day 35 in Group 3 only (day 0 *vs.* day 35: p=0.0117). Responses of Ag85A-specifc CD4+ T cells positive for g+17+ in Group 2 were never above baseline (all p>0.05).

The pattern of responses for Ag85A-specific CD4+ T cells for the t+2+ and g+t+2+ phenotype was similar: peaking on day 28 for Group 1, peaking on day 35 for Groups 2 and 3, and still elevated above baseline on day 168 in all three groups (day 0 *vs.* day 168: p<0.05 for all, Figure 4.9)

The Ag85A-specific CD4+ T cell responses for the g+t+ phenotype increased on day 7 for all three groups (day 0 *vs.* day 7: Group 1 p=0.0005, Group 2 p=0.0078, Group 3 p=0.0010) and remained higher than baseline up until day 168 (day 0 *vs.* day 168 Group 1 p=0.0005; Group 2 p= 0.0391; Group 3 p= 0.0039). The peak response compared to baseline, in Group 1 was on day 7 (p=0.0005), for Group 2 on day 35 (p=0.0078), and for Group 3 levels were similar in magnitude across days 7, 28 and 35 (p<0.001 for all 3 timepoints). The latter two Ag85A-specific polyfunctional CD4+T cell phenotypes (g+t+2+ and g+t+) were highest in magnitude.

Figure 4.9 ***Whole blood ICS Ag85A-specific polyfunctional CD4+ T cells*** *(detectable functional phenotypes), x= timepoints in days, % of CD4T cells, Cytokines: g= IFN-γ, t= TNF alpha, 2= IL-2, 17= IL- 17. Statistical significant differences shown: p>0.05 omitted. * for p ≤ 0.05, ** for p≤0.01 and *** for p≤0.001*



4.3.3.2 MVA-specific Polyfunctional CD4+ T cells

As before, only detectable functional phenotypes are summarised (Figure 4.10). Phenotypes of MVA-specific polyfunctional CD4+ T cell responses, when detectable, were of highest frequency in Group 2 on day 42 (Figure 4.10). However, none of the MVA-specific polyfunctional CD4+ T cell responses in Group 2 were significantly higher on day 42 (2 weeks following aerosol boost vaccination) compared to day 14 or day 28 (following intradermal prime vaccination); i.e. not boosted (data not shown).

Responses for the g+t+2+17+, g+t+17+, g+17+ and t+17+ phenotypes of MVA-specific CD4+ T cells were never above day 0 baseline for any of the groups and there were no significant differences between the groups for any of the phenotypes (Figure 4.10).

For the g+t+2+ phenotype, MVA-specific CD4+ responses above day 0 baseline were detected in Group 2 on day 42 (day 0 *vs.* day 42 p=0.0078) and in Group 3 on day 14 (day 0 *vs.* day 14 *p*=0.0425, Figure 4.10).

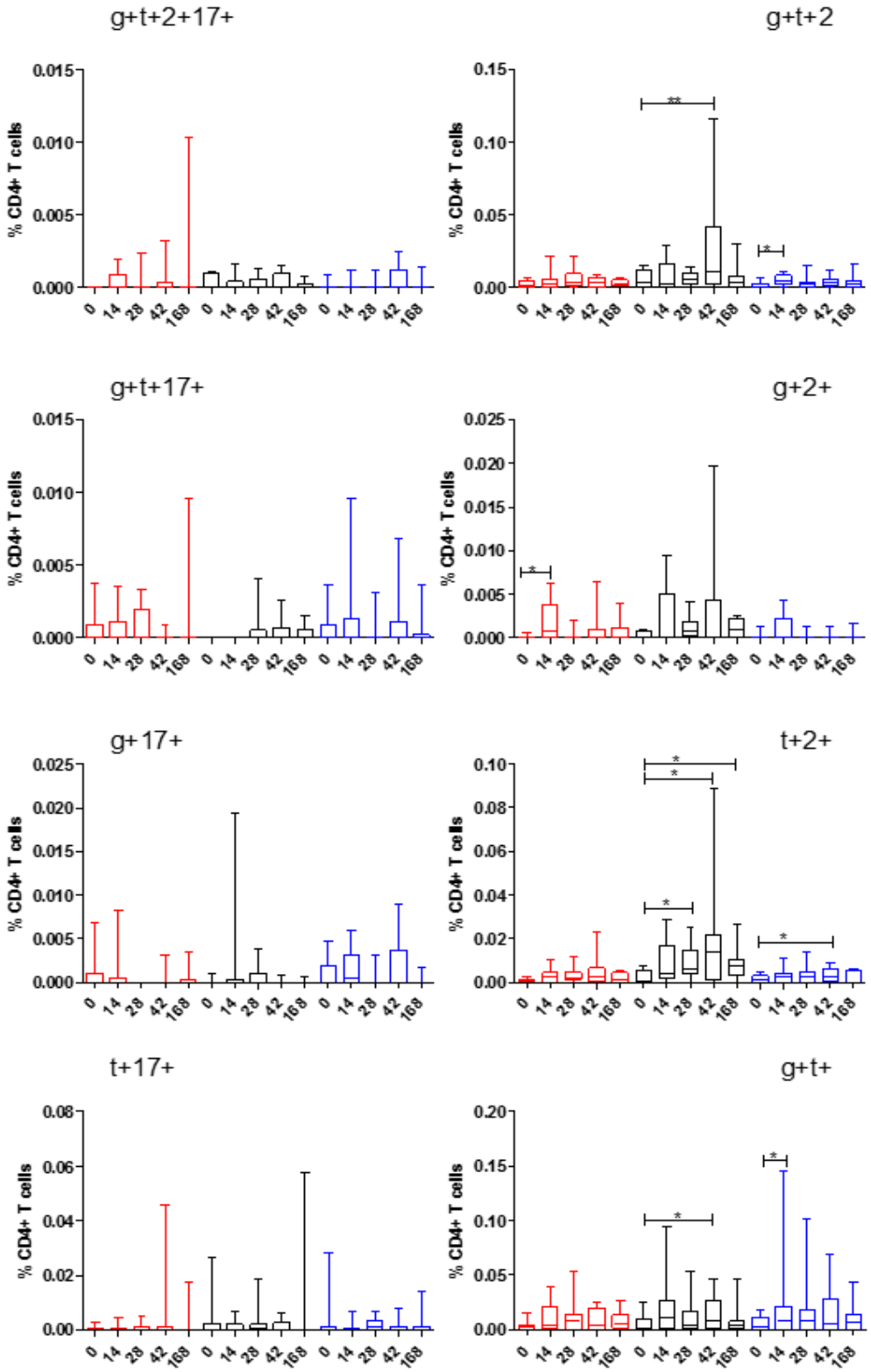
For the g+2+ phenotype of MVA-specific CD4+ T cells, responses were only significantly above baseline for Group 1 on day 14 (day 0 *vs.* day 14: p=0.0313). For Groups 2 and 3, MVA-specific CD4+response of the g+2+ phenotype were detectable on day 14 and day 42 for Group 2 and day 14 for Group 3; but none were significantly above baseline (all $p>0.05$, Figure 4.10).

For the t+2+ phenotype of MVA-specific CD4+ T cells, responses were above baseline from day 28 until day 168 for Group 2 (day 0 *vs.* day 168 p=0.0156) and on day 42 only for Group 3 (day 0 *vs.* day 42: *p*=0.0137, Figure 4.10).

For the g+t+ phenotype of MVA-specific CD4+ T cells, responses were above baseline on day 42 for Group 2 (day 0 *vs.* day 42 p=0.0313) and day 14 for Group 3 (day 0 *vs.* day 14: p=0.0488). No significant differences between the three groups were detected for any of the MVA-specific CD4+ polyfunctional T cell phenotypes.

Correlation of day 14 or day 28 WB ICS MVA-specific CD4+ IFN-γ *vs.* day 35 WB ICS Ag85A-specific CD4+ IFN-γ responses showed marginally significant correlations between day 14 MVA- and day 35 Ag85A- specific responses for Group 3 (r=0.5845, p=0.0489; data not shown), and between day 28 MVA-and day 35 Ag85A- specific for Group 2 (r=0.7137, p= 0.0369; data not shown).

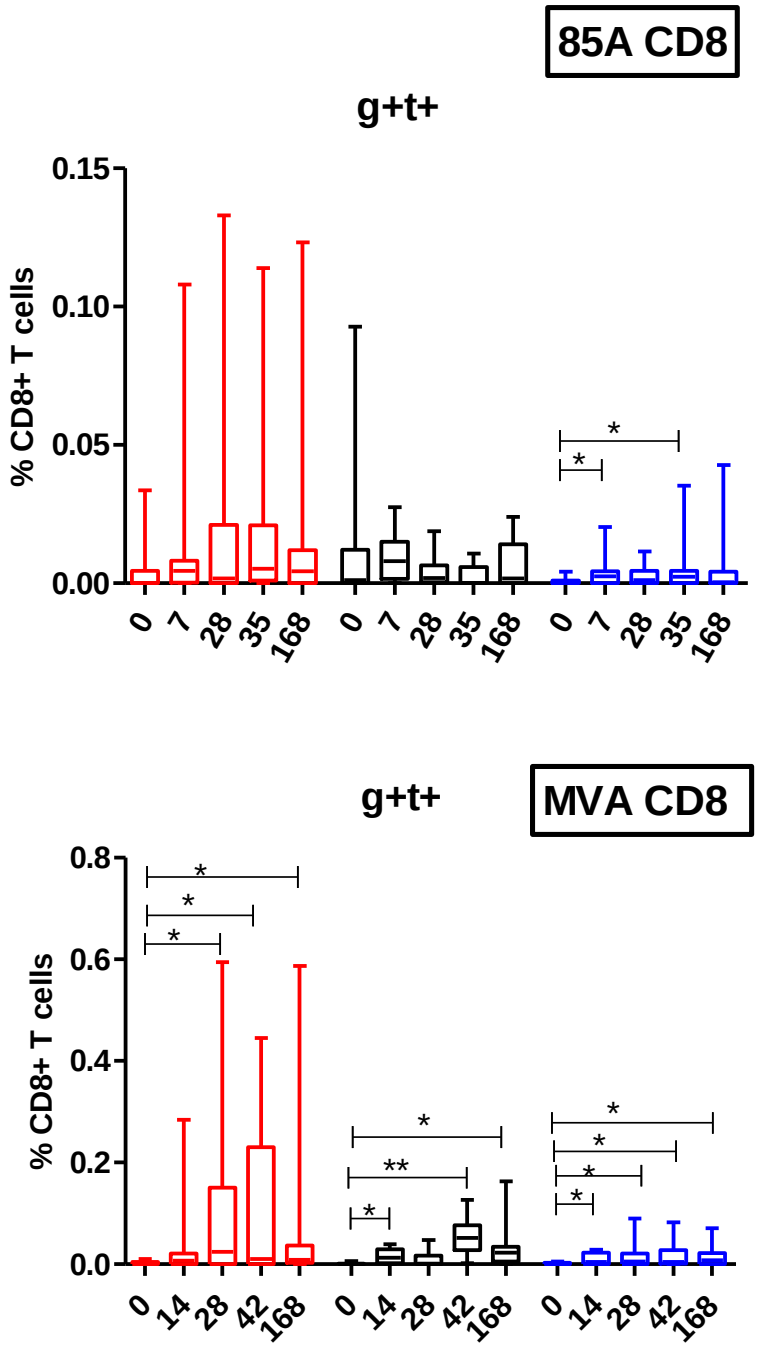
Figure 4.10 ***Whole blood ICS MVA specific polyfunctional CD4+ T cells*** ***(****detectable functional phenotypes). x= timepoints in days, % of CD4T cells, Cytokines: g= IFN-γ, t= TNF alpha, 2= IL-2, 17= IL- 17. Statistical significances shown: p>0.05 omitted. * for p ≤ 0.05, ** for p≤0.01 and *** for p≤0.001*



4.3.3.3 Antigen-specific Polyfunctional CD8+T cells

Ag85A-specific and MVA-specific polyfunctional CD8+ T cells detected in WB ICS are summarised in Figure 4.11. The only detectable functional phenotype was g+t+. There were no significant differences between the three groups. Levels of g+t+ Ag85A-specific polyfunctional CD8+ T cells were detectable in all 3 groups but were only marginally significant above baseline in Group 3 on day 7 and day 28 (day 0 *vs.* day 7 p=0.0391 and day 0 *vs.* day 28 p=0.0391). For MVA-specific g+t+ CD8+T cells, responses in Group1 were above baseline from day 28 until day 168 (day 0 *vs.* day 28 p=0.0117; day 0 *vs.* day 168 p=0.0137); in Group 2, the responses increased on day 7, peaked on day 42 and were still above baseline on day 168 (day 0 *vs.* day 7 p=0.0313, day 0 *vs.* day 42 p=0.0078, day 0 *vs.* day 168 p=0.0156). In Group 3 responses were above baseline from day 7 until day 168 (day 0 *vs.* day 7 p=0.0391, day 0 *vs.* day 168 p=0.0137).

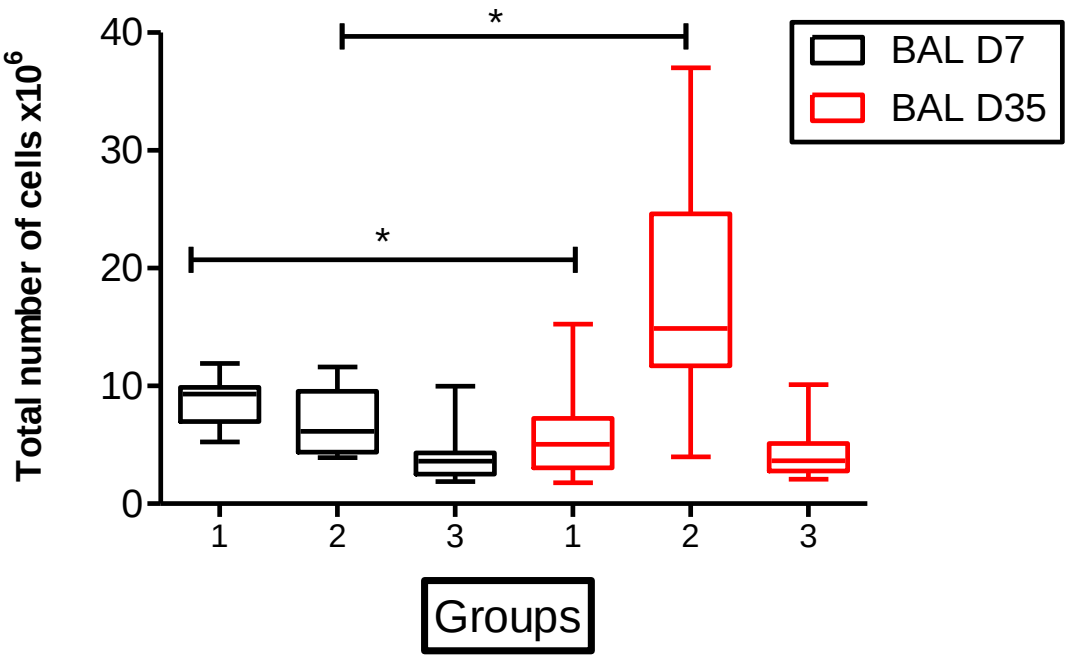
Figure 4.11 ***Whole blood ICS Ag85A (top) and MVA (bottom) specific polyfunctional CD8+ T cells (****detectable functional phenotypes). Cytokines: g= IFN-γ, t= TNF alpha. Statistically significant differences from baseline shown. * for p ≤ 0.05, ** for p≤0.01 and *** for p≤0.001. No difference between the groups.*



4.4Bronchoalveolar lavage ICS

BAL was processed and stimulated fresh as described in Chapter 2: Materials and methods (p.79). Total BAL cells were counted prior to stimulation and the comparative counts are presented in the following figure (Figure 1.12). BAL cell counts were higher on day 7 than day 35 in Group 1, higher on day 35 than day 7 in Group 2 and unchanged in Group 3 (day 7 *vs.* day 35: Group 1 *p*=0.0376, Group 2 *p*=0.0117, Group 3 *p*=0.1763; Figure 1.12). There were no differences in BAL volumes from day 7 to day 35 between the groups (day 7 *vs.* day 35: Group 1 *p*=0.4142, Group 2 *p*=0.5143, Group 3 *p*=0.4800; data not shown).

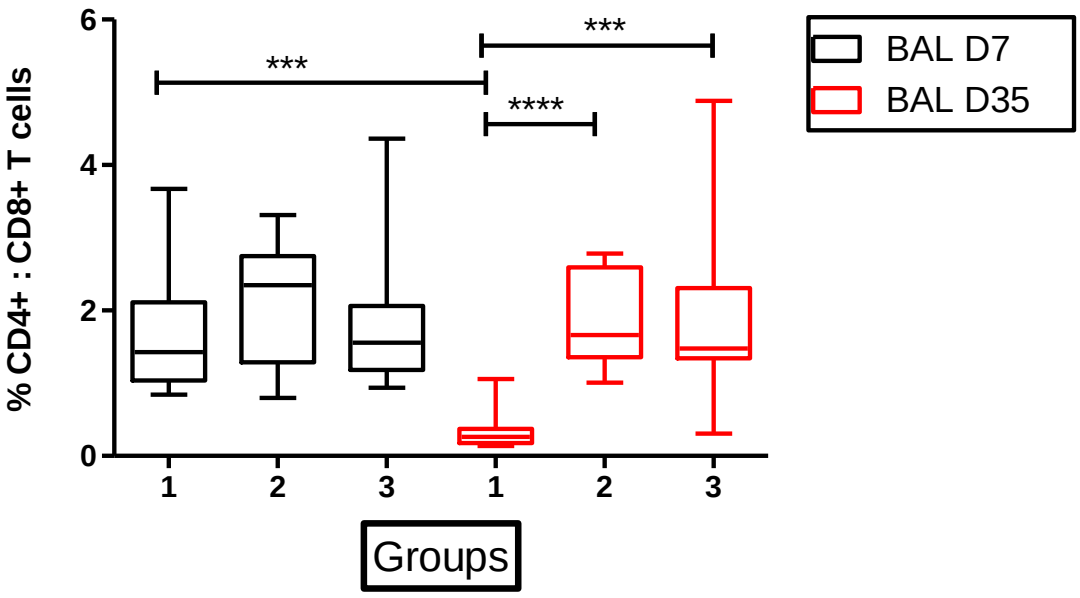
Figure 4.12 ***Total number of cells recovered from the BAL at day 7 and day 35 by group.*** *BAL was spun and resuspended in fixed volume for counting. Black day 7 BALs (Groups 1-3), Red Day 35 BAL (Groups 1-3)*



There were no correlations between volume of BAL fluid obtained, lung function of the volunteers and BAL cell counts (data not shown, corrected for multiple comparisons).

The ratio of total percentages of CD4+ to CD8+ T cells in the different groups were also examined (Figure 4.13). The CD4:CD8 ratio in Group 1 dropped significantly on day 35 compared to day 7 (day 7 *vs.* day 35 p=0.0005) due to an increase in the percentage of CD8+ T cells and also a reduction in the percentage of CD4+ T cells (day 7 *vs.* day 35: CD8+ p=0.0005, CD4+ p=0.0005, data not shown). The CD4:CD8 ratio in Group 1 on day 35 was significantly lower than that in Groups 2 and 3 on day 35 (p <0.0001 *vs.* Group 2, p=0.0002 *vs*. Group 3).

Figure 4.13 ***Ratio of percentages of total CD4+ and CD8+ T lymphocytes*** *detected on day 7 and day 35 in BAL, by group*



4.4.1 Antigen specific BAL ICS

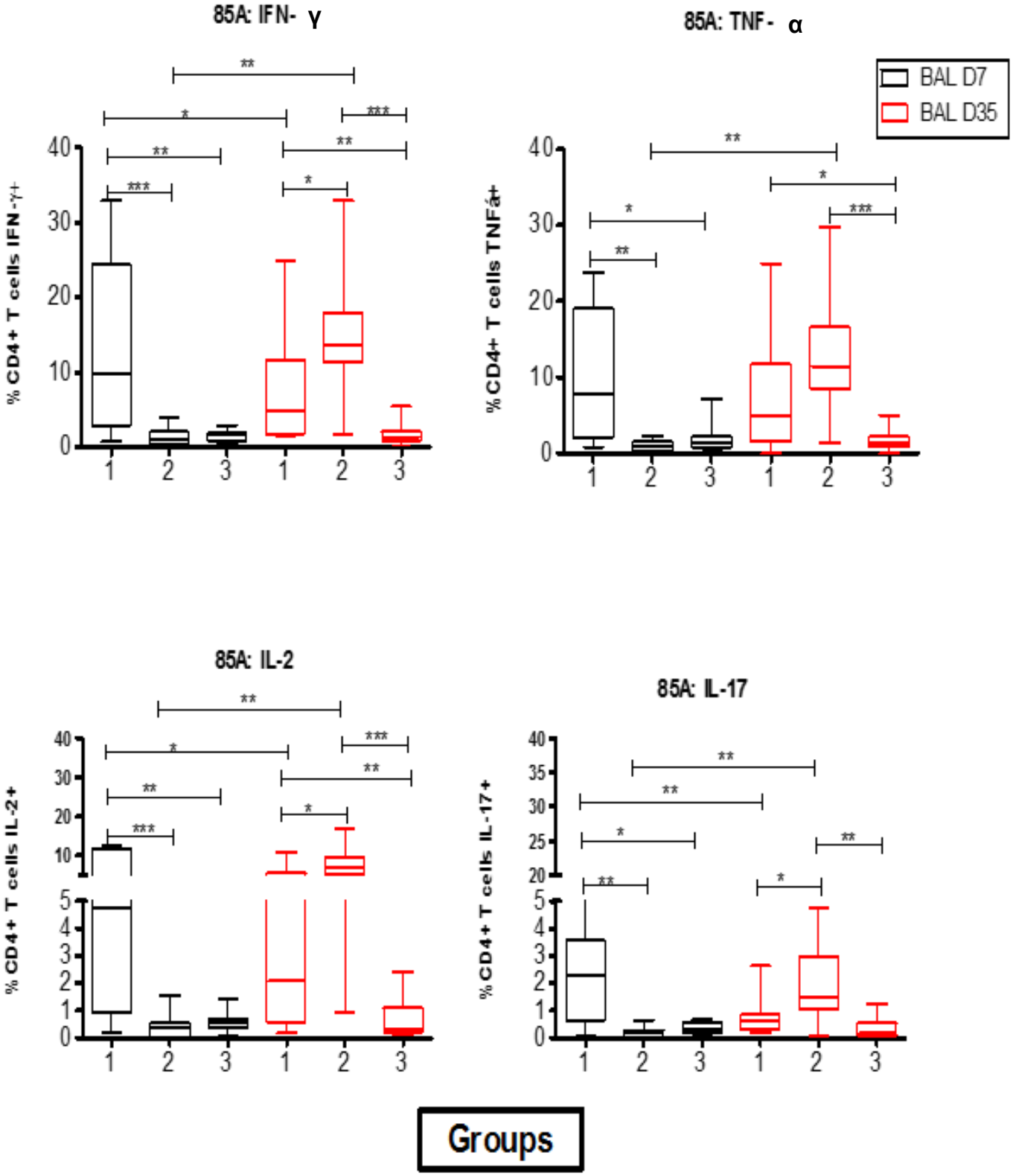
Next, summarised are Ag85A-specific CD4+ T cell responses in BAL positive for IFN-γ, TNF-α, IL-2 and IL-17 (Figure 4.14).

Comparison of day 7 BALs between the three groups, 1 week after the first vaccination, showed that Group 1 had the highest percentage of Ag85A-specific CD4+ T cells for all cytokines; significantly higher than those in Group 2 or Group 3 (Group 1 *vs.* Group 2 p=0.0009 for IFN-γ, p=0.0014 for TNF-α, p=0.0007 for IL-2, p=0.0016 for IL-17; Group 1 *vs.* Group 3 p=0.0020 for IFN-γ; p=0.0102 for TNF-α, p=0.0014 for IL-2, p=0.0102 for IL-17; corrected for multiple comparisons).

Following the second vaccination, comparing BALs on day 35, the percentages of cytokine positive Ag85A-specific CD4+ T cells were highest in Group 2; higher than Groups 1 and 3 for IFN-γ, IL-2 and IL-17 and higher than Group 3 for TNF-α (Group 2 *vs.* Group 1 p=0.0428 for IFN-γ, p= 0.0209 for IL-2, p= 0.0302 for IL-17; Group 2 *vs.* Group 3 p=0.0003 for IFN-γ, p=0.0002 for IL-2, p=0.0030 for IL-17, p=0.0004 for TNF-α; corrected for multiple comparisons).

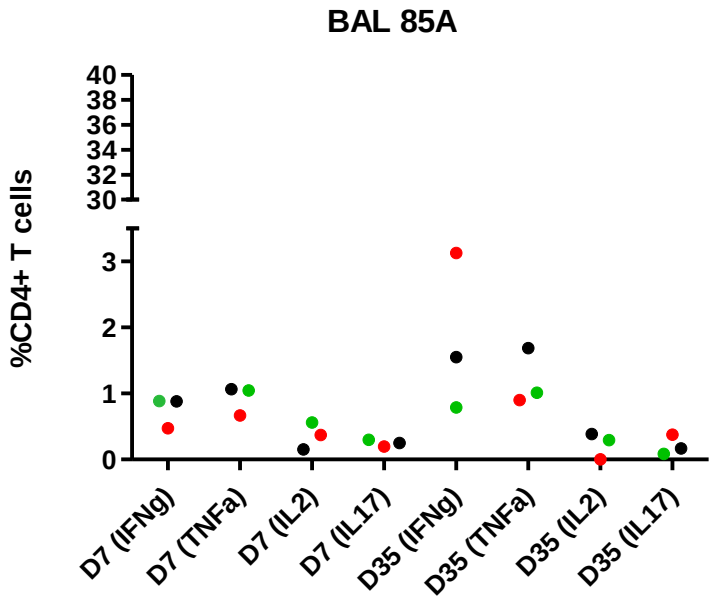
Also, day 35 BAL cytokine positive Ag85A-specific CD4+ T cell responses in Group 2 were higher than those on day 7 (day 7 *vs.* day 35 p=0.0039 for all 4 cytokines).

Figure 4.14 ***Ag85A specific CD4+ T cell responses detected by BAL ICS positive for IFN-γ, TNF-a, IL-2 or IL-17.*** *Statistically significant differences from baseline and between groups shown. Non-significant differences (p>0.05) omitted for clarity. * for p ≤ 0.05, ** for p≤0.01 and *** for p≤0.001*



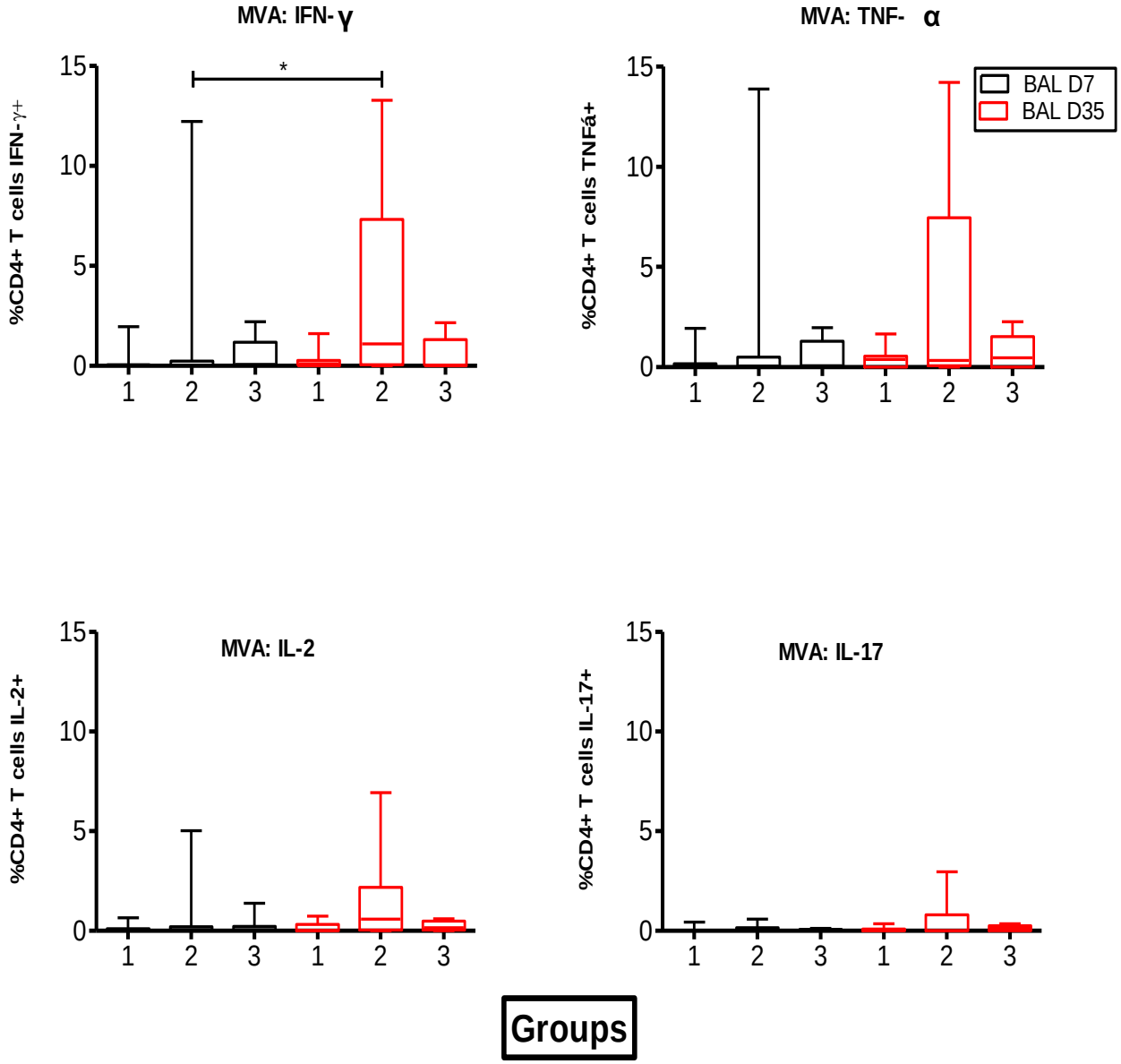
Three volunteers enrolled into Group 2 received an intradermal prime vaccination but no boost vaccination due to an urgent safety protocol amendment. However, these volunteers still underwent a bronchoscopy with BAL on day 35. Shown here are their Ag85A-specific CD4+ T cell responses (Figure 4.15). Responses for IFN-γ and TNF-α positive Ag85A-specific CD4+ T cells were slightly higher on day 35 than day 7 despite not having received any boost vaccination on day 28 (sample size too small for statistical comparison).

Figure 4.15 ***Ag85A specific CD4+ T cell responses detected in BAL ICS on day 7 and day 35 in Group 2 volunteers who received intradermal prime vaccination but no boost vaccination on day 28 (n=3),*** *Each of the three volunteers represented in a different colour; from left to right day 7 BAL (IFN-γ, TNF-α, IL-2, IL-17) and day 35 BAL (IFN-γ, TNF-α, IL-2, IL-17)*



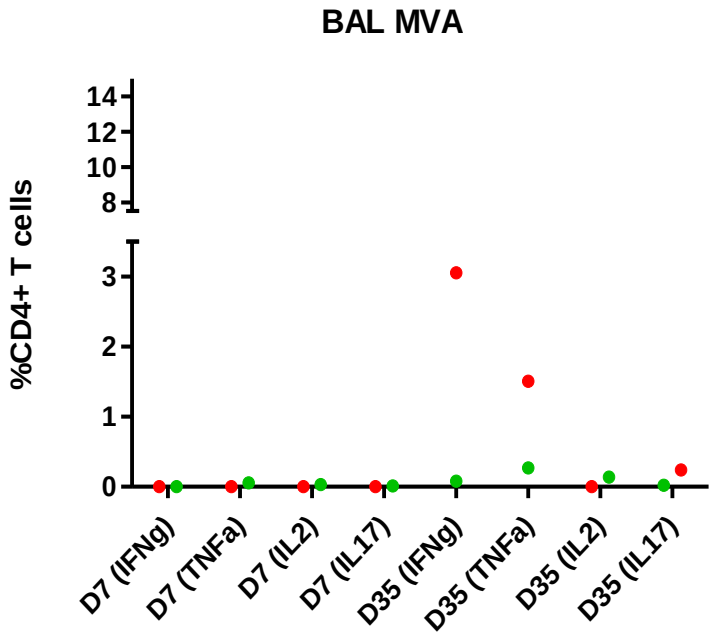
Next, shown are MVA-specific CD4+ T cell responses detected by BAL ICS positive for IFN-γ, TNF-α, IL-2 and IL-17 (Figure 4.16). The magnitude of responses overall was lower than Ag85A-specific CD4+ BAL T cell responses (Figure 4.14 and 4.16). The responses in Group 2 were significantly higher on day 35 compared to day 7 for IFN-γ (day 7 *vs.* day 35: p=0.0156) and this followed a similar pattern for the other 3 cytokines; although this was not statistically significant.

Figure 4.16 ***MVA specific CD4+ T cell responses detected by BAL ICS positive for IFN-γ, TNF-α, IL-2 or IL-17.*** *Statistically significant differences from baseline and between groups shown. Non-significant differences (p>0.05) omitted for clarity. * for p ≤ 0.05, ** for p≤0.01 and *** for p≤0.001*



Comparison of responses in the three volunteers in Group 2, who were subjected to the urgent safety amendment and only received an intradermal prime but no boost vaccination, was only possible for two of the three volunteers, as one volunteer did not have a high enough cell count for BAL cells to be stimulated with MVA (Figure 4.17). One of the two volunteers had a higher MVA-specific CD4+ T cell response on day 35 compared to day 7 for IFN-γ and TNF-α.

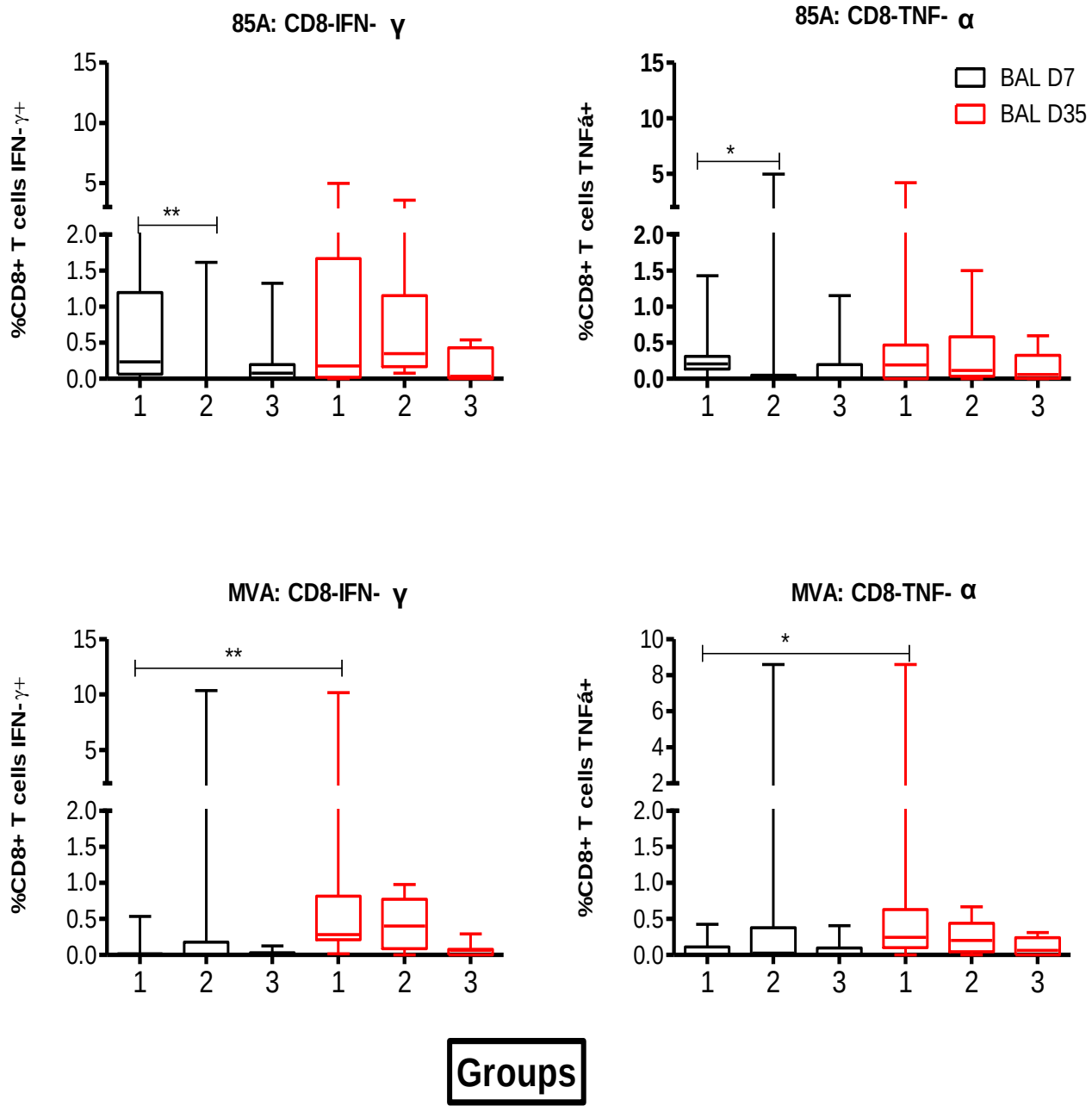
Figure 4.17 ***MVA-specific responses detected in BAL ICS on day 7 and day 35 in Group 2 volunteers who received intradermal prime vaccination but no boost vaccination on day 28 (n=3),*** *Each of the three volunteers represented in a different colour*



For Ag85A-specific and MVA-specific CD8+ BAL T cell responses only IFN-γ and TNFα positive CD8+ BAL T cells and no IL-2 and IL-17 positive CD8+ BAL T cells were detected (Figure 4.18). Following the first vaccination Ag85A-specific IFN-γ and TNF-α CD8+ T cell responses in the day 7 BAL were highest in Group 1 compared to the other two groups, but were only significantly different to Group 2 (p=0.0034, Figure

4.18). Ag85A-specific TNF-α CD8+ T cell responses in the day 35 BALs following the second vaccination were slightly higher in Groups 1 and 2 than Group 3, but this was not statistically significant. MVA-specific CD8+ T cell responses in Group 1 were higher on day 35 compared to day 7 for both IFN-γ and TNF-α (p=0.0068 and p= 0.137 respectively, Figure 4.18).

Figure 4.18 ***CD8+ T cell responses detected by BAL ICS. Ag85A specific (top row) and MVA specific (bottom row).*** *Positive for IFN-γ, TNF-a. Statistically significant differences from baseline and between groups shown. Non-significant differences (p>0.05) omitted for clarity. * for p ≤ 0.05, ** for p≤0.01 and *** for p≤0.001*



4.4.2 Polyfunctional T cell responses in BAL measured by ICS

Ag85A-specific CD4+ T cells in BAL were also examined for their ability to secrete multiple cytokines, given the significance of polyfunctional T cells in mycobacterial immunity as described in the Introduction (Chapter 1, p.36). Summarised here are detectable functional phenotypes of Ag85A-specific polyfunctional CD4+ T cells in BAL (Figure 4.19).

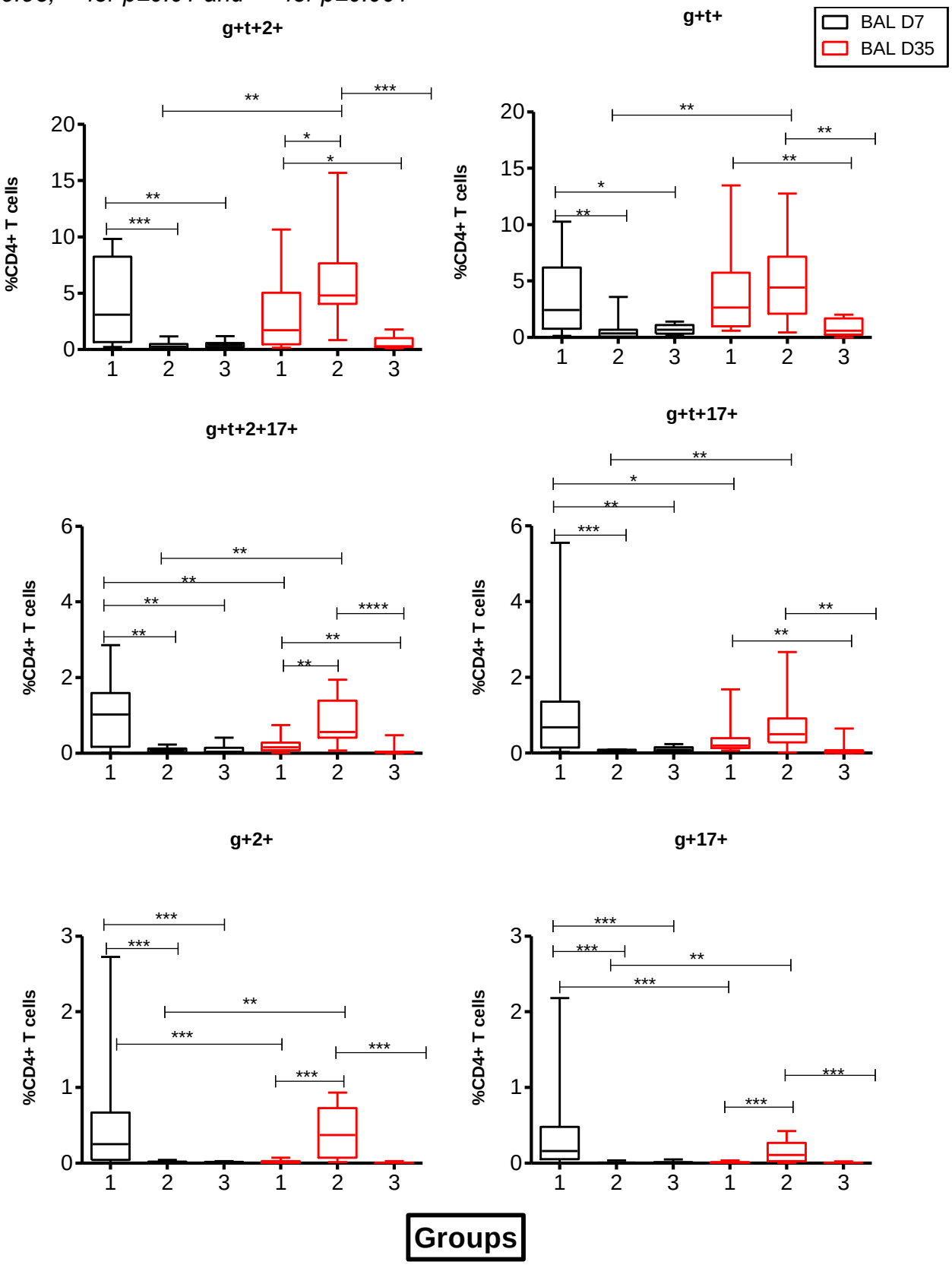
Following the first vaccination, polyfunctional CD4+ T cells were only detected in BAL of Group 1 on day 7. At this day 7 time point, these BAL responses in Group 1 were significantly higher than those in Groups 2 or Group 3 for all functional phenotype (Group 1 *vs.* Group 2: p=0.0006 for g+t+2+, p=0.0042 for g+t+, p=0.0024 for g+t+2+17+, p=0.0006 for g+t+17+, p=0.0003 for g+2+, p=0.0005 for g+17+; Group 1 *vs.* Group 3: p=0.0014 for g+t+2+, p=0.0102 for g+t+, p= 0.0047 for g+t+2+17+, p=0.0043 for g+t+17+, p=0.0001 for g+2+, p=0.0006 for g+17+; corrected for multiple comparisons).

After the second vaccination, polyfunctional CD4+ T cells in BALs on day 35 were significantly higher in Groups 1 and 2 than those in Group 3 for four subsets: g+t+2+, g+t+, g+t+2+17+ and g+t+17+; and higher in Group 2 than Groups 1 and 3 for the remaining two subsets: g+2+ and g+17+ (P values for the first four subsets respectively are: p=0.0134 for Group 1 *vs.* Group 3 and p=.0.0002 for Group 2 *vs.* Group 3 for g+t+2+; p=0.0076 for Group 1 *vs.* Group 3 and p=0.0021 for Group 2 *vs.* Group 3 for g+t+; p= 0.0074 for Group 1 *vs.* Group 3 and p<0.0001 for Group 2 *vs.* Group 3 for g+t+2+17+, p=0.0041 for Group 1 *vs.* Group 3 and p=0.0062 for Group 2

vs. Group 3 for g+t+17+; and for the remaining two subsets are: p=0.0006 for Group 1 *vs*. Group 2 and p=0.0004 for Group 2 *vs*. Group 3 for g+2+, p=0.0007 for Group 1 *vs.* Group 2 and p=0.0004 for Group 2 *vs.* Group 3 for g+17+, corrected for multiple comparisons).

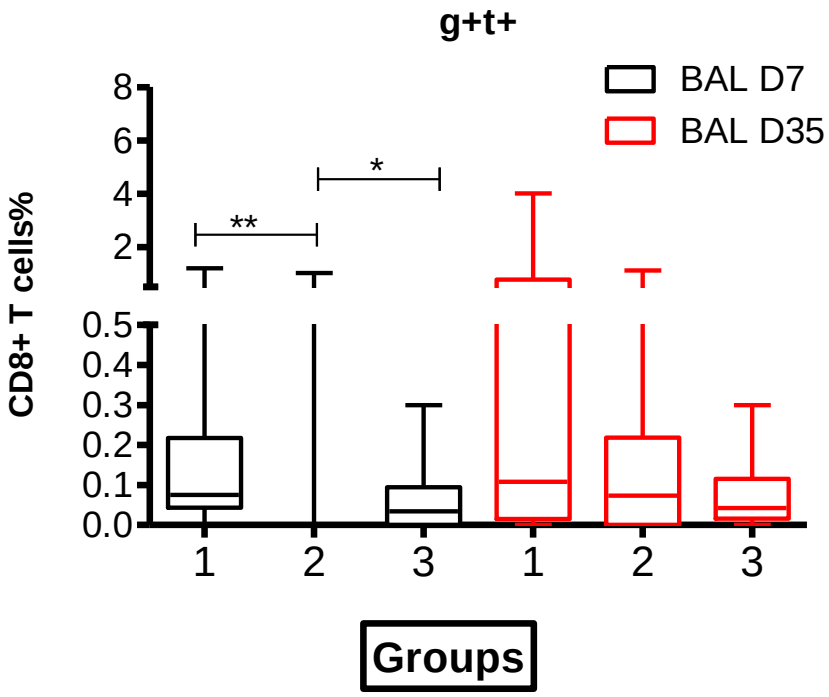
Some polyfunctional CD4+ T cell phenotypes on day 35 (e.g. quadruple cytokine positive) were almost found exclusively in Group 2 at that timepoint.

Figure 4.19 ***Ag85A-specific polyfunctional CD4+ T cells detected in BAL ICS.*** *Only detectable functional phenotypes of polyfunctional CD4+ T cell shown. Cytokines: g= IFN-γ, t= TNF alpha, 2= Interleukin 2, 17= Interleukin 17. * for p ≤ 0.05, ** for p≤0.01 and *** for p≤0.001*



Polyfunctional Ag85A-specific CD8+ T cells were also examined. As previously mentioned, IL-2 and IL-17 could not be detected. The only detectable functional phenotype was Ag85A-specific g+t+ CD8+ T cells (Figure 4.20). The highest percentage following the first vaccination in day 7 BALs was in Group 1, which was higher than that in Group 2 and 3; although only reaching statistical significance compared to Group 2 (p=0.0032). The percentage of Ag85A-specific g+t+ CD8+ T cells in Group 3 was marginally higher than that in Group 2 (p=0.0493). For day 35 BALs g+t+ CD8+ T cells, the trend was higher in Groups 1 and 2 (following a similar pattern to polyfunctional CD4+ T cells in BAL on day 35) but not statistically significant.

Figure 4.20 ***Ag85A-specific polyfunctional CD8+ T cells*** *g= IFN-γ, t= TNF-α*
*Only significant differences shown: * for p ≤ 0.05, ** for p≤0.01*



4.4.3 Correlation of vector and insert BAL T cell responses

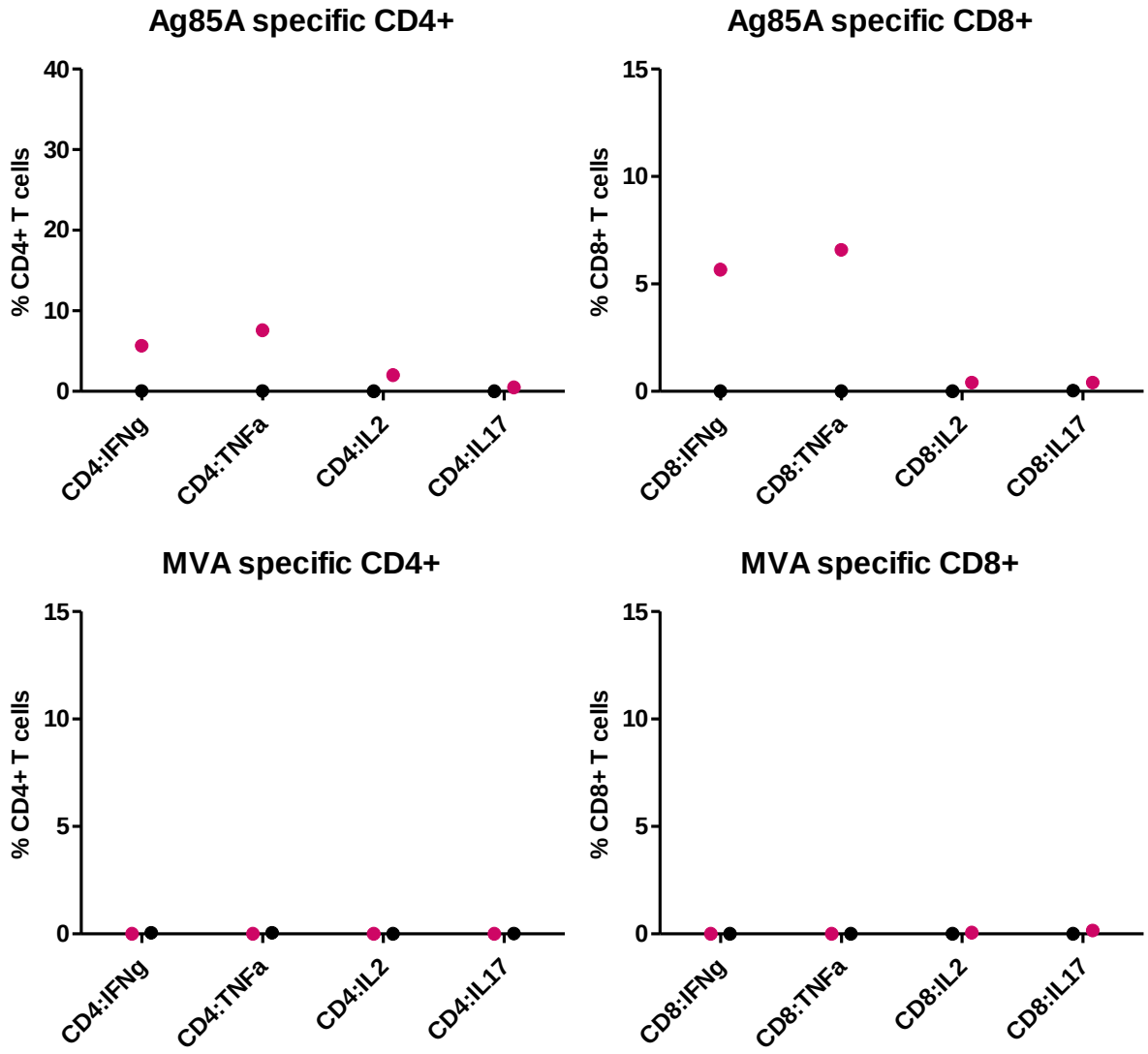
Given this clinical trial aimed to assess the functional relevance of any MVA vector-specific responses induced by a priming vaccine on the magnitude of insert immunogenicity after subsequent boosting (by heterologous routes in Group 1 and 2 or homologous routes in Group 3), the following correlation was examined: the impact of MVA-specific responses induced in BAL (day 7) by the priming vaccine on Ag85A-specific responses in the BAL after boosting (day 35). MVA-specific CD4+ and CD8+ IFN-γ BAL T cell responses on day 7 did not correlate with Ag85A-specific CD4+ and CD8+ IFN-γ BAL T cell responses on day 35 in any group (all $p>0.05$, data not shown). Furthermore, MVA-specific CD4+ and CD8+ WB ICS responses on day 28 did not correlate with Ag85A-specific CD4+ CD8+ IFN-γ BAL responses on day 35 in any group, either, i.e. no impact of anti-MVA cellular immune responses induced in the systemic compartment (WB ICS) after prime vaccination, on Ag85A-specific cellular responses in the lung mucosal compartment detected (all $p>0.05$, data not shown).

4.4.4 Unrelated BAL samples from NHS patients to determine baseline of unvaccinated population

Whilst there was a baseline for all systemic immunogenicity measures (WB ICS and IFN-γ ELISPOT) there was no baseline BAL for these volunteers and this was one of the limitations of this clinical trial. At the outset of the clinical trial, it was felt that a third (baseline) bronchoscopy was not justifiable given these were healthy volunteers and the bronchoscopy an invasive procedure. However, an attempt was made to obtain unrelated NHS patient BALs *in lieu* of baseline bronchoscopies, with the

potential confounding that these were patients and not healthy volunteers. Due to logistical and practical difficulties only two patient BALs were obtained before the end of the clinical trial. Both patients were being considered for a diagnosis of sarcoidosis. Antigen-specific CD4+ BAL T cell response are summarised in Figure 4.21. The two different volunteers are distinguished by colour and scales are matched to those of clinical trial volunteers in Figures 4.14 and 4.16 for comparison. One of the two NHS patients (in black) has no Ag85A-specific or MVA-specific CD4+ responses. The other volunteer (in magenta) had low level of Ag85A specific CD4+ and CD8+ responses but these were several fold lower in magnitude compared to the ones detected in vaccine recipients enrolled in this clinical trial (Figure 4.14).

Figure 4.21 ***Antigen specific CD4+ and CD8+ T cells detected in BAL of two NHS patients,*** *later diagnosed to have sarcoidosis. Scaled comparable to clinical trial volunteers data.*



4.5 Discussion

Presented here were the cellular immune responses to insert Ag85A and vector MVA in two different compartments measured by three different readouts: BAL ICS, WB ICS and PBMC IFN-γ ELISPOT.

Enrolled volunteers were all historically BCG-vaccinated and therefore assumed to have a certain degree of central memory responses to Ag85A, which was the rationale for boosting with MVA85A[289]. The peak of the Ag85A-specific response as measured by PBMC IFN-γ ELISPOT and WB ICS has previously been found to occur one week following intradermal and aerosol vaccination[247, 266].

Figure 4.2 highlighted that the aerosol route of vaccination induced higher magnitude Ag85A-specific IFN-γ ELISPOT responses as these were higher in Group 1 on day 7 following aerosol prime vaccination (significant compared to Group 2 alone or when Group 2 and Group 3 were combined) and higher in Group 2 (compared to the other two groups) following aerosol boost vaccination. IFN-γ ELISPOT responses on day 7 and day 35 demonstrated the potency of the aerosol route with the highest Ag85A-specific responses seen in Group 1 on day 7 (following aerosol prime) and in Group 2 on day 35 (following aerosol boost), i.e. high systemic responses in groups who had received vaccination by the aerosol route 7 days prior. The differences between the groups were not significant by day 168 but this could be because the IFN-γ ELISPOT measures antigen-specific circulating effector T cell responses, and not central memory T cell responses[92, 290].

Ag85A-specific IFN-γ ELISPOT responses were only boosted by the second vaccination in Group 2, not Groups 1 and 3.

Ag85A-specific WB ICS responses on day 7 in Group 1 following aerosol prime vaccination, mirrored the IFN-γ ELISPOT responses and again highlighted the potency of the aerosol route, as responses in Group 1 on day 7 were significantly higher than baseline and significantly higher than in those who received the intradermal prime vaccination (i.e. Groups 2 and 3 on day 7) for IFN-γ, TNF-α and IL-17.

Ag85A-specific CD8+ T cells in WB by ICS were as expected from previous studies lower in magnitude than CD4+ T cell responses[247]. Notably, Ag85A-specific CD8+ T cells positive for IFN-γ were most detectable in Group 1 especially on day 7 (although not significant for all timepoints/cytokines, Figure 4.6). Therefore, the aerosol route still appeared potent in this context also.

These observations pose the possibility of the aerosol route being potentially dose-sparing. One explanation for this effect could be the large surface area of the lung, which is an estimated 50-75 square metres[291-293]. A measles vaccine delivered by the aerosol route yielded greater humoral immunogenicity when administered by the aerosol route even at 1/5th of the systemic dose equivalent[294]. The potential for dose-sparing was probably underestimated, as not all of the administered dose will ultimately have reached the target site, i.e. the lung mucosa[277]. A study using *in vivo* real-time scintigraphy to monitor the fate of aerosolised radiolabelled NYVAC and MVA-HPV delivered to anaesthetised non-human primates, demonstrated consistent delivery with approximately 4% reaching the lungs[259]. The device used, the rate of production of aerosol, the inspiratory flow tidal volume during the aerosol inhalation and other factors that were not measured here are likely to have influenced how much reached the target site[273].

In terms of polyfunctional Ag85A-specific CD4+ T cells measured in WB ICS, the highest responses for all detectable polyfunctional phenotypes were in Group 1 on day 7 following aerosol prime vaccination. Also, there was a tendency (although not always significant) for higher levels of polyfunctional Ag85A-specific CD4+T cells on day 35 in Group 2 following aerosol boost vaccination (e.g. t+2+ and g+t+ and g+t+2+) although not true for all depicted polyfunctional cell phenotypes. Therefore, an aerosol MVA85A vaccination given as a prime or boost (i.e. Group 1 or 2 respectively) in this clinical trial tended to have a higher capacity at inducing certain Ag85A-specific polyfunctional CD4+ T cells. Furthermore, the aerosol route of vaccination was also the most potent at inducing both Ag85A-specific CD4+ T cell responses in BAL and Ag85A-specific polyfunctional T cells in BAL. The ability of a candidate TB vaccine to induce mycobacterial antigen-specific polyfunctional T cells, in systemic and lung mucosal compartment, is likely to add to the functional benefits desired in protective TB immunity, given the presumed importance of these cells as described in Chapter 1: Introduction (p.36)[132-134].

MVA-specific CD4+ and CD8+ IFN-γ ELISPOT and WB ICS responses in previous studies were shown to peak 2 weeks after systemic vaccination[247, 248]. A similar pattern was mostly seen here for prime vaccinations in all three groups with the exception of MVA CD4-specific responses as measured by IFN-γ ELISPOT in Group 1, which peaked earlier, on day 7 response following the (aerosol) prime vaccination. This was the first time that two MVA85A vaccinations were given by heterologous routes and the first time that the effect of the MVA-specific priming responses on boosting could be observed in such a regimen. Interestingly, in addition to the Ag85A-specific boosting observed in Group 2 after the second vaccination (as measured by IFN-γ ELISPOT), there was also boosting of MVA CD4-specific and MVA CD8-specific

IFN-γ ELISPOT responses in Group 2; MVA CD4- and CD8-specific IFN-γ ELISPOT responses were increased on day 42 (i.e. 2 weeks following second vaccination) compared to day 28 in Group 2. This boosting of MVA-specific responses after the second vaccination (as measured by IFN-γ ELISPOT) was also observed for MVA CD4 epitopes in Group 1 and for MVA CD8 epitopes in Group 3 (as measured by IFN-γ ELISPOT). Therefore, Group 2, the only group to boost Ag85A-specific responses after the second vaccination was also the only group who boosted both MVA CD4-specifc and MVA CD8-specific responses (as measured by IFN-γ ELISPOT). This indicates that boosting of Ag85A-specific responses after a second vaccination can occur in parallel to boosting of MVA-specific responses (as measured by IFN-γ ELISPOT). These observations also indicate that heterologous routes MVA85A vaccination regimens (Groups 1 and 2) boosted MVA-specific CD4+cellular immunity but only intradermal-aerosol or intradermal-intradermal boosted MVA-specific CD8+ cellular immunity (as measured by IFN-γ ELISPOT).

MVA- specific CD4+ T cell responses as measured by WB ICS showed no boosting of responses in any of the groups but responses were higher on day 42 when compared with day 0 (i.e. 2 weeks after the boost) in Group 2 and 3 who had the intradermal prime vaccination. Plus, MVA-specific CD8+ IFN-γ+ T cell responses as measured by WB ICS were boosted in Group 2 only (day 42 compared to day 28). Therefore, the intradermal prime vaccination was effective at inducing priming vector responses as measured by WB ICS. In terms of MVA-specific polyfunctional CD4+ WB ICS responses the magnitude of detectable functional phenotypes was highest (compared to day 0) for Group 2 at day 42 (i.e. 2 weeks following aerosol boost vaccination); but not boosted beyond effects of the prime (i.e. compared to day 28).

Considering that boosted vector responses were predominantly seen in Group 2, one hypothesis could be whether the clinical phenotype observed in this group following the aerosol boost vaccination was attributable to a heightened reaction to the MVA vector. In further support of this, MVA-specific CD4+ BAL ICS responses for IFN-γ and TNFα were highest in the day 35 BALs of Group 2 volunteers. MVA-specific IFN-γ ELISPOT responses, comparing day 42 to day 28 responses, were also boosted in Groups 2; and boosting of both MVA CD4-specific and MVA-CD8-specific IFN-γ ELISPOT responses was exclusive to Group 2. The safety of MVA as a vaccine vector has been studied extensively in clinical trials and expected adverse events are dose-dependent comprising flu-like symptoms in the first 48 hours following vaccination suggesting innate immune response[295, 296]. In fact, the adverse events in Group 2 that prompted the safety measure usually commenced within 24 hours of vaccination and were transient with full recovery in all volunteers within 2-4 days. Therefore, if boosting of vector-specific cellular immune responses had contributed to clinical adverse events, the time course the symptoms occurred would suggest maybe innate immune response activation.

The overall BAL cell count in Group 1 was lower on day 35 (compared to day 7, Figure 4.12). At the same time, the CD4:CD8 ratio in Group 1 decreased, due to a relative increase in the percentage of total CD8+ T cells and a concurrent relative decrease in the percentage of CD4+ T cells from day 7 to day 35 BAL. This was an unexpected finding. The reduction in the percentage of CD4+ T cells from first (day 7 after aerosol prime) to second (day 35, i.e. 7 days after intradermal boost) BAL, maybe suggests migration of cells away from the lung mucosal compartment. The increase in the percentage of CD8+ T cells may have been due to a relative increase in vaccine induced CD8+ responses after the second vaccination or contraction of another cell

compartment. Another question is whether any increase in vaccine-induced CD8+ responses in the BAL has happened at a cost of vaccine induced CD4+ responses, as the percentage of CD4+ T cells was reduced from the first to the second vaccination in Group 1. In Group 2, the total BAL cell count was higher on day 35 compared to day 7 (Figure 4.12) but this was not accompanied by any change in CD4+:CD8+ ratio.

As mentioned earlier, the aerosol route of vaccination was the most favourable at inducing Ag85A-specific CD4+ T cell responses in BAL. The aerosol route of vaccination was also more potent at inducing Ag85A-specific polyfunctional T cells as measured by BAL ICS (alike WB ICS as outlined earlier).

Apart from the magnitude of measured Ag85A-specific cellular immune responses there might also be an anatomical and physiological advantage of local vaccine responses on the lung mucosa rather than in the systemic compartment alone. However, for the local responses to be recruited in the lung mucosal compartment during a subsequent exposure to *M.tb* by inhalation of aerosolised droplets, these responses need to be durable. Durability in terms of memory phenotyping of Ag85A-specific cells was not studied in this trial in either systemic or lung mucosal compartment (and there was no BAL beyond day 35), but this would be an important consideration for future studies for any candidate TB vaccine delivered by aerosol.

The cohort of the 3 volunteers who did not receive a boost vaccination demonstrated stable responses in two volunteers 35 days after intradermal priming. The decision to proceed with the bronchoscopy in these Group 2 volunteers who had not received a day 28 vaccination was for macroscopic assessment of the lung mucosa on day 35; and this was also a unique opportunity to assess magnitude of BAL responses 5 weeks after intradermal priming vaccine. The data from this small sample subset

demonstrated durable BAL responses following intradermal priming, in the absence of boosting.

As baseline bronchoscopies were not justifiable in this trial, baseline levels of Ag85A-specific lung mucosal immunity was not known. This was one limitation of this study design. Two BAL samples from NHS patient were too small a sample size to conclusively comment on significant differences. One patient clearly displayed no antigen specific responses. The other patient had very low level of Ag85A-specific BAL CD4+ responses that would be considered at the magnitude of background compared to the BAL observed in this clinical trial. This latter patient also had high PPD BAL responses (data not shown) which might have contributed to these non-specific background responses. For both patients, sarcoidosis was being considered as a diagnosis. It is therefore difficult to ascertain how much was attributable to background immune activation related to the disease process rather than antigen-specific responses[297]. Other factors that could have played a role in non-specific immune responses in volunteers of the trial as well as these patients are circulating respiratory pathogens, non-tuberculous mycobacteria or allergens. A larger sample size of unvaccinated healthy volunteers would be required to describe antigen-specific BAL responses meaningfully. A pre-vaccination BAL in future aerosol vaccine studies would be ideal. This could also address the effect of any possible seasonal variation. Alternative ways of immunomonitoring that are less invasive than a bronchoscopy procedure and hence more acceptable to be repeated more frequently, e.g. induced sputum, were discussed in the previous chapter (Chapter 2: Clinical Results, p.126).

Other limitations in this clinical trial included the partial blinding, but standardised immunological techniques and readouts in part compensated for this in the phase I

clinical trial presented here. Also, the study of lung mucosal responses in this trial was limited due to the invasive nature and logistics. Apart from the limited number of BALs performed (i.e. the lack of baseline BALs discussed earlier), the number of BAL cells obtained was another limiting factor and only allowed limited phenotyping of BAL cells. An interesting aspect in future studies would be the study of vaccine response in alveolar macrophages given their importance in control of *M.tb.* The effect of an aerosol vaccine on lung epithelium, given the important role of these cells in health and disease would be another very important aspect to study, especially in the context of smoking and mucosal inflammation, because smokers and patients with airway disease would also represent part of the target population for a candidate TB vaccine[30, 298, 299]. Studying the effects of innate immune cells and also immunomodulatory and unconventional T cell subsets such as regulatory T cells, MAIT cells and γδ T cells , which may be relevant in mycobacterial immunity, would also be important[30, 43, 82, 83, 101, 113].

Aerosol administration is likely to have delivered some vaccine to other mucosal surfaces such as oro- and nasopharyngeal space but also the gastrointestinal tract[277]. The amount and effect is unknown but would be interesting, in particular in the context of inducing immunological tolerance for instance[281].

This chapter summarised the cell-mediated immune response to Ag85A and MVA following prime and boost vaccinations. Boosting of Ag85A-specific responses was seen in one of the heterologous route regimens as measured by IFN-γ ELISPOT (Group 2: aerosol-intradermal); and this group also achieved the highest magnitude of Ag85A-specific CD4+ T cell responses in the BAL.

The induction of anti-MVA cellular immunity and boosting of anti-MVA responses after the second vaccination was evident in all groups (as measured by either IFN-γ ELISPOT or WB ICS or BAL ICS) and might have contributed to the lack of boosting of insert Ag85A-specific cellular responses in some regimens/compartments. However, Group 2, who boosted Ag85A-specific cellular immune responses in both compartments, also had the highest MVA- specific vector responses in WB and BAL, and was the only one to boost both MVA CD4-specific and MVA-CD8 specific IFN-γ ELISPOT responses.

5 Results: Humoral Immunity

5.1Enzyme-Linked Immunosorbent Assay

Enzyme-Linked Immunosorbent Assay (ELISAs) were performed in batches using thawed serum samples and data presented as optical density (OD) measured at 405nm absorbance, as described in Chapter 2: Materials and Methods(p.83). Antibodies against whole MVA virus and recombinant Ag85A were measured for isotypes IgA, IgM, IgG and IgE. All IgE ELISAs to insert Ag85A and vector MVA were negative (data not shown). ELISAs were also performed on thawed BAL fluid but no responses were detectable (data not shown).

To ensure stringent analysis and avoid false estimation from the three Group 2 volunteers who received a prime intradermal vaccination but then no boost vaccinations (volunteers 74, 77, 81), these volunteers were excluded from this immunogenicity analysis beyond day 28.

5.1.1 Anti-Ag85A serum ELISAs

Shown below are anti-Ag85A IgG, IgM and IgA serum ELISAs for all three groups (Figure 5.1). The pattern of responses was similar for IgG, IgM and IgA isotypes. There were no significant differences between the three groups in pre-existing baseline levels of anti-Ag85A antibodies of any of the three isotypes (data not shown, all $p>0.05$).

In Group 1 (aerosol-intradermal) there was no increase in anti-Ag85A antibodies of any isotype with the exception of day 42 for IgM (day 0 *vs.* day 42, $p = 0.0269$, Figure 5.1 first column).

In Group 2 (intradermal-aerosol) there was a significant increase in anti-Ag85A IgG antibodies on day 28 (i.e. 4 weeks after intradermal prime, day 0 *vs.* day 28, *p*=0.0039) and this was detected up until day 35 (i.e. 1 week after aerosol boost, day *0 vs.* day 35, *p*=0.0273) before it plateaued (Figure 5.1, second column).

In Group 3, anti-Ag85A IgG antibodies were also significantly above baseline at day 28 (day 0 *vs.* day 28 *p*=0.0005). This was similar to Group 2 who had also received an intradermal priming immunisation on day 0. There was no significant increase in anti-Ag85A antibody of the IgM isotype above baseline at any of the post-vaccination time points for either Group 2 or 3 (Figure 5.1, second column).

There were no significant differences in anti-Ag85A IgA from baseline at any of the post-vaccination time points in any of the three study groups for (data not shown, all *p*>0.05).

Comparison of day 28 anti-Ag85A IgG between the groups indicated significant differences between Group 1 (4 weeks after aerosol prime) compared to Groups 2 and 3 (4 weeks after intradermal prime; Group 1 *vs*. Group 2 p=0.0062, Group 1 *vs*. Group 3 *p*=0.0051, Group 1 *vs*. both Groups 2 & 3 combined, i.e. aerosol *vs*. intradermal *p*=0.0011, corrected for multiple comparisons, Figure 5.2).

Therefore, intradermal but not aerosol prime vaccination induced a significant level of anti-Ag85A IgG antibodies peaking at 4 weeks following intradermal vaccination (Figures 5.1 and 5.2).

In addition, to the primed anti-Ag85A IgG seen in Groups 2 and 3 on day 28, Group 3 anti-Ag85A IgG levels remained above baseline following the second vaccination until day 42 (day 0 *vs.* day 42: *p*=0.0005). These day 42 anti-Ag85A IgG levels in Group 3

were higher than on day 28, indicating boosting of anti-Ag85A IgG responses following the second intradermal (i.e. homologous route) vaccination in this group (day 28 *vs.* day 42 p=0.0024, Figure 5.2).

Therefore, boosting of anti-Ag85A IgG responses (detected on day 42) following the second vaccination was unique to Group 3 (intradermal-intradermal, Figure 5.2).

Also, these boosted day 42 anti-Ag85A IgG responses in Group 3 were higher than in Group 1 (p=0.0020 corrected for multiple comparison). There were no other significant differences in day 42 IgG responses between the other groups (all p>0.05, Figure 5.2).

Figure 5.1 ***Serum Ag85A-specific ELISAs for IgG (top row), IgM (middle row), IgA (bottom row) for Groups 1, 2, 3*** *(from left to right); x-axis: OD read at 405nm absorbance; y-axis: timepoints in days.. Non- significant differences (p>0.05) omitted for clarity. * for p ≤ 0.05, ** for p≤0.01 and *** for p≤0.001*

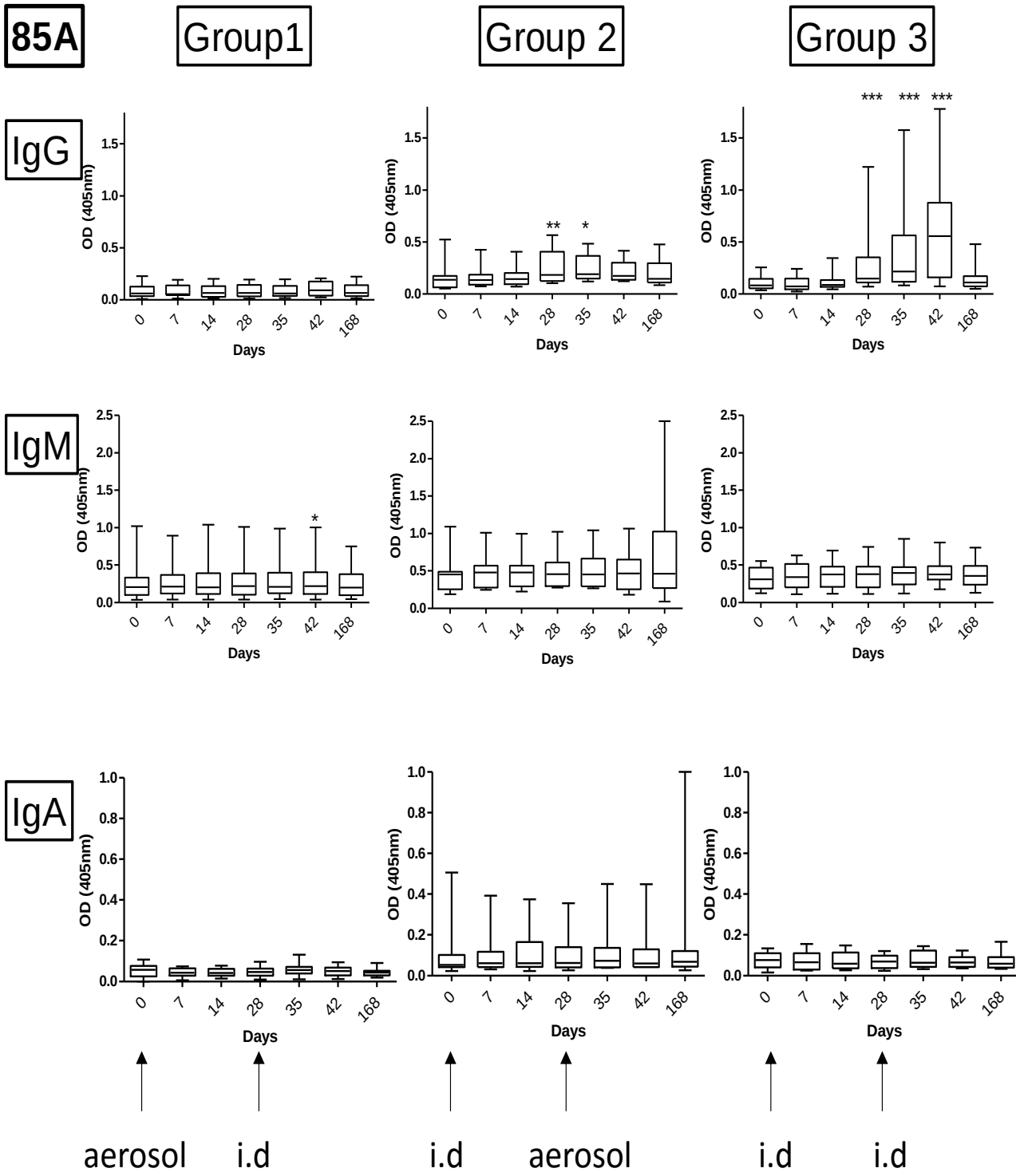
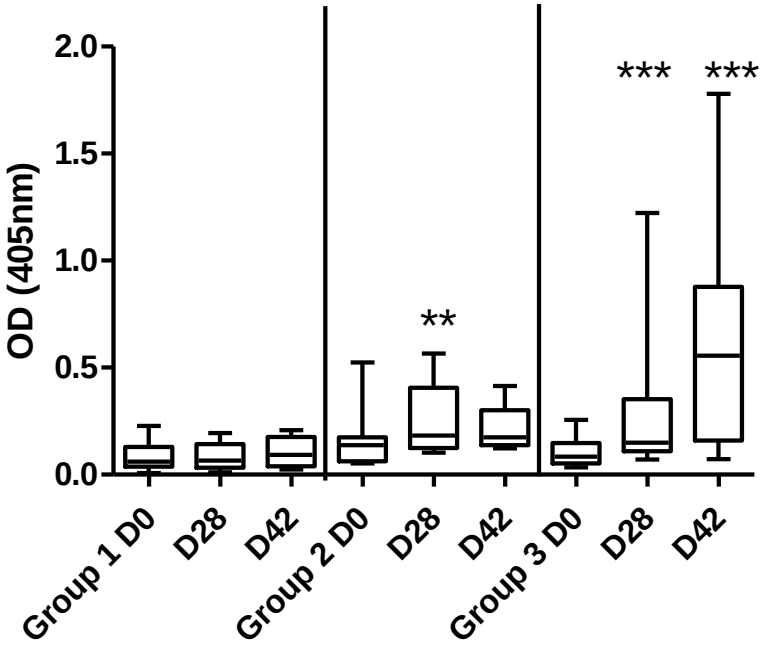


Figure 5.2 ***Serum Anti-Ag85A IgG antibodies (as OD) at peak timepoints*** *for Groups 1, 2 and 3 (from left to right), x axis: OD, y-axis: timepoints. Shown are significant differences from baseline (day 0). Non-significant differences (p>0.05) omitted for clarity. * for p ≤ 0.05, ** for p≤0.01 and *** for p≤0.001*



5.1.2 Anti-MVA serum ELISAs

Next, summarised are serum anti-MVA IgG, IgM and IgA antibody responses for all three groups (Figure 5.3).

In Group 1, anti-MVA IgG levels did not increase from baseline after the prime aerosol vaccination; but increased from day 35, i.e. 1 week following the intradermal boost vaccination, peaking on day 42 (day 0 *vs*. day 42 p=0.0005, Figure 5.3). These day 42 anti-MVA IgG levels in Group 1 were higher than on day 28, i.e. there was *de novo* induction of anti-MVA IgG following the intradermal boost vaccination (day 28 *vs.* day 42 p=0.0004). Anti-MVA IgG levels in Group 1 remained above baseline, still detectable by day 168 (day 0 *vs*. day 168 p=0.0039, Figure 5.3).

In Groups 2 and 3, anti-MVA IgG antibodies were detected from day 14, peaked on day 28 and then plateaued, still significantly above baseline by day 168 (day 0 *vs*. day 28 Group 2: p=0.0039 and Group 3: p=0.0005; day 0 *vs.* day 168 Group 2: p=0.0039 and Group 3: p=0.0005; Figure 5.3). In Groups 2 and 3, day 28 anti-MVA IgG levels were significantly higher than on day 14 highlighting that peak responses following intradermal prime vaccination were at 4 weeks post-vaccination (day 14 *vs.* day 28 Group 2: p=0.0391 and Group 3: p=0.005 respectively). Comparison of day 28 anti-MVA IgG levels between Group 1 (after aerosol prime) against Groups 2 and 3 (after intradermal prime) showed significant differences (Group 1 *vs*. Group 2 p=0.001 and Group 1 *vs*. Group 3 p=0.0002 and *vs.* Group 2&3 combined p<0.0001; corrected for multiple comparison, Figures 5.3 and 5.4) underlining that intradermal but not aerosol prime vaccination induced anti-MVA IgG which peaked at 4 weeks.

In Group 3, day 42 anti-MVA IgG levels were significantly above baseline but also significantly higher than levels on day 28; which was the day of the second vaccination (day 0 *vs.* day 42 p=0.0005; day 28 *vs.* day 42 p=0.005, Figures 5.3 and 5.4). Therefore, anti-MVA IgG levels in Group 3 were boosted following the second vaccination. This contrasts with Group 2, where day 42 anti-MVA IgG levels were not significantly higher than day 28 levels, but were only higher than baseline (Group 2 day 28 *vs*. day 42 p=0.8203, day 0 *vs*. day 42 p=0.0039, Figures 5.3 and 5.4).

Comparison of day 42 responses (2 weeks after boost vaccination) between the three groups revealed that anti-MVA IgG levels in Group 2 and 3 were higher than those in Group 1 (Group 1 *vs.* Group 2 p=0.0209 and Group 1 *vs.* Group 3 p=0.0073 on day 42, corrected for multiple comparisons). Day 42 MVA IgG levels between Groups 2 and 3 were not significantly different (p=0.5940; Figure 5.4)

Anti-MVA IgM levels demonstrated the same pattern of response to that seen with anti-MVA IgG, in that intradermal but not aerosol vaccination induced anti-MVA antibodies. However, the peak of anti-MVA IgM in Groups 2 and 3 occurred 2 weeks following intradermal prime vaccination (IgM day 0 *vs.* day 14 Group 2 p=0.0039 and Group 3 p=0.0010) and in Group 1 on day 42, i.e. 2 weeks following the intradermal boost vaccination (day 0 *vs*. day 42 Group 1 p=0.0005). Therefore, the peak anti-MVA IgM response occurred 2 weeks earlier than the peak anti-MVA IgG response following intradermal prime or boost vaccinations.

Anti-MVA IgA responses mirrored anti-MVA IgM responses peaking 2 weeks after intradermal MVA85A vaccinations, i.e. in Group 1 on day 42 (day 0 *vs*. day 42 p=0.0005) and in Groups 2 and 3 on day 14 (day 0 *vs*. day 14 Group 2: p=0.0039 and

Group 3: p=0.0015). Anti-MVA IgM and IgA responses on day 42 were never higher than on day 28, i.e. no boosting beyond the priming response.

Figure 5.3 ***Serum MVA-specific ELISAs for IgG (1st row), IgM (2nd row), IgA (3rd row) antibodies for Groups 1, 2, 3*** *(from left to right), x axis= timepoint in days, y axis= OD 405nm, Non-significant differences (p>0.05) omitted for clarity. * for p ≤ 0.05, ** for p≤0.01 and *** for p≤0.00*

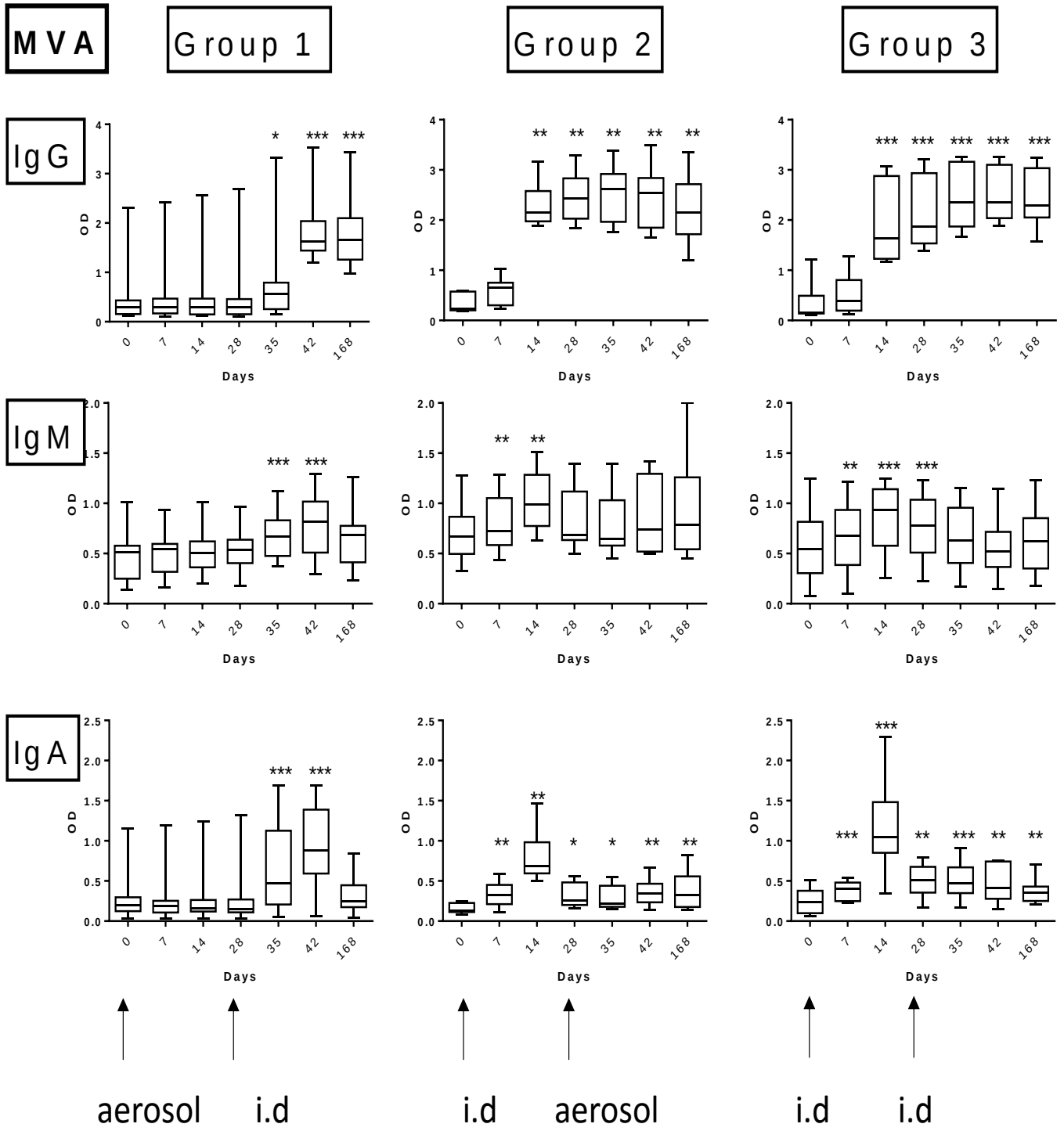
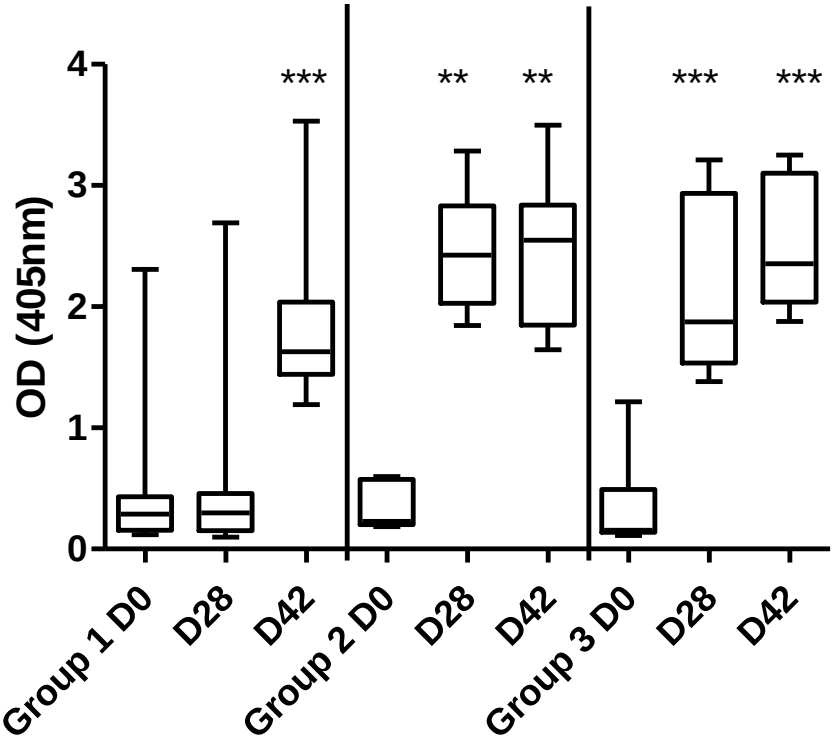


Figure 5.4 ***Serum Anti-MVA IgG antibodies at peak timepoints*** *for Groups 1, 2 and 3 (from left to right), x axis: OD, y-axis timepoints. Shown are significant differences from baseline (day 0). Non-significant differences (p>0.05) omitted for clarity. * for p ≤ 0.05, ** for p≤0.01 and *** for p≤0.001*



5.2 ELISA vector-insert correlations

One of the secondary objectives of this study was to evaluate the functional relevance of any humoral anti-vector immunity induced by aerosol or systemic MVA85A-prime on a subsequent MVA85A-boost vaccination by alternative routes, as measured by the magnitude of insert Ag85A immunogenicity. Therefore, relevant vector-insert correlations were examined. Figure 5.5 summarises the correlation of anti-MVA IgG on day 28 (peak anti-MVA IgG response and baseline for the second vaccination) against anti-Ag85A IgG on day 42, which was nearest to the presumed anti-Ag85A IgG peak (day 56), because serum 4 weeks following boost vaccination was not available. There were no correlations between anti-MVA IgG on day 28 and anti-Ag85A IgG on day 42 in any of the groups.

Other ELISA correlations examined were the effect of day 7 and day 14 MVA IgG on day 42 Ag85A IgG, neither of which were significant (p>0.05, data not shown).

The next correlation, summarised in Figure 5.6, examined the effect of humoral anti-MVA immunity induced by the prime vaccination on the cell-mediated immunity readout of insert Ag85A responses after the second vaccination, i.e. anti-MVA IgG on day 28 vs. day 35 IFN-γ ELISPOT responses. Day 28 anti-MVA IgG did not correlate with day 35 Ag85A IFN-γ ELISPOT responses in Groups 1 and 2 but positively correlated in Group 3 (r= 0.6014, p=0.0428, Figure 5.6).

Figure 5.5 Correlation between anti-MVA IgG responses (as OD) on day 28 and anti-Ag85A IgG responses (as OD) on day 42, Spearman correlation coefficient (r). All p>0.05= not statistically significant

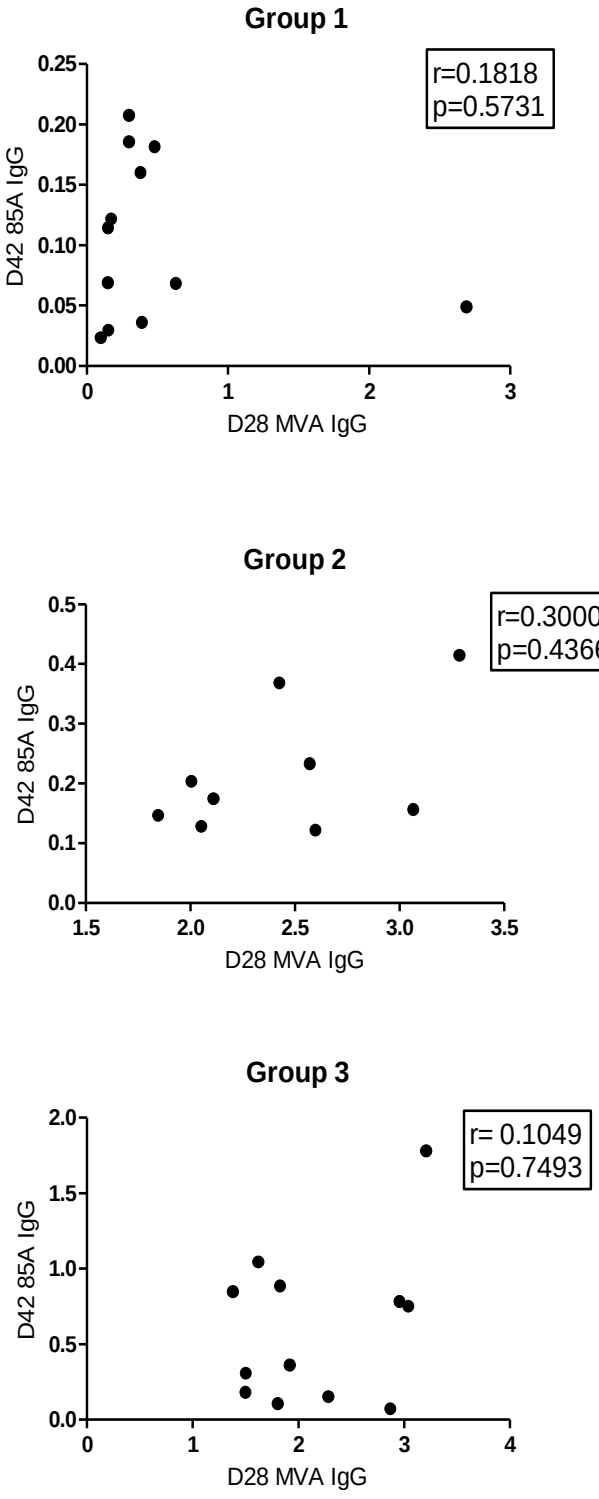
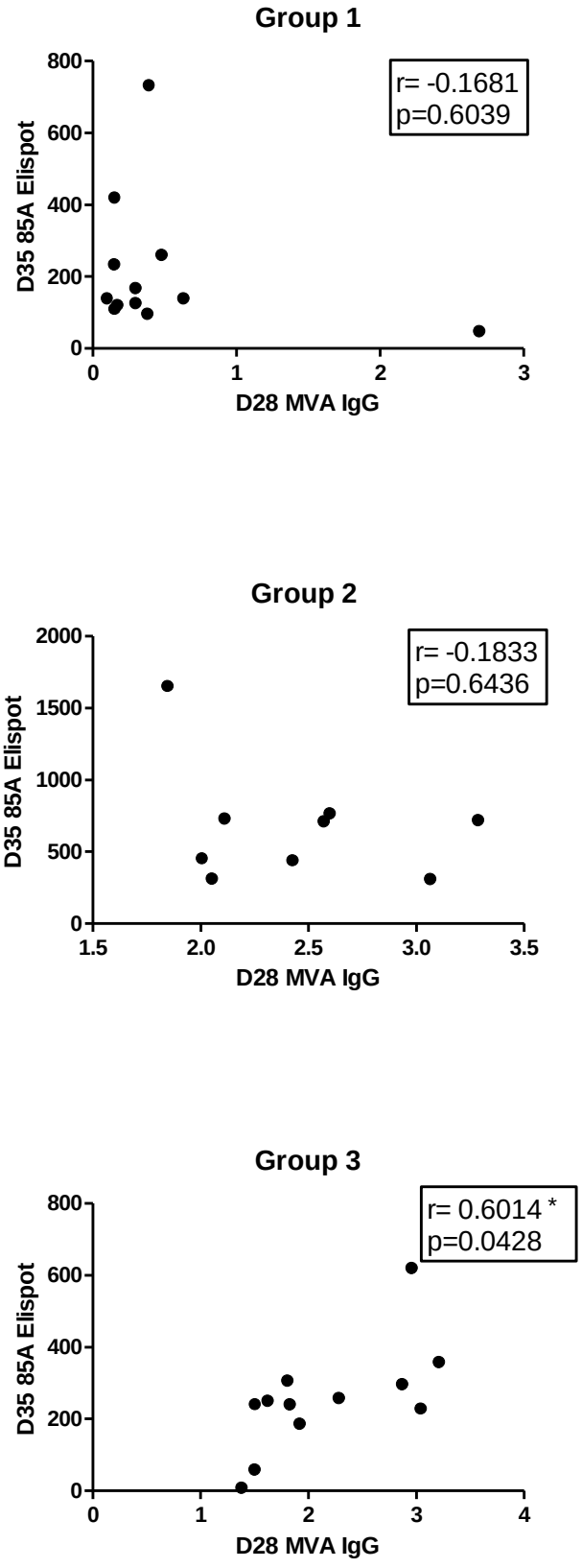


Figure 5.6 Correlation between anti-MVA IgG responses (as OD) on day 28 and Ag85A ELISPOT responses (as spots per million PBMC) on day 42 Spearman correlation coefficient (r) and p-value summarised.



5.3 Discussion

As seen in three previous MVA85A vaccine studies[248] (Huygen and McShane, unpublished data; Dheenadhayalan and McShane, unpublished data), the anti-Ag85A IgG response to intradermal vaccination peaked at 4 weeks (seen here following the prime vaccination in Groups 2 and 3).

Anti-Ag85A antibodies were only induced in Groups 2 and 3, who had both received intradermal prime vaccination. Whilst the aerosol route of vaccination induced Ag85A-specific lung mucosal and systemic cellular immune responses which were comparable to those induced by intradermal vaccination (as described in Chapter 3: Cell Mediated Immunity and in the first aerosol MVA85A study)[247], the aerosol prime vaccination in Group 1 did not induce any humoral anti-Ag85A immunity in the serum. Furthermore, the intradermal boost on day 28 did not result in any humoral anti-Ag85A antibodies, either. One explanation for the lack of anti-Ag85A IgG seen after intradermal boost vaccination in Group 1 could have been that the peak (expected at 4 weeks following vaccination, i.e. day 56) was missed between the day 42 and day 168 timepoints. If this was the case, then the peak anti-Ag85A IgG responses seen in Group 3 on day 42 following the second intradermal vaccination was likely to have been an underestimate.

This is the first time that routes of aerosol and intradermal vaccinations were combined. Whilst the anti-Ag85A IgG levels were boosted on day 42 in Group 3 (2 weeks following the homologous intradermal boost), there was not any boosting of anti-Ag85A IgG levels in Group 2 following their second vaccinations by aerosol boost. This suggests that a homologous intradermal second vaccination was required for a

boosting of a serum anti-Ag85A IgG response beyond that induced by priming; i.e. higher than levels on day 28.

One explanation for this is, that induction and boosting of anti-Ag85A IgG could be dose-dependent, i.e. the level of anti-Ag85A IgG responses measured in Group 3 following prime and boost was due to the cumulative dose administered to the systemic compartment in the intradermal-intradermal vaccine regimen. Equivalently, the levels of anti-Ag85A IgG following the first vaccination in Group 2 due to the single intradermal priming dose, was sufficient for induction but not for boosting of serum anti-Ag85A IgG antibodies. Either way, only intradermal vaccination with MVA85A was capable of inducing serum anti-MVA IgG; and only the intradermal-intradermal vaccination regimen was able to boost anti-Ag85A IgG responses. Whilst MVA is recognised as a vector capable of enhancing T cell responses, evidence on its ability to induce or boost antibody responses is scarce[196, 203]. However, a recent Ebola vaccine trial where MVA was used to boost responses, which had been primed by a chimpanzee adenoviral vector vaccine, enhancement of antibody and T cell responses were seen[196].

A previous clinical trial examined a fowl pox-vectored candidate TB vaccine also expressing Ag85A (FP85A) alone and in combination with MVA85A using the same dose of MVA85A and the same boost interval as in this trial design[248]. Interestingly, anti-Ag85A IgG levels did not increase in a FP85A-MVA85A or a MVA85A-FP85A boost regimen. This is in contrast to the observation of boosted anti-Ag85A IgG levels in Group 3 around day 42 in this current study. One explanation for this could be that in humans FP85A used as a prime or boost vaccination showed comparatively low T cell immunogenicity, which is why this vaccine did not progress through further

development. Given that T cell help is required for effective B cell responses this might be an explanation[248].

In the first clinical trial of low dose MVA85A delivered by the aerosol route to humans, no anti-Ag85A antibodies were detected in either the group vaccinated by the aerosol route or the group vaccinated intradermally[247] (McShane, unpublished data). This is in contrast to the three earlier studies with higher intradermal doses of MVA85A which induced Ag85A IgG at 4 weeks post-vaccination[248] (Huygen and McShane, unpublished data; Dheenadhayalan and McShane; unpublished data). Therefore, considered together the observation of the highest (boosted) anti-Ag85A IgG responses in this current study being noted in Group 3 who received two intradermal doses is in keeping with dose-dependent induction of serum Ag85A IgG. If induction of Ag85A antibodies in the systemic compartment (i.e. serum) required a threshold dose of MVA85A being delivered to the same compartment, then it would be very interesting to compare this with the Ag85A antibodies in the BAL and whether a single aerosol dose of $5x10^7$ MVA85A as was given in Groups 1 and 2 reached threshold for Ag85A induction in the lung mucosal compartment; and furthermore, if an aerosol-aerosol regimen would achieve boosting (like Group 3 for intradermal route). However, in this trial presented here, BAL anti-Ag85A or anti-MVA antibodies of any isotype could not be measured and therefore conclusions on the ability of aerosol immunisation to induce mucosal humoral immunity cannot be drawn.

The failure to detect any Ag85A- antibodies in the BAL fluid could be attributable to the inadequacy of the assay rather than no antibodies being present. For instance, the titre induced by one dose of $5x10^7$pfu MVA85A might have been below the limit of detection in this assay or the BAL too dilute (in comparison to serum). In keeping with

this, anti-Ag85A antibodies could not be detected in serial BALs from macaques who received aerosolised MVA85A, nor in human BAL from the first study of aerosolised MVA85A, either (Sharpe, McShane, unpublished data[247]). As there was no aerosol-aerosol group in this study, no data was available on a cumulative dose (twice $5x10^7$) delivered to the lung mucosal compartment, equivalent to that in Group 3 delivered to the systemic compartment. However, in the literature induction of antibodies in the mucosal department by mucosal administration of vaccine has previously been reported. Administration of aerosolised measles vaccine in children was able to induce antibodies of the IgA and IgG isotype in serum as well as nasal secretions[300]. The magnitude of these antibodies in both compartments (serum and nasal mucosa) was higher in the group who received the aerosolised vaccine compared to those who received the vaccine by the systemic route; and the difference between the groups in magnitude was more marked in nasal secretions than serum[300]. Another example was a more recent trial of intranasally administered HIV vaccine which reported induction of both systemic and mucosal humoral responses, including antibodies at distal sites (rectum)[301]. These studies suggest that antibodies were likely to have been induced by the aerosol route of vaccination- although not detected, maybe due to the titre being below the limit of detection.

In terms of durability of the serum anti-Ag85A IgG response, levels were not elevated above baseline by day 168 in any of the three groups. Even in Group 3, which was the only group whose serum anti-Ag85A IgG antibody responses were boosted after the second vaccination, this was the case.

As outlined in Chapter 1: Introduction, a recent case-control analysis of BCG vaccinated infants from the MVA85A efficacy trial, revealed that Ag85A-specific IgG

antibodies were associated with a reduced risk of TB disease. This finding suggests that a durable anti-Ag85A IgG response would be desirable. Given that even in Group 3, who had the boosted serum anti-Ag85A antibodies, levels contracted by day 168 would suggest that this regimen would not lead to a durable antibody response. This also raises the question what the significance of any mucosal Ag85A-specific antibodies would be in terms of reduced risk correlation.

In terms of anti-MVA IgG responses, previous studies showed levels to increase from two weeks and peak at four weeks following intradermal vaccination, consistent with the findings presented here[247, 248]. Two studies of aerosolised MVA85A, one in macaques and one in humans, demonstrated induction of serum anti-MVA antibodies by intradermal but not aerosol vaccination[239, 247], also consistent with the findings presented here.

As mentioned before, measurement of BAL antigen-specific antibodies in this trial was not possible and therefore no comment can be made on the ability of aerosol immunisation with MVA85A to induce mucosal humoral anti-MVA immunity. If the hypothesis (based on observations in the systemic compartment in macaques and humans) that induction of anti-MVA IgG is compartment specific is correct, then one would expect anti-MVA IgG to be present in BAL of the groups who received aerosol, i.e. in Groups 1 and 2 but not in Group 3. A study in non-human primates using *in vivo* real-time scintigraphy to monitor the fate of aerosolised radiolabelled NYVAC noted anti-vector specific IgA antibodies in vaginal secretions of 3 out of 5 female animals; which was not observed after intramuscular immunisation[259]. No insert specific antibodies were found in the secretions[259].

The functional relevance of anti-MVA antibodies on predicting insert Ag85A immunogenicity is not known. The observations of compartment-specific induction of anti-MVA antibodies in the two studies of aerosolised MVA85A in macaques and humans[239, 247], supported design of this trial evaluating homologous boosting by heterologous routes with the hypothesis that switching of vaccination compartments might enhance Ag85A immunogenicity possibly by circumventing local anti-MVA immunity in the primed compartment. Boosting of a viral vector vaccine by heterologous combined aerosol and intradermal routes was not evaluated before in humans. Hence, this was the first opportunity to assess functional impact of anti-vector immunity induced by a prime immunisation, on magnitude of insert responses following boosting. The lack of correlations of anti-MVA IgG on day 28 (peak timepoint following prime vaccination and baseline for second vaccination) *vs.* anti-Ag85A IgG on day 42 suggested that there was no functional difference in boosting of humoral insert responses between the groups, i.e. anti-MVA IgG on day 28 was not inversely correlated with anti-Ag85A IgG on day 42. The caveat to the correlation between anti-MVA and anti-Ag85A IgG was that the peak timepoint of anti-Ag85A IgG occurred 4 weeks following vaccination and as a day 56 serum sample was not available (day 42 nearest to expected peak of anti-Ag85A IgG used instead), an inverse correlation with peak Ag85A response could have been missed. Also, the effect of humoral mucosal anti-vector immunity on boosting is uncertain.

Lack of correlation of anti-MVA IgG on day 28 *vs.* Ag85A IFN-γ ELISPOT responses on day 35 for Groups 1 and 2 also suggested that anti-MVA IgG did not inversely affect Ag85A cellular immune responses (measured by IFN-γ ELISPOT), i.e. no functional relevance of anti-vector IgG on insert IFN-γ ELISPOT responses can be concluded, either. The exception was Group 3 for which anti-MVA IgG on day 28 correlated

marginally with Ag85A IFN-γ ELISPOT responses on day 35. Despite this statistically significant association it is difficult to conclude that this correlation was functionally meaningful, because if so, it should have been observed in Group 2 as well; i.e. if anti-MVA IgG induced by intradermal prime (which was the conditional variable in this correlation) directly impacted on Ag85A measured by IFN-γ ELISPOT this would have been seen for both Groups 2 and 3.

To evaluate whether the magnitude of anti-MVA antibodies induced by the second vaccination could explain the concurrent lack of boosting of anti-Ag85A antibodies in Groups 1 and 2, the peak anti-MVA IgG levels following the second vaccination was examined alone; and in relation to insert Ag85A responses following the second vaccination. The closest timepoint to expected peak of anti-MVA IgG response following the second vaccination was examined, i.e. day 42. Whilst in Group 2 day 42 anti-MVA IgG levels were significantly higher than baseline, these were not significantly different from day 28, i.e. not boosted by second vaccination. In contrast, in Groups 1 and 3 on day 42, anti-MVA IgG levels increased compared to day 28. In Group 1 this was *de novo* induction of anti-MVA IgG as this group did not have any anti-MVA IgG induced by the aerosol prime vaccination. In Group 3 this was boosting of anti-MVA IgG beyond what had been induced after the prime intradermal vaccination. Considered together these findings suggest that prime and boost vaccinations by homologous intradermal routes boosted anti-MVA IgG (as seen in Group 3), whereas a second vaccination delivered by the heterologous aerosol route did not boost levels beyond those induced by an intradermal prime (as seen in Group 2); at least not in the systemic compartment (i.e. serum).

Interestingly, despite boosting of anti-MVA IgG responses in Group 3 (evident on day 42), this was the only group to have had concurrent boosting of anti-Ag85A responses, i.e. in functional terms serum anti-MVA IgG antibodies induced by the intradermal prime vaccination did not impede boosting of anti-Ag85A IgG levels in Group 3. In fact, boosting of anti-Ag85A IgG responses following the second vaccination occurred in parallel to boosted anti-MVA IgG.

This lack of inverse correlation between vector and insert responses and the lack of functional consequence of humoral anti-vector immunity on insert responses are consistent with recent findings of a phase 1 trial using the human type 5 adenovirus-vectored vaccine of AdHu5Ag85A. In this study pre-existing anti-AdHu5 total IgG and neutralising antibodies pre-vaccination did not inversely affect insert Ag85A immunogenicity as measured by IFN-γ ELISPOT[193]. A similar observation was made for an AdHu-5 based malaria vaccine where pre-existing neutralising antibodies did not attenuate insert immunogenicity[195]. Circumventing of pre-existing inhibitory humoral responses in a different context has also been observed in studies delivering aerosolised measles vaccine: In an infant study the aerosol route of vaccination was more likely to overcome resistance to seroconversion in young infants in whom maternal measles-specific IgG antibodies were present at time of vaccination[302].

Anti-MVA IgG antibodies observed in this trial were durable, still being above baseline in all 3 groups by day 168. This was in contrast to the anti-Ag85A IgG mentioned earlier, which were not durable.

IgG has mainly been used for comparison to other studies and correlations for several reasons. Firstly, the main body of data available on MVA85A antibody responses was of the IgG isotype, so permitted comparison[247, 248]. Secondly, similar to the observation

in this trial, in the first trial of aerosolised MVA85A in humans, which also examined anti-MVA antibodies of the IgM and IgA isotypes, responses of these two isotypes mirrored the pattern seen in anti-MVA IgG in that intradermal (but not aerosol) vaccination induced anti-MVA antibodies; and any effect was highest and most sustained for the IgG isotype[247].

IgM expressed on naïve B cells initiate the primary humoral response to foreign antigen[303, 304]. Upon antigen encounter these B cells proliferate and differentiate initiating the humoral adaptive immune response[303, 304]. Engagement of CD40 on B cells (through binding of CD40L expressed on activated T cells) initiates the process known as class switching ultimately resulting in the production of high affinity antibodies of the IgG, IgA and IgE isotypes[305]. Class switching occurs in lymph node germinal centres[305]. Therefore, IgM plays an important role in the first line of defence through formation of immune complexes to activate the adaptive immune responses, recruiting antigen to germinal centres[303-305]. The initial production of short lived plasma cells producing IgM with a short serum half-life aid antigen clearance[306, 307]. Long term humoral immunity is represented by memory B cells, terminally differentiated antibody secreting plasma cells, and IgG. IgG, which has a longer serum half-life than IgM, is characteristic of the secondary humoral response[306, 307]. In the context of this study, the time difference between the peak of anti-MVA IgM and anti-MVA IgG responses observed in this study, i.e. the earlier peak of anti-MVA IgM (day 14) compared to anti-MVA IgG antibodies (day 28), might be attributable to the natural timeline of affinity maturation and class switching seen in these vaccinia naïve individuals.

Similarly, the relative lack of anti-Ag85A antibodies of the IgM isotype and presence of anti-Ag85A of the IgG isotype at 4 weeks following vaccination could possibly be

explained by the fact that these volunteers were previously vaccinated with BCG, i.e. anti-Ag85A IgG antibodies seen here were result of a secondary response. To my knowledge, there is no published data in human vaccine studies regarding anti-Ag85A antibodies of the IgM and IgA isotype. However, humoral responses against Ag85A have been studied in tuberculosis patients with view to developing serodiagnostic applications[308-310]. Anti-Ag85A antibodies of all isotypes were found in patients with leprosy and tuberculosis[310]. Mean levels of anti-Ag85A IgG but not IgM antibody responses could distinguish between active TB cases and controls. Also, mean anti-Ag85A IgG levels were higher in untreated cases and those with more extensive TB disease[308-310].

The scarcity of anti-Ag85A antibody of the IgA isotype could possibly be explained by the fact that only serum samples were examined, and this isotype predominantly found on mucosal surfaces[311, 312]. As mentioned above in a study administering aerosolised measles vaccine could induce measles-specific antibodies of the IgG and IgA isotype in the serum as well as nasal secretion- although the increase in IgG was only significant in the serum and that of IgA only in nasal secretions[300]. This suggests that any anti-Ag85A IgA that would have been induced should have been more readily detectable in the BAL with an adequate assay or at a sufficiently high titre.

This initial characterisation of humoral immunity in serum described in this chapter suggests further work could be performed to aid understanding of humoral responses of viral vector vaccines delivered by the aerosol route. A more comprehensive characterisation of humoral responses such as study and correlation of antibody titre with viral neutralisation assays would potentially provide more information. Neutralisation assays, where infectious virus and serially diluted antibodies are co-

incubated and neutralising titre described as the dilution which halves the infectivity, are a functional measure of the ability of antibodies to reduce the infectivity *in vitro*[313]. Also, a method to robustly detect insert and vector-specific antibodies in BAL fluid would aid to characterising humoral immunity of this compartment, especially given IgA antibodies are the second largest category of proteins found in BAL[314]. Further study and characterisation of innate, B cell and Th2 cell mediated immune responses in systemic and lung mucosal compartments would further advance the understanding of this humoral arm of the MVA85A vaccine response in these locations. Comparison of humoral and cellular immunity to Ag85A and MVA in this study showed discordance. Whilst no serum anti-MVA antibodies were induced by aerosol vaccination, cellular MVA-specific responses were seen with both aerosol and intradermal vaccination in all groups (Chapter 3: Cellular Immunity). Also, whilst vaccination with aerosolised MVA85A was able to induce systemic Ag85A-specific cell mediated immune responses, as measured by IFN-γ ELISPOT and WB ICS, this route did not induce serum anti-Ag85A antibodies. The induction and boosting of anti-Ag85A IgG in the serum of Group 3 was encouraging; but not durable. From a vaccine perspective, increased durability of humoral responses to insert Ag85A, than that seen here in either compartments, is likely to be desirable. A humoral response induced by a candidate TB vaccine could interfere with extracellular stages of the *M.tb* infection and thereby preventing or influencing the intracellular persistence of the pathogen[120, 315].

Although as outlined in Chapter 1: Introduction, humoral immunity alone is not sufficient for protection. Therefore, optimisation of prime- boost regimens that are capable of inducing potent both cellular as well as humoral responses are being developed for example for Ebola where also no correlate of protection is known but humoral and cellular immune responses thought to be important[316]. In a recent Ebola

vaccine trial, priming with a chimpanzee adenovirus encoding an Ebola antigen induced potent virus-neutralising antibodies; and boosting with MVA enhanced virus-specific antibody titres as well as antigen-specific cellular immune responses[196]. Interestingly, vaccine-induced antibodies were detected in oral fluid obtained from gingiva of vaccinated individuals[317].

This was the first trial to combine heterologous routes of MVA85A vaccination, studying humoral immunity Ag85A and MVA in more detail. More studies are required to understand functional consequences of cellular and humoral anti-vector immunity.

6 Final Conclusions

The global burden of TB introduced in Chapter 1 of this thesis illustrated the need for new tools[8, 16]. Advances in both diagnostics and new drugs are required for the short to medium term control of this pandemic. However, the most cost-effective public health measure to control any infectious disease pandemic is with an effective vaccine. There is an urgent need for an improved prophylactic vaccination regimen for adolescents and adults to prevent pulmonary TB, thus reducing transmission. A post-exposure vaccine to prevent reactivation of LTBI or an immunotherapeutic one as adjunct to treatment in people with TB disease would facilitate control of this pandemic. Despite successful global efforts in the last decade, progress has remained slow[16, 17].

One of the difficulties in TB vaccine development is the lack of a correlate of protection and hence uncertainty about predicting whether a vaccine will be efficacious [135]. However, the first completed efficacy trials of MVA85A in infants and HIV infected adults have given us insights into the predictive value of the animal studies and immune correlates[130, 205].

Animal studies of successful delivery of vaccines by the mucosal route provided proof-of-concept and encouraged development of this route for a candidate TB vaccine[215, 236, 237, 239, 247, 318, 319]. Pre-clinical studies and one previous study in humans using a candidate TB vaccine, MVA85A, demonstrated feasibility and safety of an aerosol approach[141, 239, 247]. An aerosolised TB vaccine would offer practical advantages. The anatomical location of the aerosol route of vaccine delivery is likely to provide an immunological benefit, because local vaccine responses would be in proximity to the future site of *M.tb* exposure.

The aim of the clinical trial presented in this thesis was to combine aerosol and intradermal routes of MVA85A vaccine delivery to enhance immunogenicity to insert

Ag85A in local lung mucosal and systemic body compartments. I hypothesised that by alternating routes of vaccine delivery I would be able to enhance insert Ag85A responses in both body compartments; maybe by a mechanism that circumvented local anti-vector immunity.

6.1 Clinical Safety

The primary objective was to evaluate safety of aerosolised MVA85A and also safety of a prime-boost regimen of aerosolised and intradermal MVA85A combined.

The results of this clinical trial demonstrated that aerosol delivery of MVA85A as a priming vaccination was safe. This was the second phase I clinical trial to demonstrate this[247]. However, delivering aerosolised MVA85A as a boost vaccination one month after an intradermal prime caused an unfavourable pattern of adverse events. Although symptoms were transient and there were no serious adverse events, the profile of adverse events observed were not acceptable for a prophylactic vaccine regimen, designed to be delivered to heathy people. The aetiology of this unexpected pattern of adverse events is uncertain. Differential diagnoses considered were a predisposition to atopy, a heightened response to the MVA vector or hypersensitivity pneumonitis (HP) to MVA or Ag85A.

Atopy as a predisposition was unlikely. Subsequent clinical and immunological observations were not in support of this.

The magnitude of cellular anti-vector responses observed in Group 2, in lung and systemic compartment, would be in keeping with a heightened response to MVA on re-exposure. If this is the case then a regimen combing adenoviral and MVA vaccines

in a systemic prime-aerosol boost regimen should not cause this pattern of adverse events.

HP to MVA, Ag85A or possibly any remnant chick embryonic fibroblast protein from the MVA manufacturing process are possibilities. HP due to re-exposure to mycobacterial antigen 85A would represent an issue in people with higher mycobacterial burden, e.g. exposure to NTM or infection with *M.tb* in endemic countries. A trial evaluating aerosolised MVA85A in people with LTBI is currently ongoing (ClinicalTrials.gov Identifier: NCT02709278). HP to avian protein has been described[267, 268, 270, 271].

It is possible that amending either the dose or the boosting interval would have minimised this adverse event profile but this was outside of the scope of this trial. These could be considerations for future clinical trials exploring homologous boosting by heterologous routes.

6.2 Insert and vector immunogenicity

One of the secondary objectives was to measure lung mucosal and systemic insert (Ag85A) and vector (MVA) responses in the different vaccination regimens.

Aerosolised MVA85A was a potent route to induce insert Ag85A-specific cellular immune responses (including polyfunctional phenotypes) in both systemic and lung mucosal compartments. These observations highlighted the potency of the aerosol route in inducing systemic cellular immunity to insert Ag85A and suggested a potential for dose sparing.

The intradermal-aerosol regimen boosted Ag85A-specific systemic responses in both compartments. This suggests a mechanism whereby a systemic prime induced a

potent reservoir of Ag85A-specific cellular immune responses that were then augmented by boosting in the lung mucosal compartment.

Induction of systemic cellular immune responses to the MVA vector was demonstrated in all regimens, but highest in subjects who received an intradermal prime vaccination. This was true for lung mucosal and systemic anti-vector responses. In this context, the clinical features in Group 2 after aerosol boost might have been attributable to MVA-specific responses, as in this group MVA-specific were also boosted after the second vaccination in both systemic and lung mucosal compartments.

Serum antibodies to insert and vector were only demonstrated after intradermal vaccination. Boosting of antibody responses measured in the systemic compartment depended on repeat vaccination in the same compartment.

6.3 Effects of boosting by heterologous routes on insert responses

The other secondary objective was to evaluate the effects of a MVA85A prime vaccination (by either intradermal or aerosol route) on the magnitude of insert Ag85A responses after a second vaccination by heterologous or homologous routes. At the outset I hypothesised that the groups who received MVA85A by heterologous routes would demonstrate enhanced insert Ag85A immunogenicity.

This hypothesis held true for Group 2 (intradermal-aerosol). Ag85A-specific cellular immune responses in this group were boosted in the lung mucosal and systemic compartments following the second vaccination. The trend for the highest magnitude of insert Ag85A-specific responses overall in systemic and lung mucosal

compartments was observed in Group 2. This trend was also reflected in polyfunctional phenotypes. Group 3 who was primed and boosted by the intradermal route did not show the level of Ag85A immunogenicity as Group 2 did. Therefore, a mechanism in Group 2 whereby boosting in a different compartment after systemic priming synergised responses to Ag85A was demonstrated.

Interestingly, Group 2 was also the most productive at boosting MVA responses following the second vaccination in systemic and lung mucosal compartments. This means that boosting in heterologous compartments happened for both insert and vector.

Ag85A-specific cellular immune responses induced by aerosol prime vaccinations were stable but not boosted after the second vaccination (Group 1). Therefore, reversing the order of heterologous route boosting did not yield the boosting of Ag85A responses. Systemic priming preceding local boosting appeared beneficial.

Intradermal priming induced robust Ag85A-specific cellular immune responses in the systemic but not in the lung mucosal compartment. Responses were not boosted after a second intradermal vaccination (Group 3).

Boosting of anti-Ag85A serum antibodies was only demonstrated for the intradermal-intradermal regimen.

6.4 Functional relevance of anti-vector immunity on insert boost responses

Part of the secondary objectives was to evaluate the functional relevance of anti-vector immunity induced by the priming vaccination on the magnitude of insert Ag85A

responses after a second vaccination by heterologous or homologous routes. It was expected that anti-vector immunity induced by the first vaccination would negatively impact on generation of insert responses after the second vaccination. The findings on this were mixed.

None of the vector-insert correlations studied reached statistical significance. Therefore, no direct inverse correlation between vector and insert responses was demonstrated.

MVA-specific IFN-γ ELISPOT responses were induced by both routes of vaccination. The intradermal-aerosol group was the main regimen to boost Ag85A-specific responses in both compartments despite presence of humoral and cellular anti-vector responses. This suggests no functional relevance of priming vector responses on magnitude of insert responses after boosting. Also, comparison between Groups 2 and 3, who were both primed intradermally, suggested that systemic MVA-specific cellular immune responses permitted boosting by the aerosol route but not by the intradermal route. The findings from the WB ICS assay were not consistent with this, although the trend was similar. One possibility could be that the IFN-γ ELISPOT assay is more selective, as it quantifies the proportion of antigen-specific reactive IFN-γ secreting T cells in PBMC. Therefore, it might detect a greater dynamic range to reach statistical significance than WB ICS. The WB ICS detects markers on antigen-specific T cells in the whole blood compartment, therefore a different sample and assay. Discrepancies between IFN-γ ELISPOT and WB ICS were reported in trials of BCG vaccinated infants. BCG-specific IFN-γ ELISPOT responses were associated with reduced risk of TB in one study but another study using WB ICS IFN-γ failed to demonstrate this[129, 320].

In BAL, MVA-specific cellular immune responses were generally low after the first vaccination regardless of route. If cellular anti-vector immunity impacted directly on boosting of insert responses then any local lung mucosal anti-vector immunity induced by the prime vaccination would have affected the local (in this case aerosol) route of boosting the most (i.e. Group 2). However, boosting of Ag85A responses in BAL from day 7 to day 35 was observed in Group 2. This suggests that any MVA-specific T cell responses in BAL after the first vaccinations did not affect boosting of insert responses by aerosol. Interestingly, like in the systemic compartment, boosting of Ag85A-specific T cell responses in BAL of Group 2 was accompanied by concurrent boosting of MVA-specific cellular immune responses in the lung mucosal compartment.

Humoral responses to insert and vector followed a different pattern. Serum antibodies to insert or vector were only induced by intradermal vaccination. Boosting by homologous route (intradermal-intradermal) led to boosting of anti-Ag85A antibody responses in the serum despite the presence of anti-MVA antibodies. Therefore, serum anti-vector antibodies did not inhibit boosting of Ag85A-specific systemic humoral responses.

The inability to measure mucosal antibodies in this clinical trial meant that no conclusions could be drawn on mucosal humoral immune responses to insert or vector. Also, no comments on the effect of any mucosal anti-vector humoral immunity on the boosting of insert responses locally or systemically can be made.

6.5 Final discussion

Some pre-clinical studies have evaluated the benefits of a so called prime-pull vaccination regimen whereby systemic priming preceded local boosting. The first study to mention this term was a vaccination model in mice against genital herpes that used subcutaneous priming vaccination followed by a topical chemokine application as a pulling boost[321]. The concept behind the pull was to maintain a tissue resident memory T cell population in the genital mucosa[321]. Challenge of these mice with genital herpes demonstrated this regimen to reduce viral spread and prevent clinical disease[321]. A subsequent study used a HIV antigen intranasally followed by a chemokine pull in the vaginal mucosa to evaluate mucosal humoral responses. This showed that one of the chemokines trialled was able to induce a significant level of antigen-specific vaginal IgA once the boost interval was optimised but that this was "modest"[322]. A more recent study found different evidence demonstrating the prime-pull approach to be able to induce humoral immunity. This study used a prime-boost regimen by heterologous routes in mice, with vaccines containing a Th17-inducing adjuvant. The route of priming was subcutaneous followed by an intranasal boost. This study demonstrated induction of high antigen-specific IgA levels in the lung without any increase in total IgA[323]. Subsequent *in vivo* staining revealed a mechanism: intranasal boosting recruited IgA+ B cells and Th17 cells to the lung parenchyma[323]. Depletion of Th17 cells ablated the responses indicating that this IgA induction was Th17 dependant[323].

With regards to the clinical trial presented in this thesis, Group 2 somewhat resembled a prime-pull approach. The published observations of cellular immune responses in the mucosal compartment after prime-pull were in keeping with the potent cellular immune responses to Ag85A seen in the BAL of Group 2.

MVA85A was previously shown to induce Th17 responses[324]. If antibodies could have been measured in the lung mucosa, this pre-clinical study would suggest that Group 2 might have had vaccine induced IgA responses following the aerosol boost vaccination by a Th17 dependent mechanism. An optimised assay which is able to detect any mucosal insert and vector humoral responses will be required to conclude on antibody production in the lung mucosal compartment. This is one of the future considerations that this clinical trial highlighted.

Another consideration for a successful vaccine regimen with an aerosolised TB vaccine is optimisation of the heterologous prime-boost regimen. Such an improved regimen would utilise the most advantageous choice of vectors and maybe alternative inserts as well as dose and boosting intervals that are most beneficial. These are all questions already being addressed in vaccine development in general. The clinical trial presented here demonstrated that MVA85A by the aerosol route in BCG-vaccinated adults was able to elicit higher magnitude lung mucosal and comparable systemic insert responses compared to the intradermal route (Group1). Aerosol MVA85A given as a boost vaccination was most potent (Group 2). MVA85A was developed as a booster vaccine to BCG. MVA as a viral vector vaccine is good at boosting cellular immune responses. For instance, an adenoviral prime combined with MVA given as a boost vaccination was proven to be a promising combination in Malaria vaccine trials; and in a recent Ebola vaccine trial this regimen was shown to boost adenoviral primed cellular and humoral responses[196, 203]. A non-human primate study using mucosal (rectal) immunisation with DNA failed to induce systemic vaccine specific cytotoxic CD8+ T cells responses. However, boosting with a related MVA vaccine given as an intradermal vaccination triggered durable CD8+ T cell responses. Animals who only received the MVA vaccine (without prior mucosally delivered DNA

priming vaccine) had a low magnitude and non-durable responses[325]. This underlines the potential of MVA as a booster vaccine. Maybe use of aerosolised MVA85A to boost BCG-vaccinated or BCG plus MVA85A primed individuals in this clinical trial was an important factor in establishing the potent BAL responses seen in Groups 1 and 2 after aerosol vaccination[325]. A regimen which utilises different viral vaccine vectors and combines alternating routes might avoid any possible adverse events related to re-exposure to the vector. Any risk of immunopathology triggered by re-exposure of mycobacterial antigen by the aerosol route needs to be ruled out to make this regimen safe.

An optimal vaccine regimen does not only mean optimal immune responses but also that it is suitable and deployable in the target population. Large field clinical trials are needed to assess the suitability of an optimal vaccine, route and regimen in a representative population. For TB this means a target population living in an area where TB is endemic, resources are scarce, there is a high burden of LTBI, a high exposure to NTM as well as environmental exposure to substances such as biofuel. TB vaccine trials also require field trials for efficacy testing. Two such trials of MVA85A in infants and healthy HIV infected adults demonstrated no efficacy[130, 205]. It is important to consider the MVA85A data available from testing in pre-clinical animal models in the context of these human efficacy trials with MVA85A. This is a unique opportunity to do this comparison and understand what this implies for pre-clinical testing of TB vaccine candidates going forward. Animal models remain essential in evaluating the safety, immunogenicity as well as efficacy of TB vaccine candidates, and help to pinpoint parameters that predict responses in humans. The caveat is that it is still not known what correlates with protection from TB in humans. However, pre-clinical animal challenge studies can generate robust data on the level of protection

conferred by a vaccine regimen. Unlike influenza, malaria or typhoid, a human challenge with *M.tb* is not ethically justified to test the efficacy of a TB vaccine. A representative animal model that reflects the human response to TB vaccination and subsequently the response to *M.tb* exposure is therefore pivotal in TB. In the murine model, vaccination to the respiratory mucosa was shown to yield relatively compartmentalised vaccine responses. Intranasal vaccination with a viral vector vaccine in the murine model led to more potent responses and protection on aerosol challenge with *M.tb*; and the corresponding being true for systemic vaccination and T cell responses found in the spleen[141, 238]. A more recent study using BCG as a subcutaneous prime and then boosting with a lentiviral vector vaccine expressing Ag85A only induced minimal T cell responses in the lung and did not protect against intranasal mycobacterial challenge[326]. In a converse study where similar vaccines were administered but both by the systemic route, protective efficacy to intravenous challenge with *M.tb* was demonstrated[327]. This discrepancy between pre-clinical studies of TB vaccine candidates highlight that differences in the immune responses in murine body compartments might play a role in generating conflicting evidence depending on study design (i.e. route of vaccination, boosting and pathogen challenge). Mucosal delivery of MVA85A in mice, non-human primates and humans demonstrated notable differences between these models. In the non-human primate model, the magnitude of responses was dependent on the route of vaccination, with strongest systemic responses after systemic delivery and strongest lung mucosal responses in those vaccinated by the aerosol route. In the two clinical trials of aerosolised MVA85A stronger responses were observed in the BAL after aerosol administration. However, in humans a trend of higher responses in whole blood was also observed after aerosol administration compared to the systemic route[239, 247].

An important factor in advancing future studies is the need for a biomarker which predicts lung mucosal immune responses and permits comparison between different candidate vaccines in different models and populations. Biomarkers which best reflect vaccine-induced mucosal response would facilitate the evaluation and development of the aerosol delivery of TB vaccines. BAL samples are likely to be a representative sample of mucosal immunity induced by an aerosol vaccine but require an invasive method. The realisation of two bronchoscopies per volunteer in this trial was feasible and tolerated but a logistical challenge. As mentioned any biomarker would need to accurately reflect and be validated for its indication, i.e. in this case vaccine-induced mucosal immunity. A marker that correlates with magnitude or quality of vaccine-induced mucosal immune responses does not necessarily translate into protection.

Mucosal durability and T cell memory were not studied in this clinical trial. For a TB vaccine to confer protection from pulmonary TB in adolescents and adults, durable local lung mucosal as well as systemic memory response would need to be present. These vaccine-induced responses could then be re-initiated rapidly and vigorously on the second encounter with *M.tb.* It is pivotal to understand what combination of routes is able to establish durable antigen-specific memory T cell responses. As mentioned previously, several pre-clinical studies have demonstrated induction of antigen-specific tissue resident memory T cells by mucosal vaccination[232, 233, 235]. One of the first studies to report this ability by systemic immunisation is a study using BCG vaccination in mice which demonstrated systemic BCG vaccination being able to induce polyfunctional durable systemic and mucosal memory CD4+ T cells. On challenge with *M.bovis* this cell population expanded in the lung. More studies investigating the differential effects of vaccination route on durability and location of memory T cell responses would be useful. The extensive sample set generated in this

clinical trial allows future study of memory and homing responses which in turn can be used to inform study design of prospective clinical trials.

Future studies that examine the innate immune response in both systemic and lung mucosal compartment following aerosol vaccination are likely to complement our understanding of immune responses induced by this route of vaccination. Studying alveolar macrophages, B lymphocytes, unconventional T cells and other cell types in BAL would be very interesting, especially given the changes in BAL cell counts observed in one of the groups.

There are practical advantages associated with the aerosol route but also some challenges. The vaccine was stored frozen. To make the aerosol route of vaccination practical in the field, further work is needed on vaccine formulation and appropriate delivery devices. As mentioned, powdered BCG vaccine for inhalation has been generated by spray drying and aerosol delivery of BCG nanomicroparticles was shown to protect guinea pigs[233, 276]. The nebuliser used in this clinical trial was portable and operator friendly. The choice of device could also influence bioavailability. One study using aerosolised measles vaccine also reported better vaccine responses after increased exposure time in an age group that is more resistant to induction of seropositivity[328]. Further studies are required to determine bioavailability and hence actual dose of aerosolised MVA85A that reaches the lung mucosa. *In vivo* studies using traceable vaccine such as the one using real time scintigraphy to follow the fate of labelled NYVAC in non-human primates would be the best study design[259]. Such studies would also allow evaluation of a dose sparing potential of the aerosol route of vaccination more accurately.

In conclusion, the trials with aerosolised MVA85A to date, including the findings in this trial, suggest that this is a potent route of vaccine delivery inducing comparable systemic immunogenicity and superior lung mucosal immune responses. MVA85A given as an aerosol boost after an intradermal prime led to the most promising cellular insert Ag85A-specific immune responses in both lung mucosal and systemic compartments. However, this route did not have an acceptable safety profile. The clinical trial presented in this thesis adds to our understanding of cellular and humoral immune responses to vector and insert induced by aerosolised MVA85A as a prime or boost in a heterologous route regimen. These findings presented are important for the development of the mucosal route for viral vector vaccines against TB and other respiratory pathogens.

7 References

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8 Appendices

Appendix 1: Clinical Trial Protocol

Appendix 2: Volunteer Diary Cards

Appendix 3: Scanned bronchoscopic images

Appendix 4: Supplementary figures and tables

Appendix 1

- Clinical Trial Protocol



CLINICAL TRIAL PROTOCOL

Trial reference: TB035

Title:
A Phase I trial evaluating mucosal administration of a candidate TB vaccine, MVA85A, as a way to induce potent local cellular immune responses and avoid anti-vector immunity

Trial Reference:	TB035
Protocol Version Number:	4.1
Date:	13th November 2015
EudraCT number:	2013-002020-16
REC Reference:	13/SC/0329
Sponsor:	University of Oxford
Chief Investigator:	Prof Helen McShane
Local Safety Committee Chair:	Dr Brian Angus
Funding body:	Wellcome Trust, NIHR BRC
Author:	Dr Zita-Rose Manjaly Thomas

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."

Prof Helen McShane		14.11.15
Chief Investigator	Investigator Signature	Date

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 Independent Ethics Committee. This information cannot be used for any purpose other than the evaluation or conduct of the clinical investigation without the prior written consent of Prof Helen McShane.

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KEY ROLES AND GENERAL INFORMATION

Trial Centres:	Centre for Clinical Vaccinology and Tropical Medicine (CCVTM) & Oxford Centre for Respiratory Medicine Churchill Hospital, Old Road, Headington Oxford, OX3 7LE
Chief Investigator:	Prof Helen McShane CCVTM
Lead Investigator:	Dr Zita-Rose Manjaly Thomas CCVTM
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Monitor:	Clinical Trials & Research Governance Office Joint Research Office University of Oxford Block 60, Churchill Hospital

Old Road, Headington
Oxford OX3 7LE

LIST OF ABBREVIATIONS

AE	Adverse Event
ALT	Alanine Aminotransferase
BAL	Bronchoalveolar Lavage
BCG	Bacille Calmette-Guérin
CCVTM	Centre for Clinical Vaccinology and Tropical Medicine
CFP-10	Culture filtrate protein 10kDa
CFU	Colony-forming unit
CRF	Case Report Form
CXR	Chest X Ray
DNA	Deoxyribonucleic Acid
EC	Ethics Committee
ELISPOT	Enzyme-linked Immunospot
ESAT-6	Early-secreted antigenic target 6kDa protein
FBC	Full Blood Count
GCP	Good Clinical Practice
GMO	Genetically Modified Organism
GLP	Good Laboratory Practice
GMP	Good Manufacturing Practice
GMSC	Genetic Modification Safety Committee
GP	General Practitioner (Family Doctor)
HBsAg	Hepatitis B Surface Antigen
HBV	Hepatitis B Virus
HCG	Human Chorionic Gonadotrophin
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
ICH	International Committee on Harmonisation
ID	Intradermal
IFN-γ	Interferon Gamma
IMP	Investigational Medicinal Product
INH	Inhaled
LFT	Liver Function Test
LSC	Local Safety Committee
LSM	Local Safety Monitor
M. tb	*Mycobacterium tuberculosis*
MVA	Modified vaccinia Virus Ankara
NHS	National Health Service
PBMC	Peripheral Blood Mononuclear Cells
PFT	Pulmonary Function Tests
QP	Qualified person
SAE	Serious Adverse Event
SFC	Spot-forming cell
SOP	Standard Operating Procedure
SUSAR	Suspected Unexpected Serious Adverse Event
TB	Tuberculosis

LIST OF PROTOCOL AMENDMENTS

Protocol v4.1 Non-Substantial Amendment

Section	From	Changed To
10	Data will be stored in either paper CRFs held in a key-locked cabinet at the CCVTM, or electronic CRFs on the OpenClinica™ database (which is currently being piloted at the CCVTM and which is stored electronically on a secure encrypted University of Oxford server), or both	Data will be stored in either paper CRFs held in a key-locked cabinet at the CCVTM, or electronic CRFs on the OpenClinica™ database (which is stored electronically on secure servers outsourced by OpenClinica™), or both

Protocol v 4.0 Substantial Amendment

Section	From	Changed To
1		*For Group 2 amendment please see section 4.1*
4.1		*** Change to Group 2 vaccinations** A change has been made for group 2 volunteers due to receive their day 28 boost vaccination on or after 2 September 2015, due to a higher frequency of mild respiratory adverse events in this group. Local (skin) and systemic symptoms were as expected and consistent with data from previous studies. These volunteers will not receive the day 28 boost vaccination, but instead will receive a placebo by both intradermal and aerosol inhaled routes to maintain blinding. Additional safety and immunological information will be gained by continuing with day 35 bronchoscopy in these volunteers. Any volunteer not wishing to proceed in this manner will remain in follow up with no vaccination or bronchoscopy.
6.1	Group 2: Intradermal MVA85A 5 x 10^7 pfu (and aerosol inhaled saline placebo) on day 0, followed by homologous boosting with MVA85A 5 x 10^7 pfu aerosol inhaled on day 28 (and intradermal saline placebo)	Group 2: Intradermal MVA85A 5 x 10^7 pfu (and aerosol inhaled saline placebo) on day 0, followed by homologous boosting with MVA85A 5 x 10^7 pfu aerosol inhaled on day 28 (and intradermal saline placebo) OR, following safety amendment (see section 4.1) Intradermal MVA85A 5 x 10^7 pfu (and aerosol inhaled saline placebo) on day 0, followed by aerosol inhaled saline placebo and intradermal saline placebo on day 28

Protocol v 3.0 Substantial Amendment

Section	From	Changed To
4.3		**Restart of enrolment into Group 2** When restarting enrolment into group 2 of the trial, the first participant will be enrolled and no further enrolment will take place until they have received their second bronchoscopy. A

		second volunteer will then be enrolled and once they have received their second bronchoscopy, providing there are no safety concerns, enrolment will continue as normal.
10.2	Subjects 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).	Subjects 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, bank details request form and consent forms).
10.4	The study protocol, documentation, data and all other information generated will be held in strict confidence.	The study protocol, documentation, bank details request form, data and all other information generated will be held in strict confidence and in accordance with the Data Protection Act.

Protocol v 2.0 Substantial Amendment

Section	From	Changed To
5.2 Exclusion Criteria	Clinically significant history of skin disorder, allergy, 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	Clinically significant history of skin disorder, allergy, <u>atopy,</u> 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

Protocol v 1.3 Non-Substantial Amendment		
Section	**From**	**Changed to**
Key roles		Removal of Dr Sharon Sheehan (left department prior to start of the study)
1. Trial Summary	20ml exploratory immunology on D2 and D30	4ml of Biochemistry and 16ml of exploratory immunology i.e total volume of 20ml remains the same

Protocol v 1.2 Non-Substantial Amendment

Section	From	Changed To
Key roles		Addition of Alice Minhinnick and Morven Wilkie
8.3	All solicited AEs (listed in table 4) and any additional unexpected AEs during the diary card period will be documented in the AE line listing section of the CRF.	During the diary card period, all solicited AEs (listed in table 4) and any additional unexpected AEs are captured in the diary card and at clinic visits (CRF)

Protocol v1.1 Non-Substantial Amendment

Section	**From**	**Changed To**
Key Roles	Churchill Hospital	Oxford University Hospitals NHS Trust
1.	Churchill Hospital	Oxford University Hospitals NHS Trust

1. TRIAL SUMMARY

Trial Title	A Phase I trial evaluating mucosal administration of a candidate TB vaccine, MVA85A, as a way to induce potent local cellular immune responses and avoid anti-vector immunity
Trial Identifier	TB035
Clinical Phase	I
Active Ingredients of Vaccines	MVA85A
Finished Products	MVA85A
Dose(s)	5×10^7 pfu
Route(s)	Aerosol inhalation by nebuliser and intradermal needle injection in the deltoid region of the arm
Trial Interventions	Chest x ray at screening Pulmonary function tests at screening and after vaccination Vaccination by both intradermal and aerosol inhaled routes Venepuncture Bronchoscopy and bronchoalveolar lavage
Chief Investigator	Prof Helen McShane
Trial Centres	Centre for Clinical Vaccinology and Tropical Medicine (CCVTM), Churchill Hospital, Old Road, Headington, Oxford, OX3 7LE & Oxford Centre for Respiratory Medicine, Oxford University Hospitals NHS Trust
Planned Trial Period	Prospective start date for screening August 2013
Trial Duration	Estimated at 18 months
Primary Objectives	Safety of 5×10^7 pfu dose of MVA85A administered by aerosol and compared to the same dose administered intradermally
Secondary Objectives	- Characterise mucosal and systemic immunogenicity of viral vector (MVA) and insert (Ag85A) by comprehensive characterisation of humoral and cellular immune responses - Evaluate functional relevance of anti-vector immunity induced by aerosol and systemic immunisation in MVA85A-prime followed by homologous MVA85A-boost administered 4 weeks later
Planned Sample Size	36 healthy, BCG vaccinated adult subjects; 12 subjects per treatment arm
Allocation Method	Variable block randomisation by sequentially numbered sealed envelopes
Vaccination Schedule	Priming vaccination at day 0 (aerosol or intradermal) Homologous boosting vaccination 1 month later (aerosol or intradermal)
Follow-up Duration	24 weeks from enrolment
Blood Sampling Schedule	Safety blood tests (FBC, coagulation screen and clinical chemistry) as per schedule (table 1) HIV, hepatitis B & hepatitis C antibody blood tests at screening Exploratory immunology tests at screening, as per schedule (table 1) HLA testing at day 0
Primary Evaluation Criteria	Actively and passively collected data on adverse events
Secondary Evaluation Criteria	Laboratory markers of cell mediated immunity in blood and bronchoalveolar lavage samples

Attendance number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Timeline (days)*	S	0	2	7	14	28	30	35	42	84	168
Timeline (weeks)*	S	0		1	2	4		5	6	12	24
Time windows (days)			±1	-2+3	±4	±5	±1	-2+3	±4	±14	±21
Inclusion/exclusion criteria	X										
Review contra-indications	X	X		X		X		X			
Informed consent	X										
Medical history	X	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)
Physical examination	X			X				X			
Vital signs	X	X	X	X	X	X	X	X	X	X	X
Urinalysis	X										
Chest x ray	X										
Pulmonary function tests	X	X	X	X		X	X	X			
β-HCG urine test	X	X		X		X		X			
Vaccinations		X				X					
Bronchoscopy				X				X			
Local & systemic events/reactions		X	X	X	X	X	X	X	X	X	X
Diary cards provided		X				X					
Diary cards collected					X				X		
Biochemistry (4mL)* **	X		X	X			X	X			
Haematology (2mL)** **	X			X				X			
Coagulation (4mL)	X										
HBV, HCV, HIV (10mL)	X										
HLA typing (4mL)		X									
Exploratory immunology incl ELISPOT (20-60mL)**	X	X	X	X	X	X	X	X	X	X	X
Blood vol (mL)	**50**	**64**	**20**	**66**	**60**	**60**	**20**	**66**	**60**	**60**	**60**
Cumulative blood vol (mL)	**50**	**114**	**134**	**200**	**260**	**320**	**340**	**406**	**466**	**526**	**586**

S	Screening
X	Event scheduled to occur
(X)	If considered necessary, emphasising any complaint or change in medications
*	Timeline is approximate only, as exact timings (± time windows) of visits relate to the actual (not intended) date of the previous visit
**	Exploratory immunology blood volume will be a total of 586 ml; these samples will be processed and analysed in the Jenner Laboratories, University of Oxford
***	Sodium, Potassium, Urea, Creatinine, Liver function; these samples will be analysed at OUH
****	Full blood count; these samples will be analysed at OUH
Grey	Highlights vaccination day
Blue	Highlights bronchoscopy day

Table 1: Schedule of trial procedures

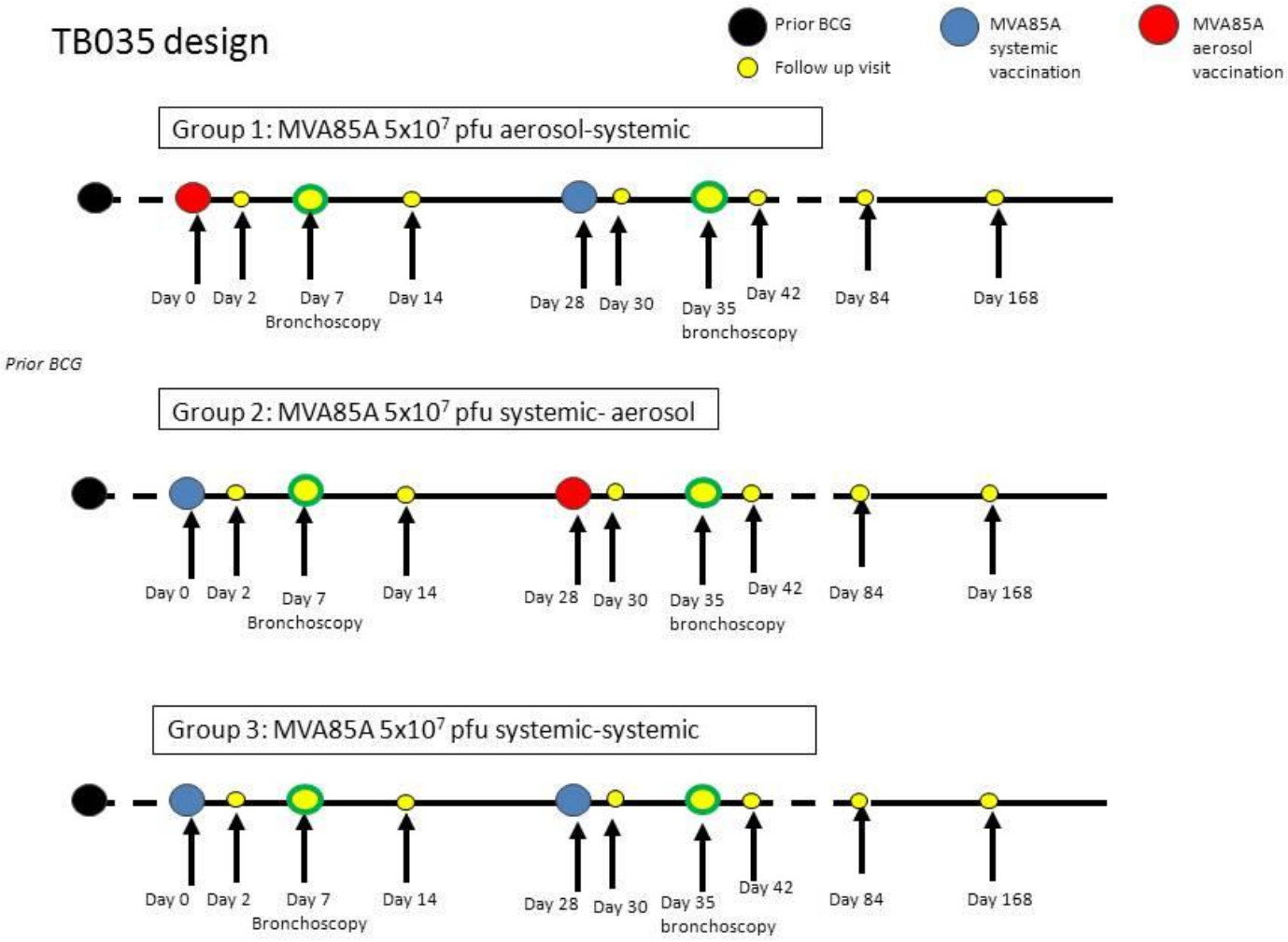


Figure 1. Trial schedule
For Group 2 amendment please see section 4.1

2. BACKGROUND AND RATIONALE

2.1 Introduction

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. A third of the world's population is latently infected with *M. tb*, these people carry a 10% lifetime risk of developing active life-threatening disease (1). Worldwide there were an estimated 8.7 million new cases of TB and an estimated 1.4 million deaths associated with TB in 2011 (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 TB vaccine and it has been administered globally to several billion people over a 70 year period (5). Although it does not protect against pulmonary TB in many parts of the world, it is effective in preventing disseminated TB disease including tuberculous meningitis in childhood (4, 6, 7). Recently heterologous "prime-boost" vaccination strategies, in which two different candidate vaccines expressing antigens in common are given weeks or months apart, have generated strong and sustained cellular immune responses correlating with a TB protective effect in preclinical animal models. In such a "prime-boost" strategy, BCG would therefore be an ideal priming vaccine.

MVA85A is a recombinant attenuated Modified Vaccinia virus Ankara expressing the *M. tb* antigen 85A. This antigen is highly conserved in all strains of BCG and *M. tb*, and is immunodominant in both animal and human studies. Developed by the University of Oxford, MVA85A has now been administered to over 2300 human individuals with an excellent safety profile. It has been shown to be highly immunogenic as a booster vaccine in BCG-primed subjects (8). A large phase IIb efficacy trial in BCG-vaccinated infants in South Africa has recently reported no enhancement of BCG-induced protection (9), however, in this trial, the immunogenicity was significantly lower than that seen in adults, and more potent routes of administration are of interest in the field. A phase IIb trial in HIV infected adults in South Africa and Senegal is ongoing (ClinicalTrials.gov reference number NCT01151189).

To date, all new TB vaccine candidates had been administered systemically. However there is data from preclinical animal models with this and other virally vectored vaccines to suggest that immunising the respiratory mucosa may give superior protection against respiratory diseases (10-13).In addition, the aerosol inhaled route of MVA85A delivery could offer practical, tolerability and safety benefits over and above needle-based methods (see below).

A Phase I safety study assessing the safety and feasibility of administering low dose MVA85A by nebuliser direct into the airways has just been completed. To our knowledge there has been no previous clinical study of any vectored vaccine delivered by aerosol except for this completed study in our group.

Enrolment for this trial was completed in November 2012, no vaccine related Serious Adverse Events were reported and aerosol immunisation was well tolerated (Meyer *et al,* personal communication). In that trial 12 volunteers have had the MVA85A as aerosol vaccination- 10 of those had the low dose (1×10^7 pfu) and 2 volunteers had the high dose which we would normally give intradermally (1×10^8 pfu). The intermediate dose of 5×10^7 pfu will be used in this trial for the first time.

Rationale for the aerosol inhaled route for TB vaccination in humans

There are strong reasons for administering a TB vaccine via the airway. Preclinical animal work increasingly indicates that the best protection occurs when the route of immunisation matches the route of infection (10-12). The most common natural route of *M. tb* infection 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 characterised by bronchus associated lymphoid tissue (BALT) which is well adapted to encounter and process antigens presenting to the respiratory mucosa. Immunising via the airway should therefore have the advantage, over other routes of eliciting protective immune responses in the lung mucosal surface. We know that BCG, administered systemically, protects most effectively against systemic disease in childhood (6, 7). It may follow that airway vaccination is needed to induce effective protection from pulmonary disease.

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. Clinical trials of aerosolised measles vaccines have shown that mass immunisation by the respiratory mucosal route in a developing country is feasible and can induce strong and long lasting immune responses (14-20). There is also valuable experience with aerosolised measles vaccine given to over 4 million schoolchildren in Mexico, where public acceptance was high, costs were low, and there were fewer side effects reported than with subcutaneous vaccination (21). A nasally administered influenza vaccine is licensed in North America (22-24) and others are in clinical trials (25, 26). Furthermore the principle of mucosal surface vaccination is well established as shown by those widely used in clinical practice against polio, typhoid, cholera and rotavirus.

There are numerous perceived advantages of aerosol inhaled vaccination: it is safe, needle-free, well-tolerated, popular, less technically demanding, economically beneficial, and allows self-administration of vaccine or administration by less technically trained community health workers (21). Furthermore, use of non-injectable vaccines reduces the likelihood of unsafe disposal and reuse of syringes in immunisation programmes (27).

A problem with all virally-vectored vaccines including MVA85A is that individuals build up immunity to the virus base (anti-vector immunity) which makes the vaccine less efficient and not suitable for re-use within short time frames. An important potential advantage of mucosal immunisation, in addition to the ones mentioned thus far, is that aerosol vaccination may minimise or avoid induction of systemic anti-vector immunity and allow homologous boosting with the same viral vector; thus minimising cost of vaccine development (28-30). Furthermore, for some vectors, e.g. human Adenovirus serotype-5, up to 80% of adults have antibodies to AdHu5 and this pre-existing immunity significantly limits the utility of this vector (31-34). Our hypothesis is that mucosal immunisation with MVA85A does not result in induction of systemic anti-vector immunity. Recent work in nonhuman-primates by our collaborators at the Health Protection Agency, comparing 2 routes of administration, showed anti-MVA antibody responses are seen in intradermally vaccinated animals but not in animals given MVA85A by aerosol (Figure 2; (35))

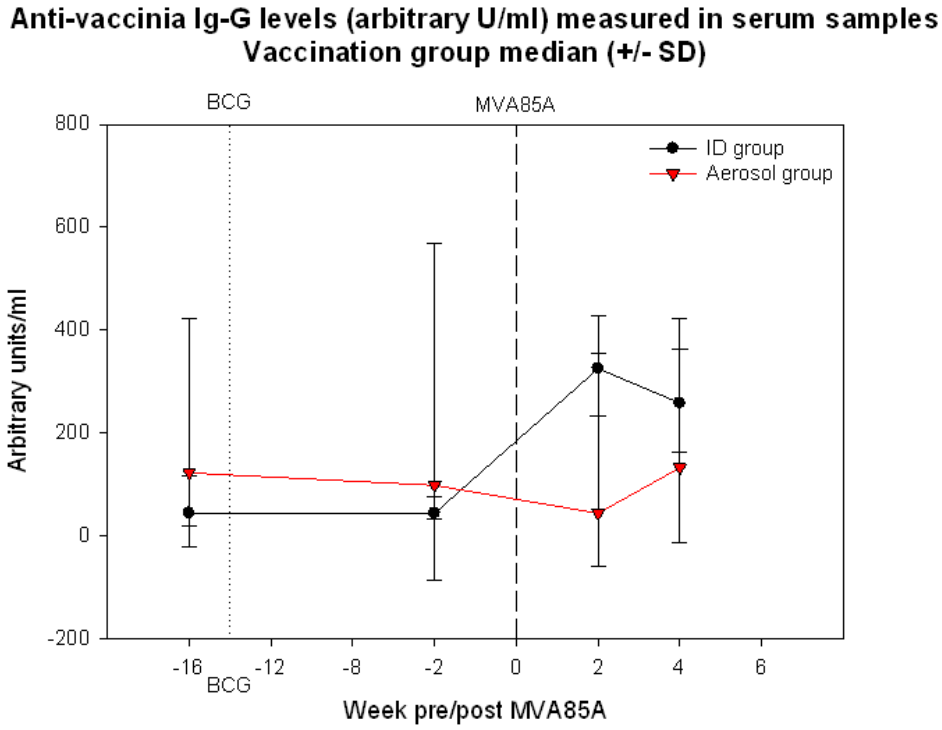


Figure 2: Vector specific IgG response induced by vaccination with MVA85A measured by ELISA. The plot shows the median change from prevaccination level for the BCG prime with MVA85A intradermal boost group with the closed circles and for the BCG prime with MVA85A aerosol boost group with triangles. Error bars are standard deviations from the median *(35).*

This effect of reducing anti-vector responses by mucosal administration has also been demonstrated in mice, rabbits and guinea pigs (12, 36, 37). There is an increased need to find strategies to overcome anti-vector immunity in vaccine development especially for vaccines that will need to be given repeatedly (e.g. seasonal influenza), so if this is also demonstrated in humans, this would be an important development for the field of viral vaccines and respiratory pathogens for which mucosal immunisation may improve efficacy (11, 12).

This project will quantify for the first time the induction of anti-vector immunity (humoral and cell-mediated) by both aerosol and systemic administration of MVA85A. The functional relevance of this anti-vector immunity will be evaluated by a second boost vaccination of MVA85A via a different administration route, one month after the first, priming immunisation. This data will have implications for the TB vaccine field but also for the wider vaccine community and for the development of mucosal vaccination strategies for other respiratory and mucosal pathogens.

Description and pre-clinical experience of MVA and MVA85A

Modified vaccinia virus Ankara (MVA) is a highly attenuated strain of vaccinia virus which cannot replicate in human cells. It is known to be highly immunogenic and is therefore suitable for use as a viral vector in new vaccine development. It has an excellent safety record as it was administered intradermally to approximately 120,000 people during the smallpox eradication campaign (38-41), and has since been used in numerous clinical trials of candidate vaccines against viral, mycobacterial and protozoal infections (42, 43). Meanwhile recombinant MVA vaccines administered by respiratory mucosal and gut mucosal routes have demonstrated strong immune responses in both rodents and non-human primates (44).

Antigen 85A is a highly conserved antigen expressed by *M. tb*, BCG, and all other mycobacterial species sequenced to date. It is a 32-kDa protein, and is an enzyme, mycolyl transferase, which is involved in cell wall biosynthesis (45). Antigen 85A is highly immunodominant in both animal and

human studies (46-48), and protects against *M. tb* challenge in mice, guinea pigs and non-human primates (49, 50). The recombinant MVA85A vaccine incorporates the 1176 base pair gene for antigen 85A into the viral DNA allowing expression of this secreted antigen.

Experiments in mice, guinea pigs, cattle, and non-human primates have shown that a prime-boost schedule of vaccination with BCG followed by MVA85A, either intradermally, intramuscularly or mucosally, can improve protective efficacy against subsequent *M. tb* challenge, compared to BCG alone (10, 50-52). A mouse GLP toxicity study using the intradermal route of administration revealed no differences from PBS-injected controls apart from irritation at the site of administration. An additional mouse GLP toxicology study of aerosol administration revealed no differences from controls given aerosolised PBS.

Clinical studies with MVA85A

Over 2300 subjects have now received MVA85A, of which 12 were by the intramuscular route, 12 by the aerosol route, and the remainder intradermally. 18 Phase I/II clinical studies of MVA85A have been completed, and a further 4 are on-going. These are summarised in Table 2.

Protocol	Phase	Population	Treatment groups[a]	N	Study Status
TB002	Phase I open label non-randomised	Healthy BCG-naïve adults, UK	5×10^7 pfu MVA85A (Days 0 & 21)	14	Completed
GM920	Phase I open label non-randomised	Healthy BCG-naïve or BCG-vaccinated adults, Gambia	5×10^7 pfu MVA85A (Days 0 & 21) BCG naïve 5×10^7 pfu MVA85A (Day 0) BCG-vaccinated	11 10	Completed
TB004	Phase I open label non-randomised	Healthy BCG-naïve adults, UK	1×10^6 pfu BCG prime (Day 0) 5×10^7 pfu MVA85A boost (after 1 month)	10	Completed
TB005	Phase I open label non-randomised	Healthy BCG-vaccinated adults, UK	5×10^7 pfu MVA85A (Day 0)	21	Completed
TB007	Phase I open label non-randomised	Healthy adults latently infected with *M. tb*, UK	5×10^7 pfu MVA85A (Day 0)	12	Completed
TB008	Phase I open label non-randomised	Healthy BCG-naïve or vaccinated adults & adolescents, South Africa	5×10^7 pfu MVA85A (Day 0) adults 5×10^7 pfu MVA85A (Day 0) adolescents	24 12	Completed
TB009	Phase I open label non-randomised	Healthy BCG-vaccinated adults, UK	1×10^7 pfu MVA85A (Day 0) 1×10^8 pfu MVA85A (Day 0)	12 12	Completed
TB010	Phase I open label non-randomised	HIV-positive adults, UK	5×10^7 pfu MVA85A (Day 0) 1×10^8 pfu MVA85A (Day 0)	10 10	Completed
TB011	Phase I open label non-randomised	Adults infected with *M. tb*, HIV, or both, South Africa	5×10^7 pfu MVA85A (Day 0) TB 5×10^7 pfu MVA85A (Day 0) HIV 5×10^7 pfu MVA85A (Day 0) TB & HIV 5×10^7 pfu MVA85A (Day 0) HIV on ARV treatment	12 12 12 12	Completed
TB012	Phase II open label non-randomised	Healthy BCG-vaccinated children & infants, South Africa	<u>Stage 1</u> EPI alone (Day 0) 2.5×10^7 pfu MVA85A with EPI (Day 0) 2.5×10^7 pfu MVA85A alone (Day 0)[b] EPI alone (Day 0) 5×10^7 pfu MVA85A with EPI (Day 0) 5×10^7 pfu MVA85A alone (Day 0)[b] <u>Stage 2</u> EPI alone 5×10^7 pfu MVA85A with EPI (Day 0) 5×10^7 pfu MVA85A alone (Day 0)[b]	 12 12 12 12 12 12 47 47 48	<u>Stage 1</u> Completed <u>Stage 2</u> Completed
TB014	Phase II open label non-randomised	Healthy BCG-vaccinated children & infants, South Africa	5×10^7 pfu MVA85A (Day 0) children 2.5×10^7 pfu MVA85A (Day 0) infants 5×10^7 pfu MVA85A (Day 0) infants 1×10^8 pfu MVA85A (Day 0) infants Prevenar (variable dose) (Day 0) infants	24 36 36 36 36	Completed

TB017	Phase I open label non-randomised	Healthy BCG-vaccinated adults, UK	5×10^7 pfu FP85A (Day 0) 5×10^7 pfu MVA85A (Day 0) then 5×10^7 pfu FP85A (Day 28) 5×10^7 pfu FP85A (Day 0) then 5×10^7 pfu MVA85A (Day 28)	12 12 7	Completed
TB018	Phase I open label non-randomised	Healthy BCG-vaccinated adults, UK	1×10^8 pfu MVA85A (Day 0) 1×10^8 pfu MVA85A (Day 0) then 1 g/kg deuterium labelled glucose (Day 4) 1×10^8 pfu MVA85A (Day 0) then 1 g/kg deuterium labelled glucose (Day 10)	4 4 4	Completed
TB019	Phase I open label non-randomised	HIV-infected adults, Senegal	1×10^8 pfu MVA85A (Day 0 & 168) 1×10^8 pfu MVA85A (Day 0 & 168) on ARV treatment	12 12	Completed
TB020	Phase II double blinded randomised	Healthy BCG-vaccinated HIV-negative infants, South Africa	1×10^8 pfu MVA85A (Day 0) Placebo	1399 1398	Completed
TB021	Phase II double blinded randomised	Healthy HIV-infected adults, South Africa & Senegal	1×10^8 pfu MVA85A (Day 0) Placebo	325 325	In follow up
TB022	Phase I open-label randomised	Healthy BCG-vaccinated adults, UK	Intramuscular 1×10^8 pfu MVA85A (Day 0) Intradermal 1×10^8 pfu MVA85A (Day 0)	12 12	Completed
TB023	Phase I open-label non-randomised	Healthy BCG-naïve or vaccinated adults, UK	$1x10^8$ pfu MVA85A (Day 0) then BCG challenge (Day 28) BCG: 100 µl ~ 2-8 x 10^5 pfu	24 24	Completed
TB026	Phase I randomised blinded	Healthy BCG-vaccinated adults, UK	$1x10^7$ pfu aerosol inhaled MVA85A with intradermal saline placebo $1x10^7$pfu intradermal MVA85A injection with aerosol inhaled saline placebo	12 12	Completed
TB029	Phase II randomized controlled trial	Infants of HIV infected mothers, South Africa	1×10^8 pfu MVA85A or Candin control at birth BCG:0.05mL 1-4 x 10^5 cfu at 8 weeks of age (Infants confirmed HIV uninfected)	170 170	Enrolling
TB032	Phase I open-label non-randomised	Healthy BCG-vaccinated adults, UK	1×10^{11} vp AERAS-402 (Day 0 and 28) followed by 1×10^8 pfu MVA85A (Day 119) 1×10^{11} vp AERAS-402 (Day 0) followed by 1×10^8 pfu MVA85A (Day 56) 1×10^{11} vp AERAS-402 (Day 0, 28 and 119)	15 15 15	Enrolling
TB034	Phase I open-label randomised trial	Healthy BCG-vaccinated adults, UK	Starter group: 5×10^9 vp ChAdOx1 85A 2.5×10^{10} vp ChAdOx1 85A 2.5×10^{10} vp ChAdOx1 85A (D0) followed by 1×10^8 pfu MVA85A (D56)	6 12 12	Enrolling

[a]Intradermal route of administration unless otherwise stated

[b]EPI deferred for 1 week

Table 2. Summary of clinical trials of MVA85A

Safety profile
The typical local reaction as a result of intradermal injection of MVA85A is temporary pain, redness and swelling at the site of the injection with a 5-10 mm central red swollen area and a paler red area ranging from 5-10 mm in diameter which peaks at 48 hours post vaccination. At seven days post vaccination, only the central area of redness remains. This fades over a few weeks and is not usually apparent eight weeks after vaccination. No signs of any Koch type reaction in subjects infected with *M. tb*, has been seen with this or any other candidate vaccine to date.

Systemic reactions to the vaccine are short-lived, and resemble influenza symptoms including fatigue, headache, malaise, feverishness, and muscle aches. These systemic symptoms are typically mild and occur in 50-90% of all subjects.

The preliminary analysis of local and systemic reactions induced by the aerosol route that were reported in the completed Phase I safety study are summarised in section 4.4 “Risks and Benefits”.

Dosing
Dose studies of MVA85A have been performed in a step-wise fashion to minimise the risk of a Koch reaction and the incidence of adverse events. The main dose-finding trial compared boosting doses of 1×10^7 pfu and 1×10^8 pfu to previous trials using boosting doses of 5×10^7 pfu. An intradermal dose of 1×10^8 pfu was found to be significantly more immunogenic than the lower doses without concomitant worsening of the adverse event profile, and has subsequently been adopted as the standard dose in all subsequent trials (53, 54). The aerosol dose administered in trial TB026, a Phase I safety study assessing the safety and feasibility of administering MVA85A by the aerosol route, was 1×10^7 pfu; safety data from this study supports dose escalating to 5×10^7 pfu is the safe and logical next step.

Immunogenicity
When evaluated by various assays of cellular immunology including interferon gamma (IFN-γ) ELISPOT and intracellular cytokine staining assays, MVA85A has induced a strong and sustained cell-mediated immune response which is known to be important for protective immunity (55). A Phase IIb efficacy trial of MVA85A in BCG-vaccinated infants in South Africa has recently been completed and work to identify correlates of protection is now underway (9).

Aerosol delivery devices

Currently the World Health Organisation (WHO) is making a major investment in developing new aerosol devices for portable, low cost vaccine delivery (56). The WHO’s Measles Aerosol Project aims to develop and license respiratory delivery for measles vaccines currently given by injectable methods. The Edmonton Zagreb strain of measles vaccine, a live virus vaccine, has been given by mesh nebuliser with excellent results. Modern devices are small, lightweight, portable, and aerosolise through a mesh to provide small and consistently-sized particles which deliver vaccine 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. MVA particles are typically around 400 nm in size and can therefore be aerosolised with minimal damage.

We demonstrated adequate delivery of our vaccine, MVA85A, by this device in preliminary plaque assays. Using a nebuliser adapted for rodent administration, we performed GLP standard toxicology studies of aerosolised MVA85A (1×10^8 pfu) in BalbC mice, demonstrating satisfactory safety and systemic immunogenicity. We further administered aerosolised MVA85A (1×10^8 pfu) by the MicroAIR NE-U22 mesh nebuliser to eight macaques, showing a good safety profile and satisfactory delivery by inhaled route (unpublished data).

Bronchoscopy and bronchoalveolar sampling

Protection from TB induced by intra-pulmonary vaccination correlates with cellular immune responses detected in the bronchoalveolar lavage (BAL) samples of immunised mice (57). In macaques receiving aerosolised MVA85A we detected significant levels of antigen-specific cellular immune responses in bronchoalveolar lavage samples (35). These pre-clinical findings provide the rationale for wishing to obtain BAL samples from our clinical trial subjects.

BAL samples are obtained by performing a bronchoscopy, which is a widely and safely used procedure in clinical research studies involving both healthy subjects and subjects with respiratory diseases such as asthma (58, 59). Clinical guidelines for performing investigative bronchoscopy in research studies are well established (60). The short procedure involves the insertion of a narrow flexible fibre-optic tube into the airway under light intravenous sedation. Under direct vision saline is delivered to a section of lung mucosa and then recollected by suction. In clinical trial TB026 bronchoscopies were well tolerated by volunteers.

Bronchoscopy is an essential component of this clinical trial because BAL samples are the ideal method to analyse the lung mucosal cellular immune responses which we intend to induce by vaccination (61). Clinical trials of intranasal vaccine candidates for other infectious diseases have analysed humoral immunity within nasal lavage, but a cellular immune response is essential for protective immunity against *M. tb* (62). It is unlikely that administering a vaccine intranasally in humans would result in the induction of a significant cell mediated immune response in the lower airways. Nasal mucosal samples are unlikely to provide adequate cellular samples as nasal lavage generally yields insufficient and variable numbers of cells (63). Furthermore as the vaccine is nebulised and inhaled orally we do not expect it to reach the nasal mucosa.

2.2 Hypothesis

Previous studies have demonstrated that a 3 week interval between homologous MVA85A–MVA85A administered systemically does not enhance responses (8). We hypothesise that responses will be boosted in the aerosol–systemic and systemic-aerosol group but not in the systemic–systemic group.

3. OBJECTIVES AND OUTCOME MEASURES/ ENDPOINTS

Objectives	Outcome Measures/Endpoints
Primary Objective To evaluate the safety of 5 x 10^7 pfu dose of MVA85A administered by aerosol and compared to the same dose administered intradermally.	Safety of 5 x 10^7 pfu dose MVA85A administered by the inhaled route through actively and passively collected data on adverse events.
Secondary Objectives To characterise mucosal and systemic immunogenicity of viral vector (MVA) and insert (Ag85A) by comprehensive characterisation of humoral and cellular immune responses Evaluate functional relevance of anti-vector immunity induced by aerosol and systemic immunisation in MVA85A-prime followed by homologous MVA85A-boost administered 4 weeks after the MVA85A-prime	1. Induction of humoral and cell-mediated immunity to MVA by aerosol and systemic immunisation 2. Induction of cell-mediated and humoral immunity to Ag85A by aerosol and systemic immunisation 3. Effect of a boosting immunisation one month after prime on humoral and cell-mediated immunity to insert (Ag85A) and vector (MVA)

4. TRIAL DESIGN

4.1 Trial Overview

This is a Phase I clinical trial where subjects are randomised to 3 different groups of homologous MVA85A boosts by either intradermal or aerosol route of vaccination to evaluate safety and immunogenicity of homologous boosting by heterologous routes of immunisation.

Trial groups

This will be a Phase I clinical trial with a standard dose of MVA85A (pfu) in 3 groups of 12 volunteers with 3 different route permutations for boosting (see Table 3).

The overall investigational approach with our MVA85A trials is to develop an effective prime-boost vaccination strategy to prevent TB infection, with BCG as the priming vaccine.

This Phase I trial will have 3 treatment arms (homologous):

Group	Route of priming MVA85A	Bronchoscopy 1 week later	Route of boosting MVA85A	Bronchoscopy 1 week later
1	Aerosol	Yes	Systemic	Yes
2	Systemic	Yes	Aerosol*	Yes
3	Systemic	Yes	Systemic	Yes

Table 3: Route of immunisations and timing of bronchoscopy

*** Change to Group 2 vaccinations**
A change has been made for group 2 volunteers due to receive their day 28 boost vaccination on or after 2 September 2015, due to a higher frequency of mild respiratory adverse events in this group. Local (skin) and systemic symptoms were as expected and consistent with data from previous studies. These volunteers will not receive the day 28 boost vaccination, but instead will receive a placebo by both intradermal and aerosol inhaled routes to maintain blinding. Additional safety and immunological information will be gained by continuing with day 35 bronchoscopy in these volunteers. Any volunteer not wishing to proceed in this manner will remain in follow up with no vaccination or bronchoscopy.

As BCG is protective in childhood, it is routinely given at birth in TB endemic countries but is also in widespread use in children and adolescents in much of the rest of the world. Therefore, as in previous trials of intradermal MVA85A, we propose to vaccinate BCG-primed subjects. These will be healthy men and women aged 18-55 years who have been previously vaccinated with BCG.

All subjects will have received BCG vaccination at least six months prior to enrolment. MVA85A (5 $x10^7$ pfu) or saline placebo will be administered by systemic (intradermal) or aerosol (Omron jet nebuliser) route (paired placebo design). The interval between the two vaccinations is one month.

In order to minimise the risk from bronchoscopy, subjects with a history or evidence of lung disease will be excluded. This will be determined by our standard screening procedures (history, examination, urine analysis, and blood tests), and, in addition, a chest x ray and pulmonary function testing.

The peak effector T-cell response in blood is ~7 days post-vaccination with MVA85A. Pulmonary mucosal responses are expected to follow a similar time course and a preclinical study has demonstrated this in non-human primates (35). Thus bronchoscopy and broncholaveolar lavage will be performed seven days after each vaccination.

4.2 Randomisation

In order to reduce bias and the influence of any difference in the baseline characteristics between the three groups, we have chosen to randomise the allocation of subjects. This aims to achieve a balance between the three groups in any important confounding factors such as levels of pre-existing antimycobacterial immunity (influenced by, for example, country of origin and length of time elapsed since prior BCG vaccination). This will be performed by variable block randomisation using sequentially numbered sealed envelopes, prepared by an independent colleague at the Centre for Statistics in Medicine, to ensure allocation concealment. Volunteers are blinded as they will receive a paired placebo at the same time of vaccination. The bronchoscopist will be blinded to the route of vaccine administration. This is intended to eliminate any bias in the reporting of the appearance of the lung mucosa and extent of airway inflammation. In addition, the postdoctoral researcher in the laboratory overseeing the immunological analyses will also be blinded to reduce any bias that could be introduced at the sample processing stage. All samples will be anonymised.

4.3 Vaccine dosage

Trials of MVA85A to date have established 1 x 10^8 pfu as the optimal dose for intradermal injection in adults. There are no published studies of dose-response via the mucosal route of vaccination. Although the fractional delivered dose of aerosolised viral vectored vaccines to the mucosal surface may be considerably less than 25%, there is evidence that strong mucosal and systemic immune responses, comparable to the injectable route, are nevertheless achieved (64, 65). The first aerosol safety study (TB026) was completed using 1 x 10^7 pfu MVA85A with no severe or serious adverse events.

In this trial we will dose escalate to a medium dose to further evaluate safety and immunogenicity. The first volunteer in the study will receive a dose of 5 x 10^7 pfu MVA85A by the aerosol route. This volunteer will be vaccinated ahead of any other volunteers and the profile of adverse events will be examined. No other volunteers will be vaccinated until at least 48 hours has elapsed following the first volunteer being vaccinated. The Chief Investigator will then decide whether it is safe to proceed to vaccinate the remaining volunteers post review of this safety data. The Chair of the Local Safety Committee will be informed if an untoward adverse event profile occurs that differs from past experience with MVA85A.

Restart of enrolment into Group 2

When restarting enrolment into group 2 of the trial, the first participant will be enrolled, and no further enrolment will take place until they have received their second bronchoscopy. A second volunteer will then be enrolled and once they have received their second bronchoscopy, providing there are no safety concerns, enrolment will continue as normal.

Time points

Prior BCG – trial enrolment time window

The specified period for prior receipt of BCG is such that no less than six months must have elapsed between prior BCG vaccination and subsequent trial enrolment. This is to allow for the peak BCG-induced immune response to have occurred, which is usually around 2-4 weeks post BCG vaccination.

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.

Follow up period

The follow up period will be five months from the last vaccination (i.e. 24 weeks from enrolment), in accordance with findings from previous trials in which adequate safety data and reliable markers of immunogenicity have been obtained in this time interval.

4.4 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 586 mL which should not compromise the health of these healthy individuals.

An intravenous cannula is routinely inserted into a peripheral (usually forearm) vein prior to bronchoscopy to administer intravenous sedation, and removed once the procedure is completed. The risks of cannulation are identical to those associated with venepuncture. Additionally cannulation carries a small risk of soft tissue infection which 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. Vaccination

MVA85A has undergone thorough pre-clinical testing and has now been administered to over 2300 healthy human individuals with no serious adverse events. The potential known adverse events associated with vaccination are:

Local reaction from intradermal vaccination
The typical local reaction as a result of intradermal injection is temporary pain, redness and swelling at the site of the injection with a 5-10 mm central red swollen area and a paler red area ranging from 5-10 mm in diameter which peaks at 48 hours post vaccination. At seven days post vaccination, only the central area of redness remains. This fades over a few weeks and is not usually apparent eight weeks after vaccination. Scaling occurs as part of the healing process, and itching is possible.

Local reaction from aerosol inhaled vaccination
Enrolment for the first ever trial administering a virally vectored vaccine by the aerosol route has just been completed in our group. The trial was single blinded paired placebo, so all volunteers received either an intradermal vaccine with saline by aerosol or vice versa. There was no significant difference between systemic adverse events, respiratory adverse events or pulmonary function tests between the two groups.

Constitutional influenza-like symptoms such as fatigue, headache, malaise, feverishness, and muscle aches can occur with any vaccination lasting for 2-3 days. In the case of MVA85A, fatigue and headache are most common, occurring in 50-90% of subjects, and these are nearly always mild. Presyncopal and syncopal episodes may occur at the time of vaccination which rapidly resolve. As with any other vaccine, temporary ascending paralysis (the Guillain-Barré syndrome, GBS) or immune mediated reactions that can lead to organ damage may occur. For influenza vaccines an excess of approximately 1 GBS case per million persons immunized has been observed. No cases were observed in people under 45 years of age. However, this has never been seen with the MVA85A vaccine or vaccines containing any of its components.

Allergic reactions
Allergic reactions from mild to severe may occur in response to any constituent of a vaccine preparation. Anaphylaxis is extremely rare but can occur.

3. Bronchoscopy

Bronchoscopy is a widely and safely used investigative procedure in clinical research studies involving both healthy subjects and subjects with respiratory conditions such as asthma and interstitial lung disease (58, 59). Clinical guidelines for performing investigative bronchoscopy in research studies are well established (60). In 98% of cases subjects have no memory of the procedure.

The bronchoscopies will be carried out in a dedicated NHS bronchoscopy suite with an excellent safety record by highly skilled and experienced respiratory physicians. Intravenous sedation and local anaesthesia are administered prior to bronchoscopy to reduce discomfort, facilitate the procedure, and remove memory of the event. 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, subjects will be excluded if they have an abnormal chest x ray, a significant smoking history, 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: infection secondary to bronchoalveolar lavage, bleeding, and respiratory depression secondary to sedation:

- Bronchoalveolar lavage is a routine procedure in investigative bronchoscopy and carries minimal bleeding risk. The risk of infection or febrile reactions will be minimised by full bronchoscope asepsis and superflush.

- Respiratory depression secondary to sedation is rare. No rescue medication for oversedation has been required in a 3 year audit period and sedation with midazolam and fentanyl removed memory of the event in over 98% of subjects (internal audit data).

- 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.

Bronchoscopies were well tolerated in the volunteers of the recently completed Phase I trial TB026. No serious adverse events were reported during or as a result of bronchoscopy.

4. Chest X-Ray

A chest x-ray is a painless routine radiological investigation which exposes subjects to approximately 0.02 milliSieverts of radiation, equivalent to around three days of natural background radiation. The additional risk of cancer due to one chest x ray is extremely small, approximately 1 in 900,000.

5. Pulmonary Function Tests

Vigorous respiratory maneouvres such as forced expiration through the spirometer can occasionally lead to coughing or lightheadedness, but these symptoms are mild and rapidly self-limiting.

Known potential benefits

Subjects are not expected to benefit directly from participation in this trial. Subjects will gain some information about their general health as a result of the screening history, examination, blood tests, urine tests, chest x-ray, and pulmonary function tests. They may also gain health information from the bronchoscopy. It is hoped that their contribution will enable the development of a safe and successful vaccine for TB and further our knowledge about mucosal vaccines.

4.5 Data Collection

The bronchoscopic procedure is not being assessed for safety as it is a standard medical technique with a known profile of expected risks as described in section 4.4. Vaccination safety data for both routes will be collected both actively and passively as follows:

- Information about local and systemic adverse events will be collected at each visit by medical history, examination and measurement of the injection site, pulmonary function testing, bronchoscopic examination of the lungs, and the use of diary cards.
- Vital signs will be recorded at each visit.
- Safety blood tests (FBC, LFT, U&E) will be collected at screening and day 7.

This information will be entered into the CRF contemporaneously at each visit. Results of laboratory investigations and immunological data will be entered into the CRF. The CRFs will be the source data for this trial.

4.6 Duration of Study

Volunteers will all be followed up to week 24, with clinic visits and blood test as outlined in table 1.

4.7 Definition of start and end of the trial

The start of trial is defined as the date of the first vaccination of the first volunteer. The end of trial is the date of the last visit of the last volunteer.

4.8 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 subjects in the trial are being exposed to undue risks as a result of administration of the IMP, or as a result of the trial procedures or follow-up schedule.
- Serious concerns about the safety of the IMP arise as a result of one or more vaccine related SAE(s) occurring in the subjects enrolled in this or any other on-going trial of the MVA85A vaccine.
- For any other reason at the discretion of the Chief Investigator.

5 SELECTION AND WITHDRAWAL OF SUBJECTS

5.1 Inclusion Criteria

Subjects must meet all of the following criteria to enter the trial:

- Healthy adult aged 18-55 years
- Resident in or near Oxford for the duration of the trial period
- No relevant findings in medical history or on physical examination
- Confirmation of prior vaccination with BCG not less than 6 months prior to projected trial vaccination date (by visible BCG scar on examination or written documentation)
- Allow the Investigators to discuss the individual's medical history with their GP

- Use effective contraception 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 subject details with a confidential database to prevent concurrent entry into clinical trials
- Able and willing (in the Investigator's opinion) to comply with all the trial requirements

5.2 Exclusion Criteria

Subjects must meet none of the following criteria to enter the trial:

- Any respiratory disease, including asthma
- Current smoker
- Clinically significant abnormality on screening chest x-ray[a]
- Clinically significant abnormality of pulmonary function tests[a]
- 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
- Laboratory evidence at screening of latent *M. tb* infection as indicated by a positive ELISPOT response to ESAT6 or CFP10 antigens[b]
- Clinical, radiological, or laboratory evidence of current active TB disease[a]
- Previous vaccination with candidate vaccine MVA85A or candidate vaccine FP85A or any other recombinant MVA vaccine
- 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
- History of serious psychiatric condition
- 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 vaccine, sedative drugs, or any local or general anaesthetic agents
- Any abnormality of screening blood or urine tests that is deemed to be clinically significant or that may compromise the safety of the subject in the trial[a]
- Positive HBsAg, HCV or HIV antibodies[a]
- Female currently lactating, confirmed pregnancy or intention to become pregnant during trial period
- Use of an investigational medicinal product or non-registered drug, live vaccine, or medical device other than the trial vaccine for 30 days prior to dosing with the trial vaccine, or planned use during the trial period
- Administration of immunoglobulins and/or any blood products within the three months preceding the planned trial vaccination date
- Any other significant disease, disorder, or finding, which, in the opinion of the Investigator, may either put the subject at risk or may influence the result of the trial or may affect the subject's ability to participate in the trial

[a]Subjects 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.

[b]Subjects discovered to have evidence of latent *M. tb* infection as defined by a positive ELISPOT test will be referred for a plain chest x-ray and reviewed with the TB nurse specialists and considered for chemoprophylaxis. If there is any evidence of active TB disease either on clinical or radiological grounds, further investigation and treatment will be offered under the supervision of a consultant physician in respiratory or infectious diseases.

5.3 Discontinuation/Withdrawal of Participants from Trial Treatment

Subjects may be withdrawn from the trial early:

- By withdrawing voluntarily
- On the decision of the Chief Investigator
- On the advice of the LSC

The Chief Investigator may withdraw the subject for any of the following reasons:

- Confirmed pregnancy during the trial
- Any adverse event which results in inability to comply with trial procedures
- Ineligibility either arising during the trial or retrospectively (having been overlooked at screening)
- Significant protocol deviation
- Subject non-compliance with trial requirements
- Loss to follow up (applies to a subject who does not return for protocol trial visits, is not reachable by telephone or other means of communication and/or is not able to be located)

The reason for withdrawal will be recorded in the CRF. Subjects withdrawn from the trial may be replaced on the decision of the Investigator. If the subject 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 trial period, and if pregnant, until pregnancy outcome.

Subjects found during bronchoscopy 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. If a volunteer withdraws from the study, blood samples collected before their withdrawal from the trial will be used/ stored unless the volunteer specifically requests otherwise

5.4 Vaccination postponement Criteria

Vaccination will not proceed on the scheduled day in any of the following situations:

- The subject has a current or recent upper respiratory tract infection, unless they have been symptom-free for at least one week
- The subject has a positive pregnancy test
- The subject has a temperature > 37.5^{o}C
- The Investigator judges the subject to have an acute moderate or severe illness (whether febrile or not)
- The subject has received a live non-trial vaccine within the preceding three weeks
- The Investigator has any other concern that vaccination may not be in the subject's best interests

In this case the subject may be vaccinated at a later date or withdrawn from the trial at the discretion of the Investigator.

5.5 Bronchoscopy postponement criteria

Bronchoscopy will not proceed on the scheduled day in any of the following situations:

- The subject has a temperature > 37.5^{o}C
- The subject has a positive pregnancy test
- The Investigator judges the subject 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 subject's best interests

In the event of postponement the bronchoscopy will be rescheduled where possible up to a 3 week window.

6 TRIAL VACCINE, MEDICINES & DEVICES

6.1 Candidate TB vaccine MVA85A

Description

The MVA85A vaccine consists of the attenuated vaccinia virus MVA vector with a 1176 base-pair insert, which is almost the complete *M. tb* gene for Ag85A, with the tissue plasminogen activator (TPA) signal sequence preceding the N terminus and a monoclonal antibody tag (pk) at the C terminus. Expression of the antigen 85A DNA sequence is regulated by the vaccinia P7.5 early/late promoter. MVA85A is manufactured under Good Manufacturing Practice conditions by IDT Biologika GmbH (IDT), Germany.

Formulation

MVA85A lot 0050811 is supplied as a sterile liquid in glass vials. Each vial contains 400 µL of vaccine at a concentration of 8.4 x 10^{8}pfu/mL in 10mM Tris buffer 140 mM NaCl, pH 7.7. The dose of MVA85A to be used in this trial will be 5 x 10^{7} pfu (60 µL) in a single intradermal injection into the deltoid region of the upper arm or diluted in saline and administered by aerosol administration. Therefore two doses may be obtained from a single vial, enabling two volunteers to be vaccinated within the same time period.

Vaccine Supply and Product storage

MVA85A will be shipped from IDT directly to the Clinical Biomanufacturing Facility (CBF) on dry ice and in the presence of a temperature logger. The vaccine will be certified and labelled for use in trial TB035 by a qualified person (QP) at the University of Oxford.

The vaccine will be stored between –70°C and –90°C, in a locked freezer, at the University of Oxford, Churchill Hospital

Dispensing and administration

All movements of vials of the trial vaccine between IDT and the University of Oxford and in or out of the locked freezer will be documented. Vaccine accountability, storage, shipment and handling will be in accordance with local SOPs and other relevant local forms. On vaccination day, vaccines will be allowed to thaw to room temperature and will be administered within 1 hour. The vaccine will be administered intradermally over the deltoid region of each upper arm, according to the site-specific SOP. The vaccine will administered by aerosol inhalation according to the site-specific SOP. Subjects will stay in the unit for 60 minutes (±10 minutes) after vaccination. During the administration of the

vaccine, monitoring equipment, oxygen, medicines including bronchodilators and resuscitation equipment will be immediately available for the management of anaphylaxis and bronchospasm according to the site SOP.

In order to minimise dissemination of the recombinant vectored vaccine virus into the environment, a number of measures will be instituted during and following vaccination:

- The intradermal injection site will be covered with a dressing after vaccination to absorb any virus that may leak out through the needle track. The dressing will be removed from the injection site after 30 minutes.
- The aerosol inhaled vaccination will take place in a closed clinic room and not opened until 10 minutes after the end of nebulisation.
- The dilution process will be carried out in a sealed vial in accordance with the site-specific SOP.
- During aerosol inhalation the Investigator will wear appropriate protective clothing as agreed with the GMSC.
- All disposable items including nebuliser parts, needles, vials, dressings, and protective clothing will be disposed of as GMO waste by autoclaving, in accordance with the current approved SOPs and standard UK practice.
- The hard surfaces within close range (1-2 metres) of aerosol vaccination will be cleaned and disinfected in accordance with GMSC recommendations.
- Non-disposable parts of the MicroAIR NE-U22 nebuliser will be cleaned and disinfected in accordance with the device documentation and GMSC recommendations.

Trial regimen

Group 1: Aerosol inhaled MVA85A 5 x 10^7 pfu (and intradermal saline placebo) on day 0, followed by homologous boosting with MVA85A 5 x 10^7 pfu intradermal on day 28 (and aerosol inhaled saline placebo)
Group 2: Intradermal MVA85A 5 x 10^7 pfu (and aerosol inhaled saline placebo) on day 0, followed by homologous boosting with MVA85A 5 x 10^7 pfu aerosol inhaled on day 28 (and intradermal saline placebo) OR, following safety amendment (see section 4.1)
Intradermal MVA85A 5 x 10^7 pfu (and aerosol inhaled saline placebo) on day 0, followed by aerosol inhaled saline placebo and intradermal saline placebo on day 28
Group3: Intradermal MVA85A 5 x 10^7 pfu (and aerosol inhaled saline placebo) on day 0, followed by homologous boosting with MVA85A 5 x 10^7 pfu intradermal on day 28 (and aerosol inhaled saline placebo)

Labelling and packaging

The vaccine, including intended route of administration, will be QP certified and labelled with Annex 13 compliant labels for trial TB035 at the CBF. The vials will be packaged into labelled cardboard boxes at the CBF.

6.2 Saline

Sterile saline (0.9%) will be used for dilution and placebo doses. The volume of saline used as placebo will be identical to the vaccine volume by the same route. For volunteers receiving the aerosol vaccination we will administer 5 x 10^7 pfu of vaccine made up to 1ml with saline and at the same time a paired placebo of 135 microlitres of normal saline for intradermal injection. For volunteers receiving the vaccine intradermally we will administer 5 x 10^7 pfu of vaccine made up with normal saline to a vaccine volume of 135 microlitres and at the same time a paired placebo of 1ml of saline inhaled. We require 60ul of MVA85A vaccine to give a dose of 5 x 10^7 pfu. For the aerosol vaccine the total volume is 1ml, i.e. ~940 microlitres of saline and 60 microlitres of vaccine. The volume for intradermal injection is 135 microlitres, i.e. ~75 microlitres of saline with 60microlitires of vaccine.

6.3 MicroAIR NE-U22

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 (66). This is the same device used for the study TB026.

6.4 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 Summary of Product Characteristics (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 Summary of Product Characteristics (SmPC).

Other licensed drugs may be used during bronchoscopy at the discretion of the Investigators.

7 TRIAL SCHEDULE

7.1 Recruitment

Subjects 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

• As a post on a Twitter account owned and operated by our group

• By e-mail distribution to a group or list only with the express agreement of the network administrator or with equivalent authorization

• On stalls or stands at exhibitions or fairs

• Via presentations (e.g. presentations at lectures or invited seminars)

• Direct mail-out. This will involve obtaining names and addresses of adults via the most recent Electoral Roll (with the contact details of individuals who have indicated that they do not wish to receive postal mail-shots already removed). 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 18-55 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 all 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 advert will be submitted as an amendment to the ethics committee for approval before use.

7.2 Screening

Subjects will be offered a screening visit if they express an interest in participating in the trial. They will be assigned a subject identifier number and provided with and asked to read the subject information sheet at least 24 hours prior to attending for screening. At the screening visit the general process of screening will be outlined and all questions about the screening process and the trial answered. The following general principles will be emphasised:

- Participation in the trial is entirely voluntary
- Refusal to participate involves no penalty or loss of medical benefits
- The subject may withdraw from the trial at any time
- The subject is free to ask questions at any time to allow him or her to understand the purpose of the trial and the procedures involved
- The trial involves administration of a unlicensed vaccine and a bronchoscopy
- There is no direct benefit from participating
- The subject's GP will be contacted to corroborate their medical history if possible

The aims of the trial and all tests to be carried out will be explained by the Investigator or the Clinical Research Nurse. The subject will be given the opportunity to ask about details of the trial, and will then have time to consider whether or not to participate. If they do decide to participate, they will sign and personally date two copies of the consent form, one for them to take away and keep, and one for the Investigator. These forms will also be signed and dated by the Investigator or Clinical Research Nurse.

Having agreed to undergo screening, a baseline medical history (including concomitant medication) and physical examination will be performed. Inclusion and exclusion criteria will be checked using a tabulated format. Demographic and occupational data relating to risk of prior TB exposure will be collected. Vital signs will be checked. Subjects will be counselled by one of the Investigators for HIV, Hepatitis B and Hepatitis C testing. Blood tests as specified in the schedule of procedures will be taken. Urinalysis and a pregnancy test will be performed. Chest x ray and pulmonary function testing will be performed on the same day if possible, or scheduled on a separate visit. The total duration of the screening visit will be approximately two hours.

Subjects will be informed that there may be leftover samples of their blood (after all testing for this trial is completed), and that such samples may be stored up to a maximum of 15 years for possible future research. Samples will be stored in liquid nitrogen storage at the Jenner Institute, University of Oxford under the approved study code "Lab T001: Finding an Immune Correlate of Protection against Tuberculosis' (Berkshire REC Reference Number: 06/Q1602/146). Subjects 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 Good Clinical Practice (GCP) and regulatory requirements.

The subject's general practitioner will be contacted with the written permission of the subject after satisfactory screening as notification that the subject is taking part in the trial and to ascertain any significant medical history.

7.3 Enrolment

Subjects who fulfil the screening criteria, satisfy all the inclusion criteria, and meet none of the exclusion criteria will be enrolled into the trial and their visits scheduled in the calendar. The ongoing eligibility of the subject will be reviewed on the day of enrolment and any new events, medications, or changes to the screening documents recorded. Subjects will be enrolled on the vaccination visit (day 0). The vaccination visit will last approximately two hours, which includes a 60 minute follow up period in the clinic for observation after vaccination. The bronchoscopy visit (day 7) will last several hours. All other pre-vaccination and follow up visits will last approximately 15 to 30 minutes.

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.

7.4 Safety Review

There will be a short visit for safety review 2 days following vaccination at which the diary card will be reviewed, pulmonary function test repeated and blood tests repeated.

7.5 Follow-up

The maximum trial duration will be around 6 months after the date of enrolment. Procedures will be performed at the time points indicated in the schedule of procedures (Table 1). Additional procedures or laboratory tests may be performed, at the discretion of the investigators if deemed clinically necessary.

8. SAFETY REPORTING

8.1 Definitions

Adverse Event (AE)	Any untoward medical occurrence in a participant to whom a medicinal product has been administered, including occurrences which are not necessarily caused by or related to that product.
Adverse Reaction (AR)	An untoward and unintended response in a participant to an investigational medicinal product which is related to any dose administered to that participant. The phrase "response to an investigational medicinal product" means that a causal relationship between a trial medication and an AE is at least a reasonable possibility, i.e. the relationship cannot be ruled out. All cases judged by either the reporting medically qualified professional or the Sponsor as having a reasonable suspected causal relationship to the trial medication qualify as adverse reactions.
Serious Adverse Event (SAE)	A serious adverse event is any untoward medical occurrence that: • results in death • is life-threatening • requires inpatient hospitalisation or prolongation of existing hospitalisation

	• results in persistent or significant disability/incapacity • consists of a congenital anomaly or birth defect. Other 'important medical events' may also be considered serious if they jeopardise the participant or require an intervention to prevent one of the above consequences. NOTE: The term "life-threatening" in the definition of "serious" refers to an event in which the participant was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe.
Serious Adverse Reaction (SAR)	An adverse event that is both serious and, in the opinion of the reporting Investigator, believed with reasonable probability to be due to one of the 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 case of a product with a marketing authorisation, in the summary of product characteristics (SmPC) for that product • in the case of any other investigational medicinal product, in the investigator's brochure (IB) relating to the trial in question.

NB: To avoid confusion or misunderstanding of the difference between the terms "serious" and "severe", 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.

8.2 Causality Assessment

The Investigator and the Local Safety Monitor both determine causality. It is expected that communication and consultation may occur in the assessment of the causality of AEs. The greatest degree of causal relationship (definite > probable > possible > unlikely related > not related) determined by either the Investigator or Local Safety Monitor after their discussions (and review of the volunteer if necessary) will determine the ultimate classification of the AE. Definite (4), probable (3) and possible (2) are considered to be related. No relationship (0) and unlikely (1) are considered to be unrelated. The following guidelines will be used to assess the relationship of all AEs to the administration of the vaccine, with the premise that all local reactions will be considered causally related to the vaccination:

0 = No relationship:
- No temporal relationship to vaccine; *and*
- Alternate aetiology (clinical state, environmental or other interventions); *and*
- Does not follow known pattern of response to the vaccine

1 = Unlikely relationship:
- Unlikely temporal relationship to vaccine; *and*
- Alternate aetiology likely (clinical state, environmental or other interventions); *and*
- Does not follow known typical or plausible pattern of response to the vaccine

2 = Possible relationship:
- Reasonable temporal relationship to vaccine; *or*
- Event not readily produced by clinical state, environmental or other interventions; *or*
- Similar pattern of response to that seen with the vaccine

3 = Probable relationship:
- Reasonable temporal relationship to vaccine; *and*
- Event not readily produced by clinical state, environment, or other interventions *or*

- Known pattern of response seen with the vaccine

4 = Definite relationship:

- Reasonable temporal relationship to vaccine; *and*
- Event not readily produced by clinical state, environment, or other interventions; *and*
- Known pattern of response seen with the vaccine

8.3 Clinical Assessment

All AEs (whether reported by the subject or solicited by the Investigator) will be clinically assessed at each visit and recorded with start and stop dates and details of any treatment undergone. Final grading of adverse events will be determined by the Investigator on the trial.

This information will be **collected** as follows:

- Subjects are asked to complete diary cards for 14 days after MVA85A vaccination by either route. They are provided with a thermometer and tape measure to enable daily recording of temperature and local redness and swelling at the intradermal injection site. They are also provided with a spirometer to record pulmonary function.
- Outside the diary card periods, expected local and systemic AEs (listed in table 4 below) will be solicited, in other words specifically asked about at each visit, and graded by severity and causality (as detailed in sections 9.2 and 9.4).
- Subjects will be given the opportunity to report any other new symptoms since the last visit with start and stop dates and any treatments undergone.
- Vital signs will be performed in the normal manner at each visit.
- The overall appearance of the lung mucosa will be assessed at the day 7 bronchoscopy.

	Adverse event
Local (intradermal route)	Pain/tenderness at the injection site
	Redness at the injection site
	Swelling at injection site
	Warmth at the injection site
	Itch at the injection site
	Scaling/pustules at the injection site
Local (aerosol inhaled route)	Cough
	Sore throat
	Wheeze
	Shortness of breath
	Sputum
	Haemoptysis
	Chest pain
Systemic	Documented fever (oral temperature > 37.5° C)
	Symptoms of feverishness
	Malaise
	Arthralgia
	Headache
	Myalgia
	Nausea / vomiting

Table 4: Routinely solicited local and systemic adverse events

The information will be **recorded** as follows:

- During the diary card period, all solicited AEs (listed in table 4) and any additional unexpected AEs are captured in the diary card and at clinic visits (CRF)
- Outside the diary card periods all AEs will be documented in the AE line listing section of the CRF with start and stop dates and details of any treatments undergone.
- Vital signs will be recorded in the CRF.

8.4 Severity Assessment

AEs will be graded by severity as follows (where 0 = absent, 1 = mild, 2 = moderate, 3 = severe):

Adverse event	Grade	Measurement
Tenderness or pain at intradermal injection site	0	No pain at all
	1	Painful on touch; easily tolerated
	2	Painful when limb is moved; interferes with daily activity
	3	Severe pain at rest; prevents daily activity
Redness at intradermal injection site	0	0 mm
	1	1 – 50 mm
	2	51 – 100 mm
	3	> 100 mm
Swelling at intradermal injection site	0	0 mm
	1	1 – 20 mm
	2	21 – 50 mm
	3	> 50 mm
Fever (oral)	0	≤37.5^{o}C
	1	37.6 – 38.0 ^{o}C
	2	38.1 – 39.0 ^{o}C
	3	>39.0 ^{o}C
Other AEs	0	Absence of symptom
	1	Awareness of symptom but tolerated; transient or mild discomfort; little or no medical intervention required
	2	Discomfort enough to cause limitation of usual activity; some medical intervention or therapy required
	3	Incapacitating, absent from work, or bed rest required; hospitalisation possible

Table 5. Severity grading for AEs

8.5 Laboratory Assessments

All laboratory results will be reviewed and recorded in the CRF after being signed by the Investigator. A note will be made of whether each result is normal, abnormal but not clinically significant, or abnormal *and* clinically significant. In the latter case trial eligibility will be reviewed and necessary referrals or tests will be offered.

The severity of laboratory test abnormalities will be assessed according to the scale in Table 6 (adapted from (67))

Laboratory Test	Grade 1	Grade 2	Grade 3
Hb (female) – decrease from testing laboratory LLN in gm/dl	>1.0 - <1.5	≥1.5 & <2.0	≥2.0
Hb (male) – decrease from testing laboratory LLN in gm/dl	≥1.5 & <2.0	≥2.0 & <2.5	≥2.5
Absolute neutrophil count (ANC, cells/mm^3)	1000-1499	500-999	<500
Leukopenia (WBC, cells/mm^3)	<3500 - ≥2500	<2500 - ≥1500	<1500
Platelets (cells/mm^3)	125,000 – 135,000	100,000 – 124,000	20,000-99,000
Bilirubin – when accompanied by any increase in Liver Function Test increase by factor	1.1 – 1.25 x ULN	1.26 – 1.5 x ULN	1.51 – 1.75 x ULN

ALT	1.25 – 2.5 x ULN	>2.6 – 5.0 x ULN	>5.0 x ULN
Creatinine	1.1 – 1.5 x ULN	>1.6 – 3.0 x ULN	>3.0 x ULN

Table 6: Severity grading criteria for clinically significant laboratory abnormalities (67)

8.6 Reporting Procedure for Adverse Events

Adverse events will be recorded in the CRF. Those adverse events likely to be related to the vaccine, whether serious or not, which persist at the end of the trial will be followed up by the Investigator until their complete disappearance. Moreover, any serious adverse event likely to be related to the vaccine and occurring after trial termination should be reported by the Investigator according to the procedure described below.

The outcome of any non-serious adverse event occurring within 30 days post-vaccination or any SAE reported during the entire trial will be assessed as:

- Recovered/resolved
- Not recovered/not resolved
- Recovering/resolving
- Recovered with sequelae/resolved with sequelae
- Fatal

Subjects who have moderate or severe on-going AEs at the completion of the trial will be advised to consult their General Practitioner (National Health Service) if the event is not considered to be related to the vaccine. A follow-up visit will be arranged to manage the problem and to determine the severity and duration of the event, if it is considered to be related to the vaccine. If appropriate, specialist review within the NHS will be arranged.

8. 7 Reporting of Serious Adverse Events

All SAEs will be documented accurately and notification deadlines respected as specified in the site-specific SOP. SAEs will be reported within 24 hours by the Investigator to the Sponsor or person with appropriate responsibility delegated from the Sponsor (Chief Investigator), and to the Local Safety Committee (see section 4.4). This will be performed by emailing an electronic version of the completed SAE Initial Report Form (CCVTM form VC004F1). Any relevant information concerning the SAE that becomes available after the SAE Initial Report Form has been sent (outcome, precise history, results of investigations, copy of hospital report, etc.) will be forwarded in a timely manner using the SAE Update Report Form (CCVTM form VC004F2). The Sponsor, or person with appropriate responsibility delegated from the Sponsor (Chief Investigator/medically qualified investigator), is responsible for commencing, maintaining and completing the SAE Sponsor Report Form (VC004F3). The anonymity of subjects shall be respected when forwarding this information.

SAEs which are not SUSARs will not normally be reported to the EC and MHRA, 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 subjects.

In addition to these reporting requirements, the Investigator shall submit once a year throughout the trial, or on request, a safety report to the EC and MHRA (and a copy to the Sponsor).

We will follow every volunteer up for adverse event until their last visit. We will finish collection of AE data at the end of the trial, last volunteer last visit.

8.8 Reporting Procedures for SUSARs

The Chief Investigator will report all SUSARs to the MHRA and ethical committee(s) within required timelines. The Chief Investigator will also inform all investigators concerned of relevant information about SUSARs that could adversely affect the safety of participants

If the SAE is a SUSAR it will be reported to the EC and MHRA within 7 days of the Chief Investigator having first knowledge of the event for fatal and life-threatening cases and within 15 days for all other SUSARs (and the report copied to the Sponsor). Any deaths occurring during the trial will be reported to the Sponsor. For all deaths, available autopsy reports will be made available for reporting to the regulatory authorities.

8.9 Local Safety Committee

The local safety committee (LSC) will provide safety oversight for this trial. Its chair will be a clinician with extensive and relevant experience of infectious disease and clinical trials work. At the time of writing the chair is Dr Brian Angus, Clinical Tutor in Medicine, Honorary Consultant Physician and Director of the Centre for Tropical Medicine at Oxford University. There will be a minimum of two other appropriately qualified committee members as specified in the site-specific SOP for safety reporting.

8.10 Development Safety Update report

A development safety update report (DSUR) for each IMP is prepared annually, and submitted within 60 days of the anniversary of the first approval date from the regulatory authority for each IMP. This will be submitted by the chief Investigator to the competent authority and ethical committee(s).

8.11 Pregnancy

Subjects who become pregnant during the trial after the vaccination may continue trial procedures including venepuncture, if appropriate, at the discretion of the Investigator. Subjects who become pregnant before MVA85A vaccination will be withdrawn and will not be vaccinated. Subjects who become pregnant between vaccination and bronchoscopy will not undergo bronchoscopy. The Investigator will collect pregnancy information on any subject who becomes pregnant while participating in this trial. The subject will be followed up to determine the outcome of the pregnancy.

9. STATISTICS

9.1 Statistics

This is primarily a trial with descriptive endpoints. Twelve subjects will be recruited into each arm of the trial (i.e. 36 in total). Our previous experience with clinical trials using MVA85A over the last 10 years suggests that this sample size is a feasible number to recruit, screen, enroll, and follow up in practical terms, whilst also allowing the determination of any substantial and clinically significant differences in safety and cell-mediated immunity between the three groups. A twofold increase in the magnitude of the cellular immune response (as measured by Elispot response to 85A peptide stimulation) is considered immunologically meaningful. Extrapolating effect size and variability from the completed initial aerosol vaccine safety study (TB026), twelve volunteers are needed to have 80% power to detect an immunologically meaningful difference in the summed peptide pools and 8

volunteers to achieve the same for single peptide pool of 85A. This sample size is appropriate for a proof-of-concept Phase I safety study.

It is anticipated that the immunological endpoints will not follow a normal distribution. A log transformation will be applied and if the resulting data are normally distributed they will be summarised by group using means and standard deviations. The one-week and six-month responses will be compared between the three groups using a t-test. If the resulting data are not normally distributed then medians and interquartile ranges will be used to summarise the data and the Mann Whitney U test will be used for statistical comparisons. An area-under-curve analysis will be used to compare the overall responses over time between the three groups. This analysis will make use of data collected at all time points.

Differences between means or medians between the three 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.

10. DATA MANAGEMENT

The Chief Investigator will be responsible for collecting, recording, analysing, and storing all the data accruing from the trial. These tasks may be delegated to other Investigators. Data will be stored in either paper CRFs held in a key-locked cabinet at the CCVTM, or electronic CRFs on the OpenClinica™ database (which is stored electronically on secure servers outsourced by OpenClinica™), or both. Some data may be duplicated anonymously into an electronic Microsoft Excel™ file on the CCVTM secure server.

10.1 Access to Data

Access to the source data documents will be provided if necessary to the EC and MHRA and to the Sponsor for trial-related monitoring and audit. All information relating to the trial and its subjects will be held in strict confidence, and in accordance with ICH E6 GCP and institutional requirements.

10.2 Data Recording and Record Keeping

Trial records will be held by the Chief Investigator for as long as required by legislation (currently until at least 2 years after the last marketing authorisation for the product or 2 years after discontinuation of clinical development of an investigational product), initially in a key-locked cabinet at the CCVTM and subsequently in a secure archive facility, and in accordance with the Data Protection Act. Subjects 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, bank details request form and consent forms).

All clinical data will be recorded on paper case report forms and the volunteer files kept securely as described (key locked cabinets at the CCVTM during trial period and secure archive facility afterwards)

10.3 Source documents

Source documents are original documents, data and records from which the volunteer`s CRF data are obtained. For this study these will include the medical records (history and examination), the volunteer consent form, the TOPS database registration receipt, the blood and microbiology results, the chest xray report, the GP response letters, copy of the bronchoscopy report and any correspondence relating to that volunteer concerning medical/clinical issues.

10.4 Data Protection

The study protocol, documentation, bank details request form, data and all other information generated will be held in strict confidence and in accordance with the Data Protection Act. No information concerning the study or the data will be released to any unauthorized third party, without prior written approval of the sponsor.

11. QUALITY ASSURANCE AND QUALITY CONTROL

11.1. 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 Chief Investigator 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 subjects' safety, the objectives of the trial and its progress or scientific value of the project. An administrative change is a non-substantial amendment and does not require EC approval.

The Chief Investigator is responsible for ensuring that changes to an approved trial, during the period for which EC approval has already been given, are not initiated without EC review and approval except to eliminate apparent immediate hazards to the subject.

Protocol deviation

All deviations from the protocol will be documented in a protocol deviation form and filed in the trial master file.

11.2. 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.

12. Ethical and Regulatory Considerations

12.1. Good Clinical Practice

This trial will be conducted in accordance with the principles of the Declaration of Helsinki as agreed by the World Medical Association General Assembly (Washington 2002), ICH Good Clinical Practice (GCP), the requirements of the Medicines for Human Use (Clinical Trial) Regulations 2004, and local regulatory requirements.

12.2. Ethical Review

A copy of the protocol, proposed informed consent form, other written subject information and the proposed advertising material will be submitted to an independent EC for written approval. The Investigators will submit and, where necessary, obtain approval from the EC for all subsequent substantial amendments to the protocol and informed consent document.

12.3. Informed Consent

Written informed consent will be obtained at screening as detailed in section 7.2.

12.4. 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 Chief Investigator 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 subjects of the trial
- the scientific value of the trial”

In the event that a serious breach is suspected, the Sponsor will be contacted as soon as possible.

13. Finance/Compensation/Insurance

13.1. Finance

This trial will be financed by research grants from The Wellcome Trust and Biomedical Research Council held by Prof Helen McShane and a NIHR BRC clinical research training fellowship held by Dr Zita-Rose Manjaly Thomas.

13.2. Compensation

Subjects will be compensated *pro rata* for their time and travel and for trial procedures while participating in the trial, amounting to a total of approximately £800, depending on the exact number of visits and whether any repeat or additional visits are necessary.

13.3. Insurance

The University has a specialist insurance policy in place which would operate in the event of any participant suffering harm as a result of their involvement in the research (Newline Underwriting Management Ltd, at Lloyd's of London, policy numbered :WD1200463).

14. 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.

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Appendix 2

- Volunteer Diary Card

Diary Card (TB035)

Study ID Number
TBT-035_ _ _

Vaccine Number

Jenner Institute



Centre for Clinical Vaccinology and Tropical Medicine
Churchill Hospital
Oxford

Do not hesitate to contact us if you have any problems

01865 857406 during office hours (9am-5pm Mon-Fri)

Out of hours call: 07917882967

Alternatively if your query is not urgent you can email
vaccinetrials@well.ox.ac.uk

Sponsored by the University of Oxford

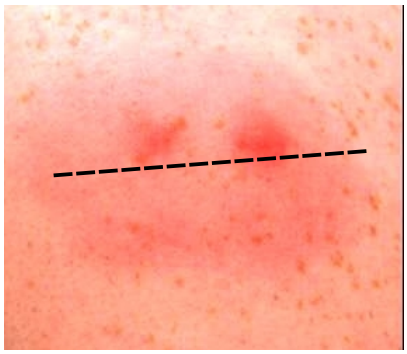
Instructions for the completion of your Diary Card

Please complete each section (**A-E**) every evening for each of the 7 days following vaccination (including day 0 which is the day of vaccination).

Observations should be completed at approximately the same time each evening.

Record your temperature using the thermometer provided, you should avoid any hot or cold drinks for at least 20 minutes before taking your temperature.

Measure the maximum diameter of any redness (**pink area**) and swelling (**raised area**) around the site of injection ensuring the tape measure goes through the centre. Please record this measurement in millimetres (**mm**) only



For each symptom listed please indicate the maximum intensity you have experienced since you left clinic or since the last time you recorded your observations, which ever is the latest. Please score all symptoms

Symptoms are scored as follows:

0 = No discomfort experienced.
1 = Causes mild discomfort. It does not limit usual activity.
2 = Causes mild to moderate limitation in activity. Some assistance may be needed.
3 = Causes marked limitation in activity

If you experience pain or feel unwell after the vaccination, we would encourage you to take simple painkillers (paracetamol and/ or ibuprofen) to try to relieve these symptoms, as long as you do not have any allergies to the medications.

Paracetamol can be taken at a dose of 1g up to 4 times a day (every 4-6 hours)
Ibuprofen can be taken at a dose of 400mg up to 3 times a day (every 6-8 hours)

Both of these medications can be bought over-the-counter at supermarkets or pharmacies. You can take both of them at the same time.

If you are unsure about taking medication please contact a member of the study team on the number(s) provided on the front of this diary card.)

If you take any Paracetamol or Ibuprofen (e.g. Nurofen™) please tick the box corresponding to the symptom for which you took it, more than one box may be ticked if the same drug was taken for multiple reasons. Record the dose and how often you took these drugs in **Table C**.

If you have experienced any other symptoms please record these in **Table B**

If you have taken any other drugs not listed above during this period please complete **Table D**

If you have any concerns or are worried by any of the symptoms please call the on-call clinician night or day on the **Emergency telephone** number shown on the front.

For routine matters e.g. appointments etc, please contact us at the office number shown on the front cover

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Day 0 (Evening)

Date
Time (hh:mm)

A. Measurement/Score of symptoms

0 = No discomfort experienced.
1 = Causes mild discomfort. It does not limit usual activity.
2 = Causes mild to moderate limitation in activity. Some assistance may be needed.
3 = Causes marked limitation in activity
(Please complete all rows: enter the measurement or tick the highest value that applies for each symptom).

			Paracetamol taken?	Ibuprofen taken?
1	Your temperature(°C)	.....	☐	☐
2	**FEV1**	.....	**Best of 3**	
3	**FVC**	.....	**Best of 3**	
	Local Reactions			
4	Diameter of redness	.....	**mm**	
5	Diameter of swelling	.....	**mm**	
6	Scaling present	0☐ 1☐	**0 = No 1 = Yes**	
7	Pain at the site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
8	Itching at site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
9	Warmth at the site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
	Generalised Symptoms (Symptoms that occur in other parts of the body not at site of vaccination)			
10	Feelings of feverishness	0☐ 1☐ 2☐ 3☐	☐	☐
11	Muscle aches	0☐ 1☐ 2☐ 3☐	☐	☐
12	Joint aches	0☐ 1☐ 2☐ 3☐	☐	☐
13	Headaches	0☐ 1☐ 2☐ 3☐	☐	☐
14	Fatigue	0☐ 1☐ 2☐ 3☐	☐	☐
15	Nausea	0☐ 1☐ 2☐ 3☐	☐	☐
16	Generalised discomfort, illness, or lack of well-being *(but not listed above)*	0☐ 1☐ 2☐ 3☐	☐	☐
	Respiratory Symptoms			
17	Any Cough?	0☐ 1☐ 2☐ 3☐		
18	Sore throat?	0☐ 1☐ 2☐ 3☐		
19	Wheeze?	0☐ 1☐ 2☐ 3☐		
20	Shortness of breath?	0☐ 1☐ 2☐ 3☐		
21	Coughing up phlegm?	0☐ 1☐ 2☐ 3☐		
22	Coughing up blood?	0☐ 1☐ 2☐ 3☐		
23	Chest pain?	0☐ 1☐ 2☐ 3☐		
	Any other symptoms not listed above *(please give brief description)*			
24	.....	0☐ 1☐ 2☐ 3☐	☐	☐
25	.....	0☐ 1☐ 2☐ 3☐	☐	☐
26	.....	0☐ 1☐ 2☐ 3☐	☐	☐
27	.....	0☐ 1☐ 2☐ 3☐	☐	☐
28	.....	0☐ 1☐ 2☐ 3☐	☐	☐

Day 0 (Evening)

Subject ID

C. Paracetamol/Ibuprofen

If you have ticked to say you have taken either of these drugs please indicate the dose you have taken and the number of times it was taken during this period.

	Dose taken	Number of times this dose was taken	Which lines in the table opposite do these refers to.
Paracetamol			
Paracetamol			
Ibuprofen			
Ibuprofen			

D. Other Medication

If you have taken any other medication during this period please record it here.

Name	Dose taken	Number of times this dose was taken	Which lines in the table opposite do these refers to.

Day 1 (Morning)

Date
Time (hh:mm)

A. Measurement/Score of symptoms

0 = No discomfort experienced.
1 = Causes mild discomfort. It does not limit usual activity.
2 = Causes mild to moderate limitation in activity. Some assistance may be needed.
3 = Causes marked limitation in activity
(Please complete all rows: enter the measurement or tick the highest value that applies for each symptom).

			Paracetamol taken?	Ibuprofen taken?
1	Your temperature(°C)	.....	☐	☐
2	**FEV1**	.....	**Best of 3**	
3	**FVC**	.....	**Best of 3**	
Local Reactions				
4	Diameter of redness	.....	**mm**	
5	Diameter of swelling	.....	**mm**	
6	Scaling present	0☐ 1☐	**0 = No 1 = Yes**	
7	Pain at the site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
8	Itching at site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
9	Warmth at the site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
Generalised Symptoms (Symptoms that occur in other parts of the body not at site of vaccination)				
10	Feelings of feverishness	0☐ 1☐ 2☐ 3☐	☐	☐
11	Muscle aches	0☐ 1☐ 2☐ 3☐	☐	☐
12	Joint aches	0☐ 1☐ 2☐ 3☐	☐	☐
13	Headaches	0☐ 1☐ 2☐ 3☐	☐	☐
14	Fatigue	0☐ 1☐ 2☐ 3☐	☐	☐
15	Nausea	0☐ 1☐ 2☐ 3☐	☐	☐
16	Generalised discomfort, illness, or lack of well-being *(but not listed above)*	0☐ 1☐ 2☐ 3☐	☐	☐
Respiratory Symptoms				
17	Any Cough?	0☐ 1☐ 2☐ 3☐		
18	Sore throat?	0☐ 1☐ 2☐ 3☐		
19	Wheeze?	0☐ 1☐ 2☐ 3☐		
20	Shortness of breath?	0☐ 1☐ 2☐ 3☐		
21	Coughing up phlegm?	0☐ 1☐ 2☐ 3☐		
22	Coughing up blood?	0☐ 1☐ 2☐ 3☐		
23	Chest pain?	0☐ 1☐ 2☐ 3☐		
Any other symptoms not listed above *(please give brief description)*				
24	.....	0☐ 1☐ 2☐ 3☐	☐	☐
25	.....	0☐ 1☐ 2☐ 3☐	☐	☐
26	.....	0☐ 1☐ 2☐ 3☐	☐	☐
27	.....	0☐ 1☐ 2☐ 3☐	☐	☐
28	.....	0☐ 1☐ 2☐ 3☐	☐	☐

Day 1 (Morning)

Subject ID

C. Paracetamol/Ibuprofen

If you have ticked to say you have taken either of these drugs please indicate the dose you have taken and the number of times it was taken during this period.

	Dose taken	Number of times this dose was taken	Which lines in the table opposite do these refers to.
Paracetamol			
Paracetamol			
Ibuprofen			
Ibuprofen			

D. Other Medication

If you have taken any other medication during this period please record it here.

Name	Dose taken	Number of times this dose was taken	Which lines in the table opposite do these refers to.

Day 1 (Evening)

Date
Time (hh:mm)

A. Measurement/Score of symptoms

0 = No discomfort experienced.
1 = Causes mild discomfort. It does not limit usual activity.
2 = Causes mild to moderate limitation in activity. Some assistance may be needed.
3 = Causes marked limitation in activity
(Please complete all rows: enter the measurement or tick the highest value that applies for each symptom).

			Paracetamol taken?	Ibuprofen taken?
1	Your temperature(°C)	.....	☐	☐
2	**FEV1**	.....	**Best of 3**	
3	**FVC**	.....	**Best of 3**	
	Local Reactions			
4	Diameter of redness	.....	**mm**	
5	Diameter of swelling	.....	**mm**	
6	Scaling present	0☐ 1☐	**0 = No 1 = Yes**	
7	Pain at the site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
8	Itching at site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
9	Warmth at the site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
	Generalised Symptoms (Symptoms that occur in other parts of the body not at site of vaccination)			
10	Feelings of feverishness	0☐ 1☐ 2☐ 3☐	☐	☐
11	Muscle aches	0☐ 1☐ 2☐ 3☐	☐	☐
12	Joint aches	0☐ 1☐ 2☐ 3☐	☐	☐
13	Headaches	0☐ 1☐ 2☐ 3☐	☐	☐
14	Fatigue	0☐ 1☐ 2☐ 3☐	☐	☐
15	Nausea	0☐ 1☐ 2☐ 3☐	☐	☐
16	Generalised discomfort, illness, or lack of well-being *(but not listed above)*	0☐ 1☐ 2☐ 3☐	☐	☐
	Respiratory Symptoms			
17	Any Cough?	0☐ 1☐ 2☐ 3☐		
18	Sore throat?	0☐ 1☐ 2☐ 3☐		
19	Wheeze?	0☐ 1☐ 2☐ 3☐		
20	Shortness of breath?	0☐ 1☐ 2☐ 3☐		
21	Coughing up phlegm?	0☐ 1☐ 2☐ 3☐		
22	Coughing up blood?	0☐ 1☐ 2☐ 3☐		
23	Chest pain?	0☐ 1☐ 2☐ 3☐		
	Any other symptoms not listed above *(please give brief description)*			
24	.....	0☐ 1☐ 2☐ 3☐	☐	☐
25	.....	0☐ 1☐ 2☐ 3☐	☐	☐
26	.....	0☐ 1☐ 2☐ 3☐	☐	☐
27	.....	0☐ 1☐ 2☐ 3☐	☐	☐
28	.....	0☐ 1☐ 2☐ 3☐	☐	☐

Day 1 (Evening)

Subject ID

C. Paracetamol/Ibuprofen

If you have ticked to say you have taken either of these drugs please indicate the dose you have taken and the number of times it was taken during this period.

	Dose taken	Number of times this dose was taken	Which lines in the table opposite do these refers to.
Paracetamol			
Paracetamol			
Ibuprofen			
Ibuprofen			

D. Other Medication

If you have taken any other medication during this period please record it here.

Name	Dose taken	Number of times this dose was taken	Which lines in the table opposite do these refers to.

Day 2 (Morning)

Date
Time (hh:mm)

A. Measurement/Score of symptoms

0 = No discomfort experienced.
1 = Causes mild discomfort. It does not limit usual activity.
2 = Causes mild to moderate limitation in activity. Some assistance may be needed.
3 = Causes marked limitation in activity
(Please complete all rows: enter the measurement or tick the highest value that applies for each symptom).

			Paracetamol taken?	Ibuprofen taken?
1	Your temperature(°C)	.....	☐	☐
2	**FEV1**	.....	**Best of 3**	
3	**FVC**	.....	**Best of 3**	
Local Reactions				
4	Diameter of redness	.....	**mm**	
5	Diameter of swelling	.....	**mm**	
6	Scaling present	0☐ 1☐	**0 = No 1 = Yes**	
7	Pain at the site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
8	Itching at site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
9	Warmth at the site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
Generalised Symptoms (Symptoms that occur in other parts of the body not at site of vaccination)				
10	Feelings of feverishness	0☐ 1☐ 2☐ 3☐	☐	☐
11	Muscle aches	0☐ 1☐ 2☐ 3☐	☐	☐
12	Joint aches	0☐ 1☐ 2☐ 3☐	☐	☐
13	Headaches	0☐ 1☐ 2☐ 3☐	☐	☐
14	Fatigue	0☐ 1☐ 2☐ 3☐	☐	☐
15	Nausea	0☐ 1☐ 2☐ 3☐	☐	☐
16	Generalised discomfort, illness, or lack of well-being *(but not listed above)*	0☐ 1☐ 2☐ 3☐	☐	☐
Respiratory Symptoms				
17	Any Cough?	0☐ 1☐ 2☐ 3☐		
18	Sore throat?	0☐ 1☐ 2☐ 3☐		
19	Wheeze?	0☐ 1☐ 2☐ 3☐		
20	Shortness of breath?	0☐ 1☐ 2☐ 3☐		
21	Coughing up phlegm?	0☐ 1☐ 2☐ 3☐		
22	Coughing up blood?	0☐ 1☐ 2☐ 3☐		
23	Chest pain?	0☐ 1☐ 2☐ 3☐		
Any other symptoms not listed above *(please give brief description)*				
24	.....	0☐ 1☐ 2☐ 3☐	☐	☐
25	.....	0☐ 1☐ 2☐ 3☐	☐	☐
26	.....	0☐ 1☐ 2☐ 3☐	☐	☐
27	.....	0☐ 1☐ 2☐ 3☐	☐	☐
28	.....	0☐ 1☐ 2☐ 3☐	☐	☐

Day 2 (Morning)

Subject ID

C. Paracetamol/Ibuprofen

If you have ticked to say you have taken either of these drugs please indicate the dose you have taken and the number of times it was taken during this period.

	Dose taken	Number of times this dose was taken	Which lines in the table opposite do these refers to.
Paracetamol			
Paracetamol			
Ibuprofen			
Ibuprofen			

D. Other Medication

If you have taken any other medication during this period please record it here.

Name	Dose taken	Number of times this dose was taken	Which lines in the table opposite do these refers to.

Day 2 (Evening)

Date
Time (hh:mm)

A. Measurement/Score of symptoms

0 = No discomfort experienced.
1 = Causes mild discomfort. It does not limit usual activity.
2 = Causes mild to moderate limitation in activity. Some assistance may be needed.
3 = Causes marked limitation in activity
(Please complete all rows: enter the measurement or tick the highest value that applies for each symptom).

			Paracetamol taken?	Ibuprofen taken?
1	Your temperature(°C)	.....	☐	☐
2	**FEV1**	.....	**Best of 3**	
3	**FVC**	.....	**Best of 3**	
	Local Reactions			
4	Diameter of redness	.....	**mm**	
5	Diameter of swelling	.....	**mm**	
6	Scaling present	0☐ 1☐	**0 = No 1 = Yes**	
7	Pain at the site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
8	Itching at site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
9	Warmth at the site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
	Generalised Symptoms (Symptoms that occur in other parts of the body not at site of vaccination)			
10	Feelings of feverishness	0☐ 1☐ 2☐ 3☐	☐	☐
11	Muscle aches	0☐ 1☐ 2☐ 3☐	☐	☐
12	Joint aches	0☐ 1☐ 2☐ 3☐	☐	☐
13	Headaches	0☐ 1☐ 2☐ 3☐	☐	☐
14	Fatigue	0☐ 1☐ 2☐ 3☐	☐	☐
15	Nausea	0☐ 1☐ 2☐ 3☐	☐	☐
16	Generalised discomfort, illness, or lack of well-being *(but not listed above)*	0☐ 1☐ 2☐ 3☐	☐	☐
	Respiratory Symptoms			
17	Any Cough?	0☐ 1☐ 2☐ 3☐		
18	Sore throat?	0☐ 1☐ 2☐ 3☐		
19	Wheeze?	0☐ 1☐ 2☐ 3☐		
20	Shortness of breath?	0☐ 1☐ 2☐ 3☐		
21	Coughing up phlegm?	0☐ 1☐ 2☐ 3☐		
22	Coughing up blood?	0☐ 1☐ 2☐ 3☐		
23	Chest pain?	0☐ 1☐ 2☐ 3☐		
	Any other symptoms not listed above *(please give brief description)*			
24	.....	0☐ 1☐ 2☐ 3☐	☐	☐
25	.....	0☐ 1☐ 2☐ 3☐	☐	☐
26	.....	0☐ 1☐ 2☐ 3☐	☐	☐
27	.....	0☐ 1☐ 2☐ 3☐	☐	☐
28	.....	0☐ 1☐ 2☐ 3☐	☐	☐

Day 2 (Evening)

Subject ID

C. Paracetamol/Ibuprofen

If you have ticked to say you have taken either of these drugs please indicate the dose you have taken and the number of times it was taken during this period.

	Dose taken	Number of times this dose was taken	Which lines in the table opposite do these refers to.
Paracetamol			
Paracetamol			
Ibuprofen			
Ibuprofen			

D. Other Medication

If you have taken any other medication during this period please record it here.

Name	Dose taken	Number of times this dose was taken	Which lines in the table opposite do these refers to.

Day 3 (Evening)

Date
Time (hh:mm)

A. Measurement/Score of symptoms

0 = No discomfort experienced.
1 = Causes mild discomfort. It does not limit usual activity.
2 = Causes mild to moderate limitation in activity. Some assistance may be needed.
3 = Causes marked limitation in activity
(Please complete all rows: enter the measurement or tick the highest value that applies for each symptom).

			Paracetamol taken?	Ibuprofen taken?
1	Your temperature(°C)	.....	☐	☐
2	**FEV1**	.....	**Best of 3**	
3	**FVC**	.....	**Best of 3**	
Local Reactions				
4	Diameter of redness	.....	**mm**	
5	Diameter of swelling	.....	**mm**	
6	Scaling present	0☐ 1☐	**0 = No 1 = Yes**	
7	Pain at the site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
8	Itching at site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
9	Warmth at the site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
Generalised Symptoms (Symptoms that occur in other parts of the body not at site of vaccination)				
10	Feelings of feverishness	0☐ 1☐ 2☐ 3☐	☐	☐
11	Muscle aches	0☐ 1☐ 2☐ 3☐	☐	☐
12	Joint aches	0☐ 1☐ 2☐ 3☐	☐	☐
13	Headaches	0☐ 1☐ 2☐ 3☐	☐	☐
14	Fatigue	0☐ 1☐ 2☐ 3☐	☐	☐
15	Nausea	0☐ 1☐ 2☐ 3☐	☐	☐
16	Generalised discomfort, illness, or lack of well-being *(but not listed above)*	0☐ 1☐ 2☐ 3☐	☐	☐
Respiratory Symptoms				
17	Any Cough?	0☐ 1☐ 2☐ 3☐		
18	Sore throat?	0☐ 1☐ 2☐ 3☐		
19	Wheeze?	0☐ 1☐ 2☐ 3☐		
20	Shortness of breath?	0☐ 1☐ 2☐ 3☐		
21	Coughing up phlegm?	0☐ 1☐ 2☐ 3☐		
22	Coughing up blood?	0☐ 1☐ 2☐ 3☐		
23	Chest pain?	0☐ 1☐ 2☐ 3☐		
Any other symptoms not listed above *(please give brief description)*				
24	.....	0☐ 1☐ 2☐ 3☐	☐	☐
25	.....	0☐ 1☐ 2☐ 3☐	☐	☐
26	.....	0☐ 1☐ 2☐ 3☐	☐	☐
27	.....	0☐ 1☐ 2☐ 3☐	☐	☐
28	.....	0☐ 1☐ 2☐ 3☐	☐	☐

Day 3 (Evening)

Subject ID

C. Paracetamol/Ibuprofen

If you have ticked to say you have taken either of these drugs please indicate the dose you have taken and the number of times it was taken during this period.

	Dose taken	Number of times this dose was taken	Which lines in the table opposite do these refers to.
Paracetamol			
Paracetamol			
Ibuprofen			
Ibuprofen			

D. Other Medication

If you have taken any other medication during this period please record it here.

Name	Dose taken	Number of times this dose was taken	Which lines in the table opposite do these refers to.

Day 4 (Evening)

Date
Time (hh:mm)

A. Measurement/Score of symptoms

0 = No discomfort experienced.
1 = Causes mild discomfort. It does not limit usual activity.
2 = Causes mild to moderate limitation in activity. Some assistance may be needed.
3 = Causes marked limitation in activity
(Please complete all rows: enter the measurement or tick the highest value that applies for each symptom).

			Paracetamol taken?	Ibuprofen taken?
1	Your temperature(°C)	.....	☐	☐
2	**FEV1**	.....	**Best of 3**	
3	**FVC**	.....	**Best of 3**	
	Local Reactions			
4	Diameter of redness	.....	**mm**	
5	Diameter of swelling	.....	**mm**	
6	Scaling present	0☐ 1☐	**0 = No 1 = Yes**	
7	Pain at the site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
8	Itching at site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
9	Warmth at the site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
	Generalised Symptoms (Symptoms that occur in other parts of the body not at site of vaccination)			
10	Feelings of feverishness	0☐ 1☐ 2☐ 3☐	☐	☐
11	Muscle aches	0☐ 1☐ 2☐ 3☐	☐	☐
12	Joint aches	0☐ 1☐ 2☐ 3☐	☐	☐
13	Headaches	0☐ 1☐ 2☐ 3☐	☐	☐
14	Fatigue	0☐ 1☐ 2☐ 3☐	☐	☐
15	Nausea	0☐ 1☐ 2☐ 3☐	☐	☐
16	Generalised discomfort, illness, or lack of well-being *(but not listed above)*	0☐ 1☐ 2☐ 3☐	☐	☐
	Respiratory Symptoms			
17	Any Cough?	0☐ 1☐ 2☐ 3☐		
18	Sore throat?	0☐ 1☐ 2☐ 3☐		
19	Wheeze?	0☐ 1☐ 2☐ 3☐		
20	Shortness of breath?	0☐ 1☐ 2☐ 3☐		
21	Coughing up phlegm?	0☐ 1☐ 2☐ 3☐		
22	Coughing up blood?	0☐ 1☐ 2☐ 3☐		
23	Chest pain?	0☐ 1☐ 2☐ 3☐		
	Any other symptoms not listed above *(please give brief description)*			
24	.....	0☐ 1☐ 2☐ 3☐	☐	☐
25	.....	0☐ 1☐ 2☐ 3☐	☐	☐
26	.....	0☐ 1☐ 2☐ 3☐	☐	☐
27	.....	0☐ 1☐ 2☐ 3☐	☐	☐
28	.....	0☐ 1☐ 2☐ 3☐	☐	☐

Day 4 (Evening)

Subject ID

C. Paracetamol/Ibuprofen

If you have ticked to say you have taken either of these drugs please indicate the dose you have taken and the number of times it was taken during this period.

	Dose taken	Number of times this dose was taken	Which lines in the table opposite do these refers to.
Paracetamol			
Paracetamol			
Ibuprofen			
Ibuprofen			

D. Other Medication

If you have taken any other medication during this period please record it here.

Name	Dose taken	Number of times this dose was taken	Which lines in the table opposite do these refers to.

Day 5 (Evening)

Date
Time (hh:mm)

A. Measurement/Score of symptoms

0 = No discomfort experienced.
1 = Causes mild discomfort. It does not limit usual activity.
2 = Causes mild to moderate limitation in activity. Some assistance may be needed.
3 = Causes marked limitation in activity
(Please complete all rows: enter the measurement or tick the highest value that applies for each symptom).

			Paracetamol taken?	Ibuprofen taken?
1	Your temperature(°C)	.....	☐	☐
2	**FEV1**	.....	**Best of 3**	
3	**FVC**	.....	**Best of 3**	
Local Reactions				
4	Diameter of redness	.....	**mm**	
5	Diameter of swelling	.....	**mm**	
6	Scaling present	0☐ 1☐	**0 = No 1 = Yes**	
7	Pain at the site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
8	Itching at site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
9	Warmth at the site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
Generalised Symptoms (Symptoms that occur in other parts of the body not at site of vaccination)				
10	Feelings of feverishness	0☐ 1☐ 2☐ 3☐	☐	☐
11	Muscle aches	0☐ 1☐ 2☐ 3☐	☐	☐
12	Joint aches	0☐ 1☐ 2☐ 3☐	☐	☐
13	Headaches	0☐ 1☐ 2☐ 3☐	☐	☐
14	Fatigue	0☐ 1☐ 2☐ 3☐	☐	☐
15	Nausea	0☐ 1☐ 2☐ 3☐	☐	☐
16	Generalised discomfort, illness, or lack of well-being *(but not listed above)*	0☐ 1☐ 2☐ 3☐	☐	☐
Respiratory Symptoms				
17	Any Cough?	0☐ 1☐ 2☐ 3☐		
18	Sore throat?	0☐ 1☐ 2☐ 3☐		
19	Wheeze?	0☐ 1☐ 2☐ 3☐		
20	Shortness of breath?	0☐ 1☐ 2☐ 3☐		
21	Coughing up phlegm?	0☐ 1☐ 2☐ 3☐		
22	Coughing up blood?	0☐ 1☐ 2☐ 3☐		
23	Chest pain?	0☐ 1☐ 2☐ 3☐		
Any other symptoms not listed above *(please give brief description)*				
24	.....	0☐ 1☐ 2☐ 3☐	☐	☐
25	.....	0☐ 1☐ 2☐ 3☐	☐	☐
26	.....	0☐ 1☐ 2☐ 3☐	☐	☐
27	.....	0☐ 1☐ 2☐ 3☐	☐	☐
28	.....	0☐ 1☐ 2☐ 3☐	☐	☐

Day 5 (Evening)

Subject ID

C. Paracetamol/Ibuprofen

If you have ticked to say you have taken either of these drugs please indicate the dose you have taken and the number of times it was taken during this period.

	Dose taken	Number of times this dose was taken	Which lines in the table opposite do these refers to.
Paracetamol			
Paracetamol			
Ibuprofen			
Ibuprofen			

D. Other Medication

If you have taken any other medication during this period please record it here.

Name	Dose taken	Number of times this dose was taken	Which lines in the table opposite do these refers to.

Day 6 (Evening)

Date
Time (hh:mm)

A. Measurement/Score of symptoms

0 = No discomfort experienced.
1 = Causes mild discomfort. It does not limit usual activity.
2 = Causes mild to moderate limitation in activity. Some assistance may be needed.
3 = Causes marked limitation in activity
(Please complete all rows: enter the measurement or tick the highest value that applies for each symptom).

			Paracetamol taken?	Ibuprofen taken?
1	Your temperature(°C)	.....	☐	☐
2	**FEV1**	..... **Best of 3**		
3	**FVC**	..... **Best of 3**		
Local Reactions				
4	Diameter of redness	..... **mm**		
5	Diameter of swelling	..... **mm**		
6	Scaling present	0☐ 1☐	**0 = No 1 = Yes**	
7	Pain at the site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
8	Itching at site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
9	Warmth at the site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
Generalised Symptoms (Symptoms that occur in other parts of the body not at site of vaccination)				
10	Feelings of feverishness	0☐ 1☐ 2☐ 3☐	☐	☐
11	Muscle aches	0☐ 1☐ 2☐ 3☐	☐	☐
12	Joint aches	0☐ 1☐ 2☐ 3☐	☐	☐
13	Headaches	0☐ 1☐ 2☐ 3☐	☐	☐
14	Fatigue	0☐ 1☐ 2☐ 3☐	☐	☐
15	Nausea	0☐ 1☐ 2☐ 3☐	☐	☐
16	Generalised discomfort, illness, or lack of well-being *(but not listed above)*	0☐ 1☐ 2☐ 3☐	☐	☐
Respiratory Symptoms				
17	Any Cough?	0☐ 1☐ 2☐ 3☐		
18	Sore throat?	0☐ 1☐ 2☐ 3☐		
19	Wheeze?	0☐ 1☐ 2☐ 3☐		
20	Shortness of breath?	0☐ 1☐ 2☐ 3☐		
21	Coughing up phlegm?	0☐ 1☐ 2☐ 3☐		
22	Coughing up blood?	0☐ 1☐ 2☐ 3☐		
23	Chest pain?	0☐ 1☐ 2☐ 3☐		
Any other symptoms not listed above *(please give brief description)*				
24	.....	0☐ 1☐ 2☐ 3☐	☐	☐
25	.....	0☐ 1☐ 2☐ 3☐	☐	☐
26	.....	0☐ 1☐ 2☐ 3☐	☐	☐
27	.....	0☐ 1☐ 2☐ 3☐	☐	☐
28	.....	0☐ 1☐ 2☐ 3☐	☐	☐

Day 6 (Evening)

Subject ID

C. Paracetamol/Ibuprofen

If you have ticked to say you have taken either of these drugs please indicate the dose you have taken and the number of times it was taken during this period.

	Dose taken	Number of times this dose was taken	Which lines in the table opposite do these refers to.
Paracetamol			
Paracetamol			
Ibuprofen			
Ibuprofen			

D. Other Medication

If you have taken any other medication during this period please record it here.

Name	Dose taken	Number of times this dose was taken	Which lines in the table opposite do these refers to.

Day 7 (Evening)

Date
Time (hh:mm)

A. Measurement/Score of symptoms

0 = No discomfort experienced.
1 = Causes mild discomfort. It does not limit usual activity.
2 = Causes mild to moderate limitation in activity. Some assistance may be needed.
3 = Causes marked limitation in activity
(Please complete all rows: enter the measurement or tick the highest value that applies for each symptom).

			Paracetamol taken?	Ibuprofen taken?
1	Your temperature(°C)	.....	☐	☐
2	**FEV1**	..... **Best of 3**		
3	**FVC**	..... **Best of 3**		
Local Reactions				
4	Diameter of redness	..... **mm**		
5	Diameter of swelling	..... **mm**		
6	Scaling present	0☐ 1☐ **0 = No 1 = Yes**		
7	Pain at the site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
8	Itching at site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
9	Warmth at the site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
Generalised Symptoms (Symptoms that occur in other parts of the body not at site of vaccination)				
10	Feelings of feverishness	0☐ 1☐ 2☐ 3☐	☐	☐
11	Muscle aches	0☐ 1☐ 2☐ 3☐	☐	☐
12	Joint aches	0☐ 1☐ 2☐ 3☐	☐	☐
13	Headaches	0☐ 1☐ 2☐ 3☐	☐	☐
14	Fatigue	0☐ 1☐ 2☐ 3☐	☐	☐
15	Nausea	0☐ 1☐ 2☐ 3☐	☐	☐
16	Generalised discomfort, illness, or lack of well-being *(but not listed above)*	0☐ 1☐ 2☐ 3☐	☐	☐
Respiratory Symptoms				
17	Any Cough?	0☐ 1☐ 2☐ 3☐		
18	Sore throat?	0☐ 1☐ 2☐ 3☐		
19	Wheeze?	0☐ 1☐ 2☐ 3☐		
20	Shortness of breath?	0☐ 1☐ 2☐ 3☐		
21	Coughing up phlegm?	0☐ 1☐ 2☐ 3☐		
22	Coughing up blood?	0☐ 1☐ 2☐ 3☐		
23	Chest pain?	0☐ 1☐ 2☐ 3☐		
Any other symptoms not listed above *(please give brief description)*				
24	.....	0☐ 1☐ 2☐ 3☐	☐	☐
25	.....	0☐ 1☐ 2☐ 3☐	☐	☐
26	.....	0☐ 1☐ 2☐ 3☐	☐	☐
27	.....	0☐ 1☐ 2☐ 3☐	☐	☐
28	.....	0☐ 1☐ 2☐ 3☐	☐	☐

Day 7 (Evening)

Subject ID

C. Paracetamol/Ibuprofen

If you have ticked to say you have taken either of these drugs please indicate the dose you have taken and the number of times it was taken during this period.

	Dose taken	Number of times this dose was taken	Which lines in the table opposite do these refers to.
Paracetamol			
Paracetamol			
Ibuprofen			
Ibuprofen			

D. Other Medication

If you have taken any other medication during this period please record it here.

Name	Dose taken	Number of times this dose was taken	Which lines in the table opposite do these refers to.

Day 8 (Evening)

Date
Time (hh:mm)

A. Measurement/Score of symptoms

0 = No discomfort experienced.
1 = Causes mild discomfort. It does not limit usual activity.
2 = Causes mild to moderate limitation in activity. Some assistance may be needed.
3 = Causes marked limitation in activity
(Please complete all rows: enter the measurement or tick the highest value that applies for each symptom).

			Paracetamol taken?	Ibuprofen taken?
1	Your temperature(°C)	.....	☐	☐
2	**FEV1**	.....	**Best of 3**	
3	**FVC**	.....	**Best of 3**	
Local Reactions				
4	Diameter of redness	.....	**mm**	
5	Diameter of swelling	.....	**mm**	
6	Scaling present	0☐ 1☐	**0 = No 1 = Yes**	
7	Pain at the site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
8	Itching at site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
9	Warmth at the site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
Generalised Symptoms (Symptoms that occur in other parts of the body not at site of vaccination)				
10	Feelings of feverishness	0☐ 1☐ 2☐ 3☐	☐	☐
11	Muscle aches	0☐ 1☐ 2☐ 3☐	☐	☐
12	Joint aches	0☐ 1☐ 2☐ 3☐	☐	☐
13	Headaches	0☐ 1☐ 2☐ 3☐	☐	☐
14	Fatigue	0☐ 1☐ 2☐ 3☐	☐	☐
15	Nausea	0☐ 1☐ 2☐ 3☐	☐	☐
16	Generalised discomfort, illness, or lack of well-being *(but not listed above)*	0☐ 1☐ 2☐ 3☐	☐	☐
Respiratory Symptoms				
17	Any Cough?	0☐ 1☐ 2☐ 3☐		
18	Sore throat?	0☐ 1☐ 2☐ 3☐		
19	Wheeze?	0☐ 1☐ 2☐ 3☐		
20	Shortness of breath?	0☐ 1☐ 2☐ 3☐		
21	Coughing up phlegm?	0☐ 1☐ 2☐ 3☐		
22	Coughing up blood?	0☐ 1☐ 2☐ 3☐		
23	Chest pain?	0☐ 1☐ 2☐ 3☐		
Any other symptoms not listed above *(please give brief description)*				
24	.....	0☐ 1☐ 2☐ 3☐	☐	☐
25	.....	0☐ 1☐ 2☐ 3☐	☐	☐
26	.....	0☐ 1☐ 2☐ 3☐	☐	☐
27	.....	0☐ 1☐ 2☐ 3☐	☐	☐
28	.....	0☐ 1☐ 2☐ 3☐	☐	☐

Day 8 (Evening)

Subject ID

C. Paracetamol/Ibuprofen

If you have ticked to say you have taken either of these drugs please indicate the dose you have taken and the number of times it was taken during this period.

	Dose taken	Number of times this dose was taken	Which lines in the table opposite do these refers to.
Paracetamol			
Paracetamol			
Ibuprofen			
Ibuprofen			

D. Other Medication

If you have taken any other medication during this period please record it here.

Name	Dose taken	Number of times this dose was taken	Which lines in the table opposite do these refers to.

Day 9 (Evening)

Date
Time (hh:mm)

A. Measurement/Score of symptoms

0 = No discomfort experienced.
1 = Causes mild discomfort. It does not limit usual activity.
2 = Causes mild to moderate limitation in activity. Some assistance may be needed.
3 = Causes marked limitation in activity
(Please complete all rows: enter the measurement or tick the highest value that applies for each symptom).

			Paracetamol taken?	Ibuprofen taken?
1	Your temperature(°C)	.....	☐	☐
2	**FEV1**	..... **Best of 3**		
3	**FVC**	..... **Best of 3**		
Local Reactions				
4	Diameter of redness	..... **mm**		
5	Diameter of swelling	..... **mm**		
6	Scaling present	0☐ 1☐	**0 = No 1 = Yes**	
7	Pain at the site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
8	Itching at site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
9	Warmth at the site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
Generalised Symptoms (Symptoms that occur in other parts of the body not at site of vaccination)				
10	Feelings of feverishness	0☐ 1☐ 2☐ 3☐	☐	☐
11	Muscle aches	0☐ 1☐ 2☐ 3☐	☐	☐
12	Joint aches	0☐ 1☐ 2☐ 3☐	☐	☐
13	Headaches	0☐ 1☐ 2☐ 3☐	☐	☐
14	Fatigue	0☐ 1☐ 2☐ 3☐	☐	☐
15	Nausea	0☐ 1☐ 2☐ 3☐	☐	☐
16	Generalised discomfort, illness, or lack of well-being *(but not listed above)*	0☐ 1☐ 2☐ 3☐	☐	☐
Respiratory Symptoms				
17	Any Cough?	0☐ 1☐ 2☐ 3☐		
18	Sore throat?	0☐ 1☐ 2☐ 3☐		
19	Wheeze?	0☐ 1☐ 2☐ 3☐		
20	Shortness of breath?	0☐ 1☐ 2☐ 3☐		
21	Coughing up phlegm?	0☐ 1☐ 2☐ 3☐		
22	Coughing up blood?	0☐ 1☐ 2☐ 3☐		
23	Chest pain?	0☐ 1☐ 2☐ 3☐		
Any other symptoms not listed above *(please give brief description)*				
24	.....	0☐ 1☐ 2☐ 3☐	☐	☐
25	.....	0☐ 1☐ 2☐ 3☐	☐	☐
26	.....	0☐ 1☐ 2☐ 3☐	☐	☐
27	.....	0☐ 1☐ 2☐ 3☐	☐	☐
28	.....	0☐ 1☐ 2☐ 3☐	☐	☐

Day 9 (Evening)

Subject ID

C. Paracetamol/Ibuprofen

If you have ticked to say you have taken either of these drugs please indicate the dose you have taken and the number of times it was taken during this period.

	Dose taken	Number of times this dose was taken	Which lines in the table opposite do these refers to.
Paracetamol			
Paracetamol			
Ibuprofen			
Ibuprofen			

D. Other Medication

If you have taken any other medication during this period please record it here.

Name	Dose taken	Number of times this dose was taken	Which lines in the table opposite do these refers to.

Day 10 (Evening)

Date
Time (hh:mm)

A. Measurement/Score of symptoms

0 = No discomfort experienced.
1 = Causes mild discomfort. It does not limit usual activity.
2 = Causes mild to moderate limitation in activity. Some assistance may be needed.
3 = Causes marked limitation in activity
(Please complete all rows: enter the measurement or tick the highest value that applies for each symptom).

			Paracetamol taken?	Ibuprofen taken?
1	Your temperature(°C)	.....	☐	☐
2	**FEV1**	..... **Best of 3**		
3	**FVC**	..... **Best of 3**		
Local Reactions				
4	Diameter of redness	..... **mm**		
5	Diameter of swelling	..... **mm**		
6	Scaling present	0☐ 1☐	**0 = No 1 = Yes**	
7	Pain at the site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
8	Itching at site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
9	Warmth at the site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
Generalised Symptoms (Symptoms that occur in other parts of the body not at site of vaccination)				
10	Feelings of feverishness	0☐ 1☐ 2☐ 3☐	☐	☐
11	Muscle aches	0☐ 1☐ 2☐ 3☐	☐	☐
12	Joint aches	0☐ 1☐ 2☐ 3☐	☐	☐
13	Headaches	0☐ 1☐ 2☐ 3☐	☐	☐
14	Fatigue	0☐ 1☐ 2☐ 3☐	☐	☐
15	Nausea	0☐ 1☐ 2☐ 3☐	☐	☐
16	Generalised discomfort, illness, or lack of well-being *(but not listed above)*	0☐ 1☐ 2☐ 3☐	☐	☐
Respiratory Symptoms				
17	Any Cough?	0☐ 1☐ 2☐ 3☐		
18	Sore throat?	0☐ 1☐ 2☐ 3☐		
19	Wheeze?	0☐ 1☐ 2☐ 3☐		
20	Shortness of breath?	0☐ 1☐ 2☐ 3☐		
21	Coughing up phlegm?	0☐ 1☐ 2☐ 3☐		
22	Coughing up blood?	0☐ 1☐ 2☐ 3☐		
23	Chest pain?	0☐ 1☐ 2☐ 3☐		
Any other symptoms not listed above *(please give brief description)*				
24	.....	0☐ 1☐ 2☐ 3☐	☐	☐
25	.....	0☐ 1☐ 2☐ 3☐	☐	☐
26	.....	0☐ 1☐ 2☐ 3☐	☐	☐
27	.....	0☐ 1☐ 2☐ 3☐	☐	☐
28	.....	0☐ 1☐ 2☐ 3☐	☐	☐

Day 10 (Evening)

Subject ID

C. Paracetamol/Ibuprofen

If you have ticked to say you have taken either of these drugs please indicate the dose you have taken and the number of times it was taken during this period.

	Dose taken	Number of times this dose was taken	Which lines in the table opposite do these refers to.
Paracetamol			
Paracetamol			
Ibuprofen			
Ibuprofen			

D. Other Medication

If you have taken any other medication during this period please record it here.

Name	Dose taken	Number of times this dose was taken	Which lines in the table opposite do these refers to.

Day 11 (Evening)

Date
Time (hh:mm)

A. Measurement/Score of symptoms

0 = No discomfort experienced.
1 = Causes mild discomfort. It does not limit usual activity.
2 = Causes mild to moderate limitation in activity. Some assistance may be needed.
3 = Causes marked limitation in activity
(Please complete all rows: enter the measurement or tick the highest value that applies for each symptom).

			Paracetamol taken?	Ibuprofen taken?
1	Your temperature(°C)	.....	☐	☐
2	**FEV1**	.....	**Best of 3**	
3	**FVC**	.....	**Best of 3**	
Local Reactions				
4	Diameter of redness	.....	**mm**	
5	Diameter of swelling	.....	**mm**	
6	Scaling present	0☐ 1☐	**0 = No 1 = Yes**	
7	Pain at the site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
8	Itching at site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
9	Warmth at the site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
Generalised Symptoms (Symptoms that occur in other parts of the body not at site of vaccination)				
10	Feelings of feverishness	0☐ 1☐ 2☐ 3☐	☐	☐
11	Muscle aches	0☐ 1☐ 2☐ 3☐	☐	☐
12	Joint aches	0☐ 1☐ 2☐ 3☐	☐	☐
13	Headaches	0☐ 1☐ 2☐ 3☐	☐	☐
14	Fatigue	0☐ 1☐ 2☐ 3☐	☐	☐
15	Nausea	0☐ 1☐ 2☐ 3☐	☐	☐
16	Generalised discomfort, illness, or lack of well-being *(but not listed above)*	0☐ 1☐ 2☐ 3☐	☐	☐
Respiratory Symptoms				
17	Any Cough?	0☐ 1☐ 2☐ 3☐		
18	Sore throat?	0☐ 1☐ 2☐ 3☐		
19	Wheeze?	0☐ 1☐ 2☐ 3☐		
20	Shortness of breath?	0☐ 1☐ 2☐ 3☐		
21	Coughing up phlegm?	0☐ 1☐ 2☐ 3☐		
22	Coughing up blood?	0☐ 1☐ 2☐ 3☐		
23	Chest pain?	0☐ 1☐ 2☐ 3☐		
Any other symptoms not listed above *(please give brief description)*				
24	.....	0☐ 1☐ 2☐ 3☐	☐	☐
25	.....	0☐ 1☐ 2☐ 3☐	☐	☐
26	.....	0☐ 1☐ 2☐ 3☐	☐	☐
27	.....	0☐ 1☐ 2☐ 3☐	☐	☐
28	.....	0☐ 1☐ 2☐ 3☐	☐	☐

Day 11 (Evening)

Subject ID

C. Paracetamol/Ibuprofen

If you have ticked to say you have taken either of these drugs please indicate the dose you have taken and the number of times it was taken during this period.

	Dose taken	Number of times this dose was taken	Which lines in the table opposite do these refers to.
Paracetamol			
Paracetamol			
Ibuprofen			
Ibuprofen			

D. Other Medication

If you have taken any other medication during this period please record it here.

Name	Dose taken	Number of times this dose was taken	Which lines in the table opposite do these refers to.

Day 12 (Evening)

Date
Time (hh:mm)

A. Measurement/Score of symptoms

0 = No discomfort experienced.
1 = Causes mild discomfort. It does not limit usual activity.
2 = Causes mild to moderate limitation in activity. Some assistance may be needed.
3 = Causes marked limitation in activity
(Please complete all rows: enter the measurement or tick the highest value that applies for each symptom).

			Paracetamol taken?	Ibuprofen taken?
1	Your temperature(°C)	.....	☐	☐
2	**FEV1**	.....	**Best of 3**	
3	**FVC**	.....	**Best of 3**	
Local Reactions				
4	Diameter of redness	.....	**mm**	
5	Diameter of swelling	.....	**mm**	
6	Scaling present	0☐ 1☐	**0 = No 1 = Yes**	
7	Pain at the site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
8	Itching at site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
9	Warmth at the site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
Generalised Symptoms (Symptoms that occur in other parts of the body not at site of vaccination)				
10	Feelings of feverishness	0☐ 1☐ 2☐ 3☐	☐	☐
11	Muscle aches	0☐ 1☐ 2☐ 3☐	☐	☐
12	Joint aches	0☐ 1☐ 2☐ 3☐	☐	☐
13	Headaches	0☐ 1☐ 2☐ 3☐	☐	☐
14	Fatigue	0☐ 1☐ 2☐ 3☐	☐	☐
15	Nausea	0☐ 1☐ 2☐ 3☐	☐	☐
16	Generalised discomfort, illness, or lack of well-being *(but not listed above)*	0☐ 1☐ 2☐ 3☐	☐	☐
Respiratory Symptoms				
17	Any Cough?	0☐ 1☐ 2☐ 3☐		
18	Sore throat?	0☐ 1☐ 2☐ 3☐		
19	Wheeze?	0☐ 1☐ 2☐ 3☐		
20	Shortness of breath?	0☐ 1☐ 2☐ 3☐		
21	Coughing up phlegm?	0☐ 1☐ 2☐ 3☐		
22	Coughing up blood?	0☐ 1☐ 2☐ 3☐		
23	Chest pain?	0☐ 1☐ 2☐ 3☐		
Any other symptoms not listed above *(please give brief description)*				
24	.....	0☐ 1☐ 2☐ 3☐	☐	☐
25	.....	0☐ 1☐ 2☐ 3☐	☐	☐
26	.....	0☐ 1☐ 2☐ 3☐	☐	☐
27	.....	0☐ 1☐ 2☐ 3☐	☐	☐
28	.....	0☐ 1☐ 2☐ 3☐	☐	☐

Day 12 (Evening)

Subject ID

C. Paracetamol/Ibuprofen

If you have ticked to say you have taken either of these drugs please indicate the dose you have taken and the number of times it was taken during this period.

	Dose taken	Number of times this dose was taken	Which lines in the table opposite do these refers to.
Paracetamol			
Paracetamol			
Ibuprofen			
Ibuprofen			

D. Other Medication

If you have taken any other medication during this period please record it here.

Name	Dose taken	Number of times this dose was taken	Which lines in the table opposite do these refers to.

Day 13 (Evening)

Date
Time (hh:mm)

A. Measurement/Score of symptoms

0 = No discomfort experienced.
1 = Causes mild discomfort. It does not limit usual activity.
2 = Causes mild to moderate limitation in activity. Some assistance may be needed.
3 = Causes marked limitation in activity
(Please complete all rows: enter the measurement or tick the highest value that applies for each symptom).

			Paracetamol taken?	Ibuprofen taken?
1	Your temperature(°C)	.....	☐	☐
2	**FEV1**	.....	**Best of 3**	
3	**FVC**	.....	**Best of 3**	
	Local Reactions			
4	Diameter of redness	.....	**mm**	
5	Diameter of swelling	.....	**mm**	
6	Scaling present	0☐ 1☐	**0 = No 1 = Yes**	
7	Pain at the site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
8	Itching at site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
9	Warmth at the site of vaccination	0☐ 1☐ 2☐ 3☐	☐	☐
	Generalised Symptoms (Symptoms that occur in other parts of the body not at site of vaccination)			
10	Feelings of feverishness	0☐ 1☐ 2☐ 3☐	☐	☐
11	Muscle aches	0☐ 1☐ 2☐ 3☐	☐	☐
12	Joint aches	0☐ 1☐ 2☐ 3☐	☐	☐
13	Headaches	0☐ 1☐ 2☐ 3☐	☐	☐
14	Fatigue	0☐ 1☐ 2☐ 3☐	☐	☐
15	Nausea	0☐ 1☐ 2☐ 3☐	☐	☐
16	Generalised discomfort, illness, or lack of well-being *(but not listed above)*	0☐ 1☐ 2☐ 3☐	☐	☐
	Respiratory Symptoms			
17	Any Cough?	0☐ 1☐ 2☐ 3☐		
18	Sore throat?	0☐ 1☐ 2☐ 3☐		
19	Wheeze?	0☐ 1☐ 2☐ 3☐		
20	Shortness of breath?	0☐ 1☐ 2☐ 3☐		
21	Coughing up phlegm?	0☐ 1☐ 2☐ 3☐		
22	Coughing up blood?	0☐ 1☐ 2☐ 3☐		
23	Chest pain?	0☐ 1☐ 2☐ 3☐		
	Any other symptoms not listed above *(please give brief description)*			
24	.....	0☐ 1☐ 2☐ 3☐	☐	☐
25	.....	0☐ 1☐ 2☐ 3☐	☐	☐
26	.....	0☐ 1☐ 2☐ 3☐	☐	☐
27	.....	0☐ 1☐ 2☐ 3☐	☐	☐
28	.....	0☐ 1☐ 2☐ 3☐	☐	☐

Day 13 (Evening)

Subject ID

C. Paracetamol/Ibuprofen

If you have ticked to say you have taken either of these drugs please indicate the dose you have taken and the number of times it was taken during this period.

	Dose taken	Number of times this dose was taken	Which lines in the table opposite do these refers to.
Paracetamol			
Paracetamol			
Ibuprofen			
Ibuprofen			

D. Other Medication

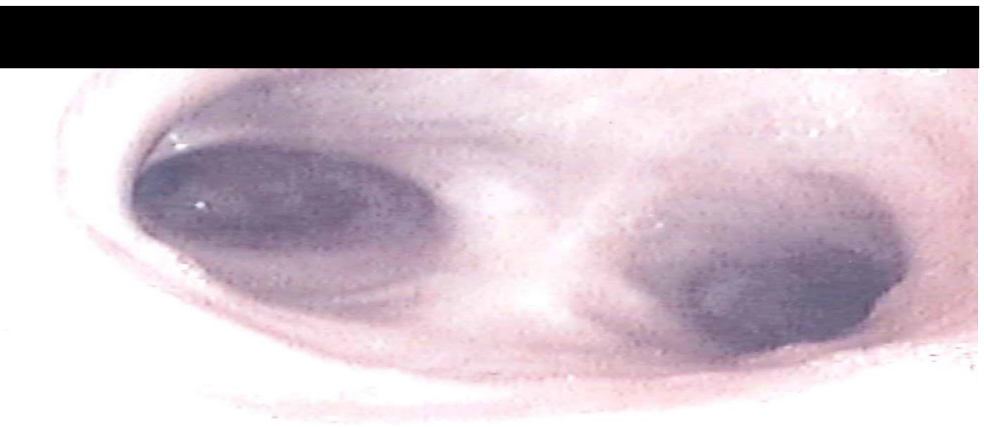
If you have taken any other medication during this period please record it here.

Name	Dose taken	Number of times this dose was taken	Which lines in the table opposite do these refers to.

Appendix 3

- Bronchoscopic Images
- Chapter 3, Section 3.4.6

Volunteer 001 (Group 1): Bronchoscopic images on day 35 after aerosol boost; reported as mild erythema throughout

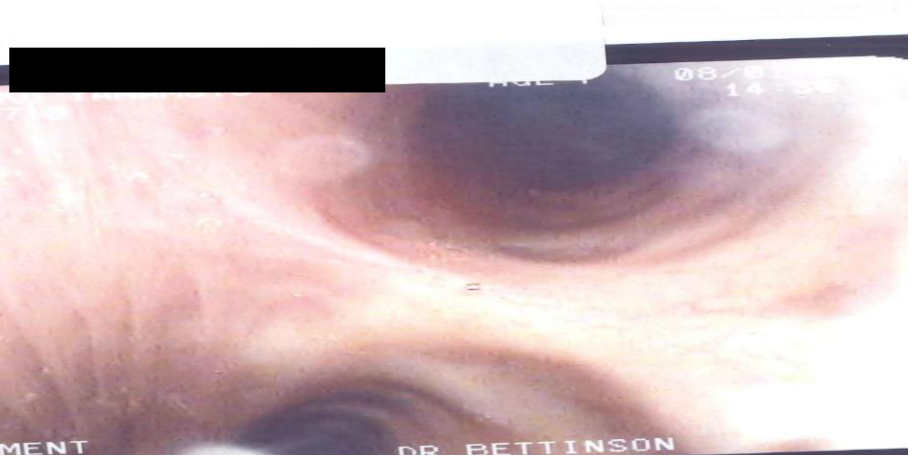


carina

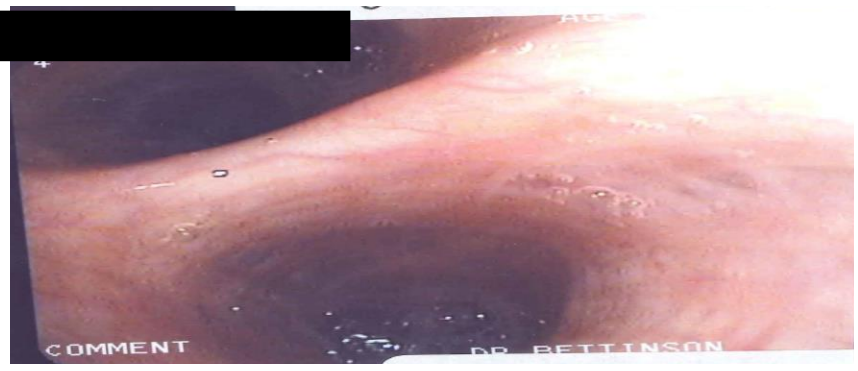


Left middle lobe bronchus

Volunteer 003 (Group 1): Bronchoscopic images on day 7 after aerosol prime; reported as normal bronchoscopic findings



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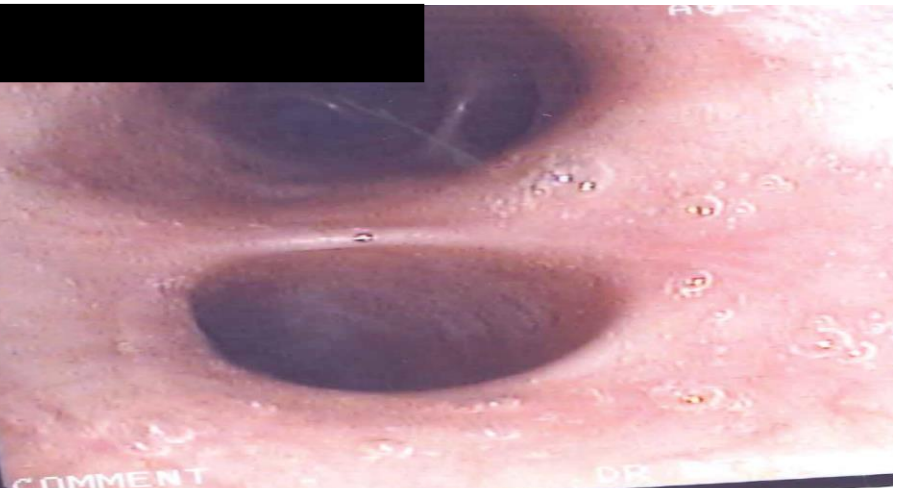


Right middle lobe bronchus

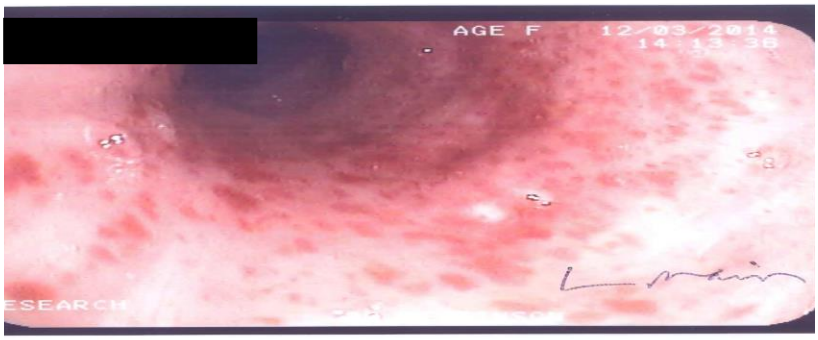
Volunteer 006 (Group 2): Bronchoscopic images on day 7 reported as mild erythema throughout



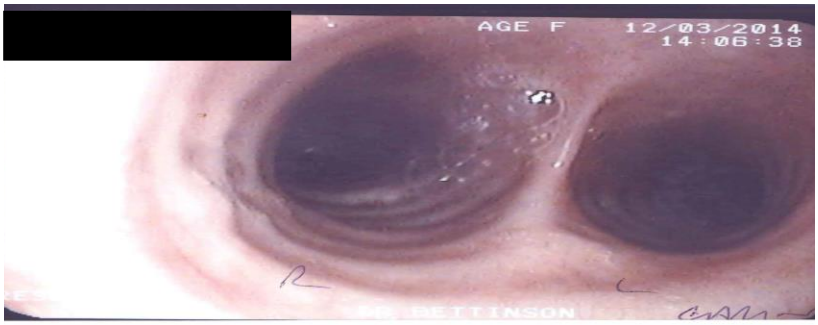
carina



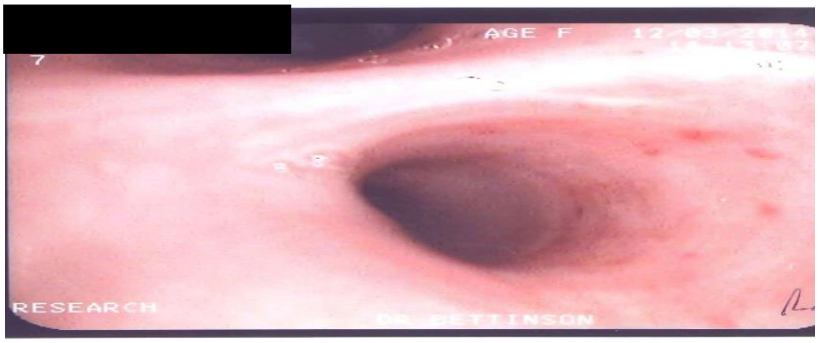
Volunteer 010 (Group 3): day 35 after intradermal boost; petechiae in left main bronchus



Left main bronchus

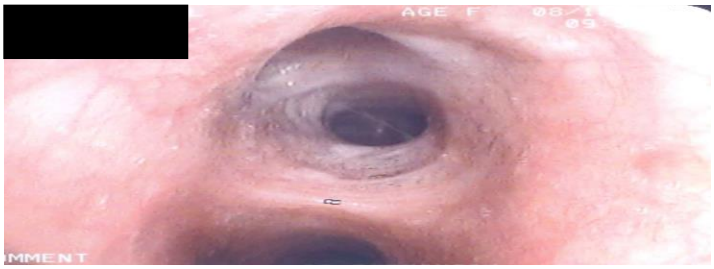


Carina



Right middle lobe bronchus

Volunteer 026 (Group 2): day 35 after aerosol boost; reported to have mucosal irregularity in left upper lobe



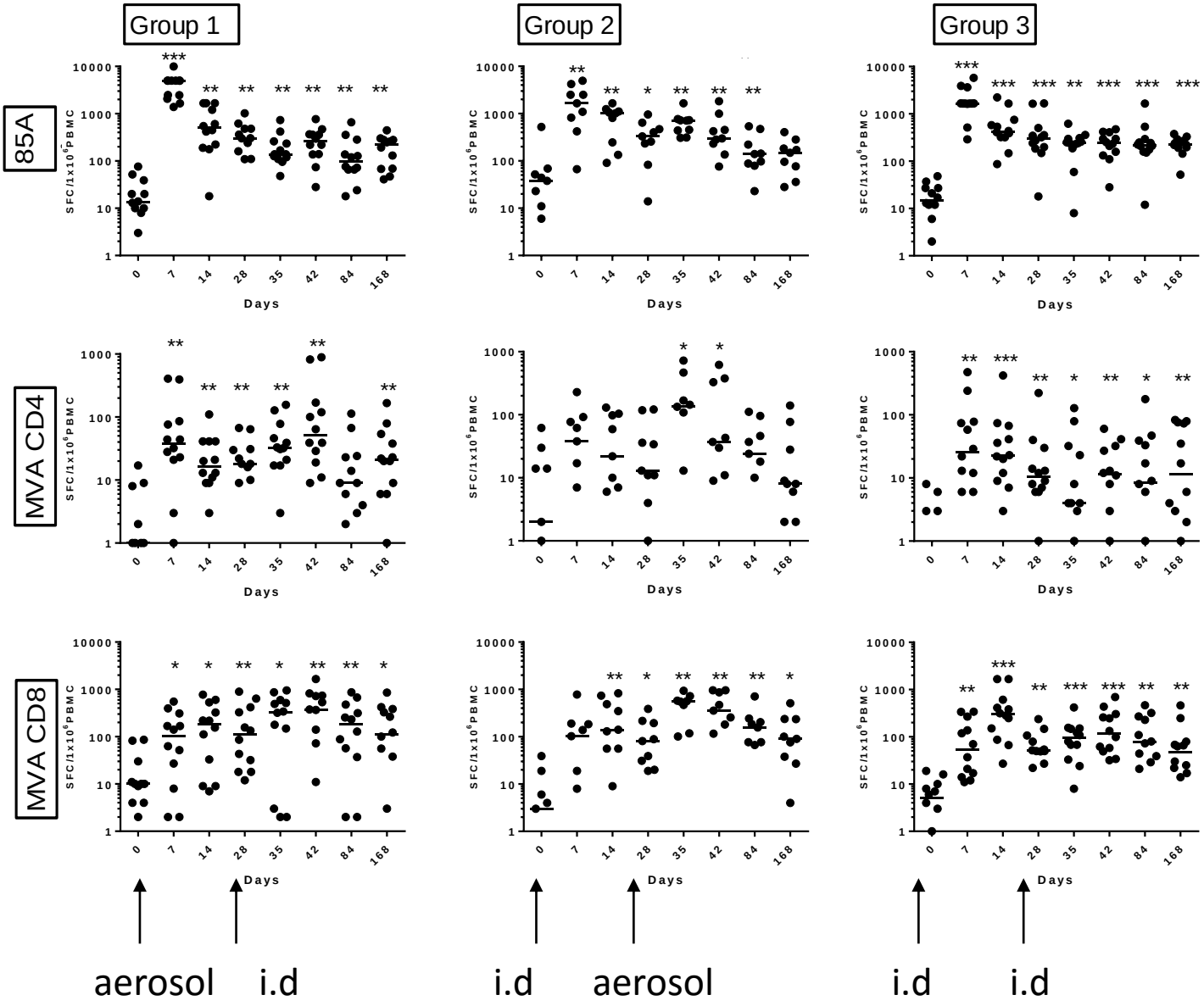
Left upper lobe bronchus



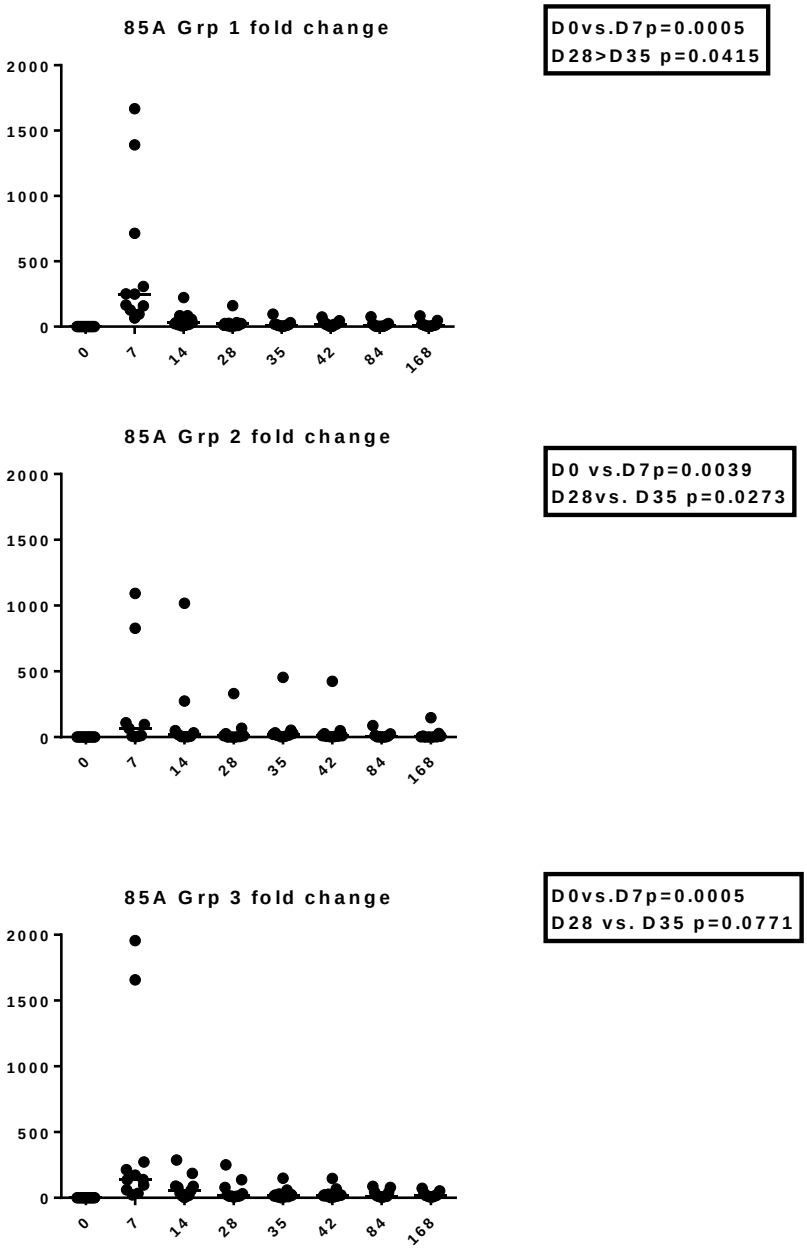
carina

Appendix 4 Supplementary Figures and Tables

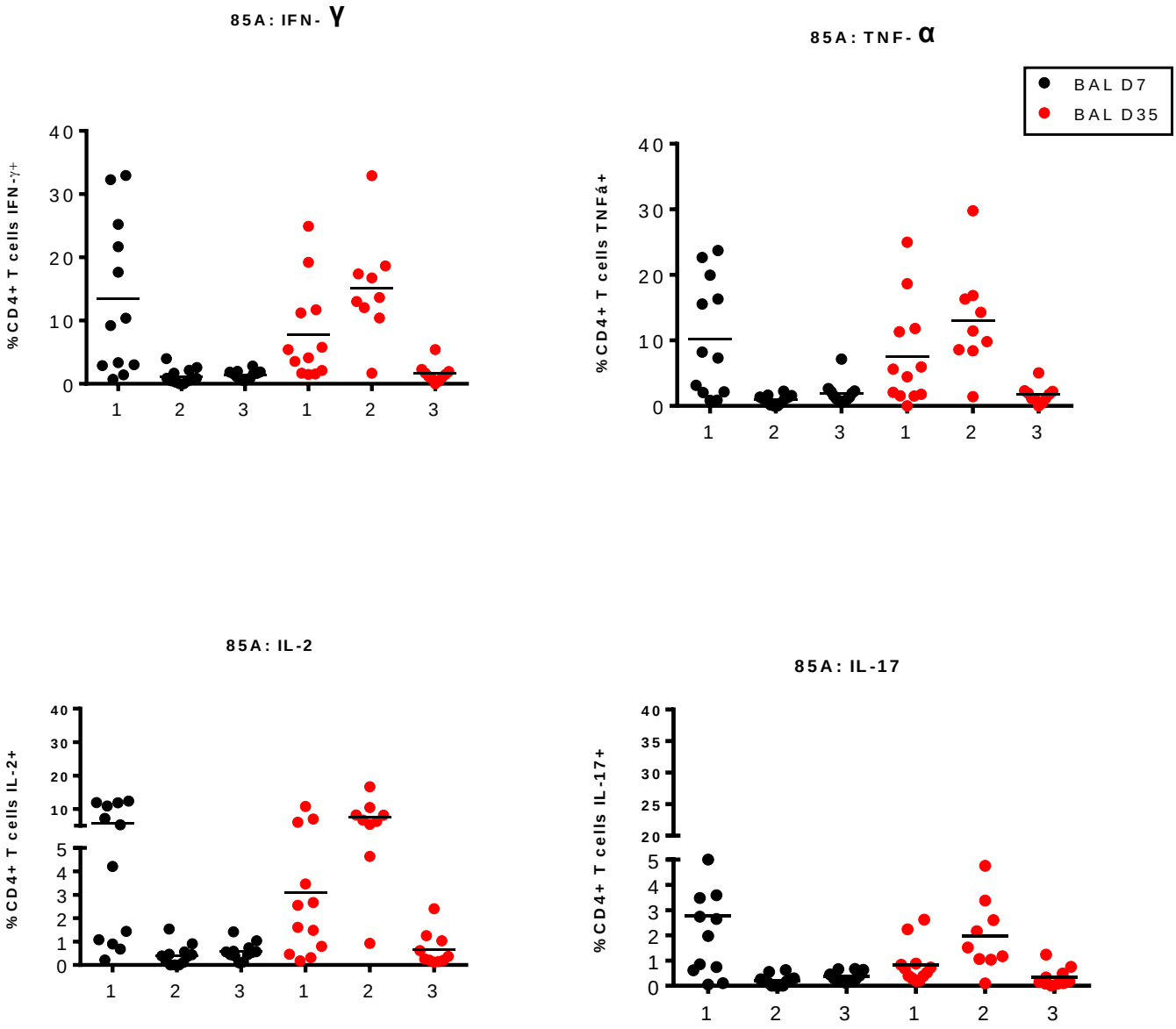
Supplementary Figure 1: **Dot plots of ELISPOT responses: alternative representation of figure 4.1. Frequency of antigen-specific IFN- γ secreting cells as measured by IFN- γ ELISPOT responses to Ag85A (top row), MVA CD4 (middle row), MVA CD8 (bottom row)** in Group 1(left column), Group 2 (middle column) and Group 3 (right column), x axis: timepoints in days, y axis: Spots per 1x10^{6}PBMC. Arrows indicate timings of aerosol/intradermal (i.d) vaccines. Shown are significant differences from baseline (day 0). Non-significant differences (p>0.05) omitted for clarity. * for p ≤ 0.05, ** for p≤0.01 and *** for p≤0.001



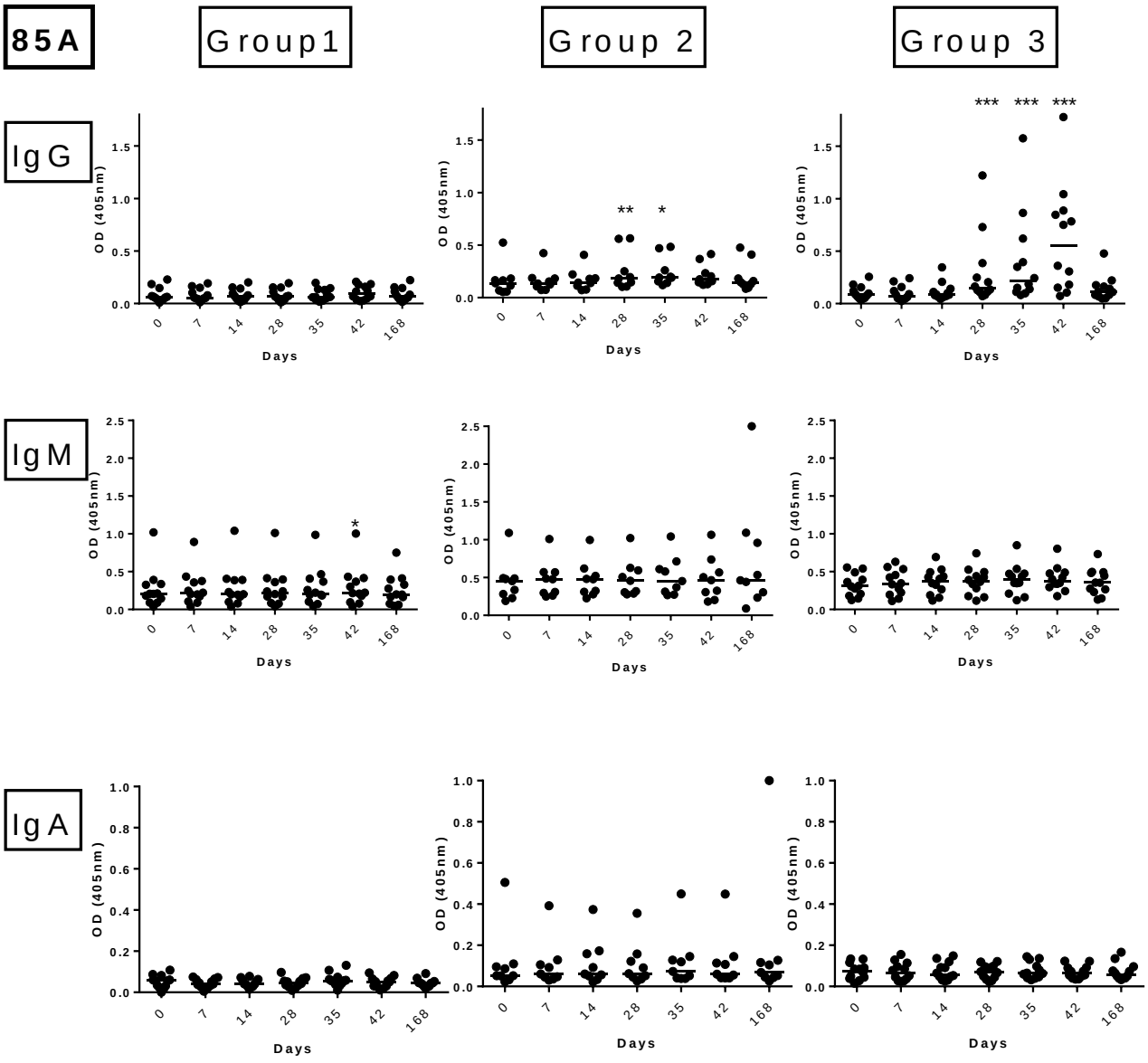
Supplementary Figure 2: **Fold change of antigen-specific IFN- γ secreting cells as measured by IFN- γ ELISPOT responses to Ag85A . Fold change calculated from baseline (day 0) values. of Spots per 1x106PBMC**



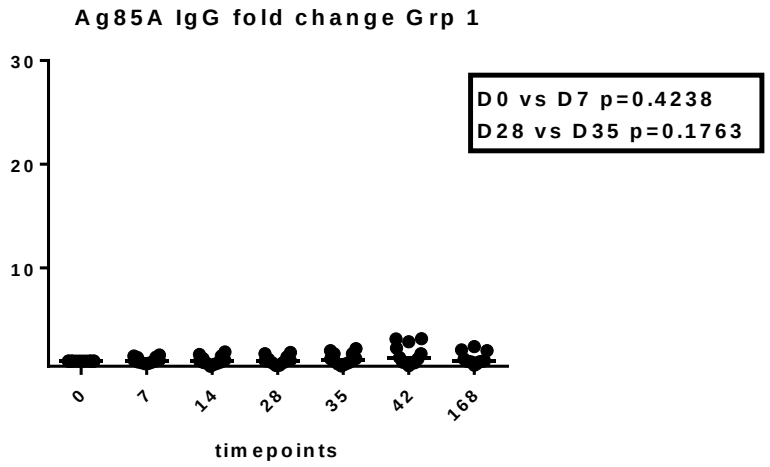
Supplementary Figure 3: Dot plots of Ag85a-specific responses in BAL. Alternative representation of figure 4.14: Dot plot

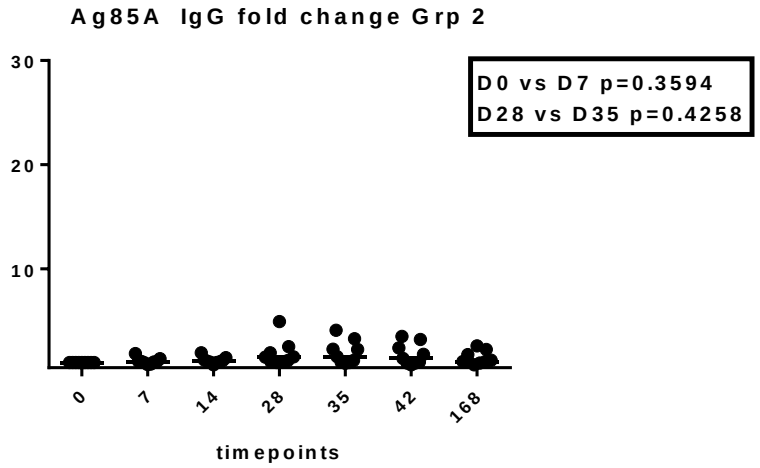


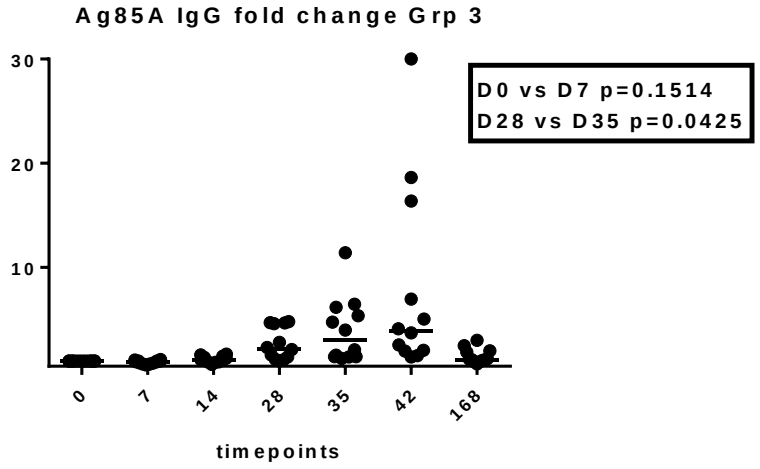
Supplementary figure 4: Dot plot of Ag85A-specific ELISA responses. Alternative representation of Figure 5.1. x-axis: timepoints, y-axis: OD (read at 405nm absorbance)



Supplementary Figure 5:Fold change of anti-Ag85A IgG responses. Fold change was calculated from baseline (day 0) of OD read at 405nm absorbance.







Supplementary table 1: Group 2 adverse events summary

	Group 2 reactions
Incidence	7 out of 9 volunteers in Group 2 were affected
Onset	Within 12 hours of receiving aerosol boost vaccination
Clinical symptoms	- Respiratory (breathlessness worse on exertion and chest tightness) were more frequent and more severe than in the other groups - Highest frequency and higher severity of systemic adverse events (myalgia, arthralgia, headache) compared to other groups/vaccinations - Highest frequence of fevers following vaccination (compared to other groups/vaccinations)
Duration of symptoms	Peak 24 hours after aerosol boost vaccination, full resolution by 72 hours following vaccination
Lung function	Significant transient decrease in FEV1 and FVC compared to other groups and vaccinations, restrictive.
Inflammatory markers	Higher range of CRP in affected volunteers in Group 22 days following aerosol boost vaccination on day 30 (between 55 and 156 mg/L; normal range 0-8mg/L) compared to range of 2.1 to 26mg/L in the other groups at that timepoint
Macroscopic appearances at bronchoscopy	Normal (7 days following aerosol boost vaccination, Day 35 of the trial)
ELISPOT responses	Boosting of Ag85A and MVA responses after aerosol boost vaccination (compared to day 28)
WB ICS responses	only group to boost IFN-g CD8 MVA responses; which are greater than in other groups on D42
BAL responses	highest percentage of Ag85A specific CD4 T cells, boosting BAL cytokine positive D35>D7
ELISA responses	Serum antibodies induced after intradermal prime

Supplementary table 2: Summary of key CMI results

			Ag85A	MVA
Group 1	**Prime**	**ES**	Peak on day 7 (higher than Groups 2 and 3)	CD4: peak on day 7, CD8: Increased on day 7
		WB	CD4: Increased on day 7 (higher than Groups 2 and 3), CD8: trend higher in Group 1 for IFN-g, Day7>day0 (till Day168) for TNF-a	CD4: Day28>day0 for TNF-a, CD8: Day14 &day28 both >day0 for IFN-g
		BAL	highest percentage of Ag85A specific CD4 T cells for all cytokines	
	Boost	**ES**	no boosting i.e Day35 not greater than day28 but Day168>Day0	CD4: Boosting Day42>day28, also Day168>day0, CD8: peak on day 28&day42, no boosting(compared to day28), Day168 >Day0
		WB	no boosting	CD8: Day42 >day0
		BAL		
Group 2	**Prime**	**ES**	peak day 7	CD4: increased on day 7 significantly higher than baseline on day 35, CD8: peak on day 28
		WB	CD4: increased on day 7	CD8: Day14>day0 for IFN-g
		BAL		
	Boost	**ES**	Boosting, i.e Day35>day28, also Day84>day0	CD4: Day42>day28, i.e boosting, then responses contracts, CD8: boosting i.e Day42>day28, also Day168>day0
		WB	CD4: no boosting	CD4: Day42>day0 for IFN-g, CD8: Day42>day28 (only group to boost IFN-g CD8 MVA responses; and these responses are greater than in other groups on day42) and Day168>day0
		BAL	highest percentage of Ag85A specific CD4 T cells, boosting BAL cytokine positive day35>day7	
Group 3	**Prime**	**ES**	peak day 7	CD4: increased on day 7, peaks day 14, CD8: peaks day14
		WB	CD4: increased on day7, CD8: Day28>day0	CD4: Day14>day0
		BAL		
	Boost	**ES**	no boosting, i.e Day35 not greater than day28 but Day168>day0	CD4: no boosting, CD8: boosting Day42>day28, also Day168>day0
		WB	CD4: no boosting	
		BAL		

Other points:

Ag85A specific ES responses peak after aerosol vaccination

Highest frequencey of Ag85A specific polyfunctional CD4 T cells in WB in Group 1 on day 7

Highest frequency of MVA specific polyfunctional T cells in Group 2 on Day 42