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INNATE IMMUNE RESPONSES TO TUBERCULOSIS VACCINES

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Abstract

Tuberculosis, caused by infection with *Mycobacterium tuberculosis (M.tb)*, remains a global health problem. Drug resistance and high rates of HIV infection have fuelled the pandemic and, although a vaccine exists, its ability to protect from pulmonary tuberculosis varies between 0 and 80%. Bacille Calmette Guerin (BCG) has been administered to billions worldwide yet its protective mechanisms remain unknown, as do the reasons for its failure to protect in many parts of the world. Modified Vaccinia virus Ankara expressing antigen 85A (MVA85A) is a novel candidate vaccine designed to boost immune responses to BCG and improve protection. An aim of this thesis has been to characterise the innate immune response to an MVA85A boosting vaccination in both UK adults and South African infants. In the former, volunteers develop a strong innate response following vaccination however this does not always translate into a robust adaptive response to antigen 85A (Ag85A), which is determined in part by Treg expansion and the nuclear protein HMGB1 signaling through the TLR1-2-6 axis. By contrast, not all South Africa infants mount a strong innate immune response to MVA85A yet this response is correlated with the magnitude of the adaptive response. The immune response to BCG in both populations is also characterised and an association found between increased production of IL-17, IL-22 and IFN-γ in response to BCG stimulation and control of mycobacterial growth. The results presented here further the knowledge on the links between innate and adaptive responses to vaccination with BCG and MVA85A and the variation in mechanisms involved in different populations.

DECLARATION

I hereby declare that the work presented in this thesis is original. With the exception of the list below, all experimental steps and analyses were conducted by the author.

I gratefully acknowledge the help of the following persons:

All the clinicians and laboratory staff involved in the primary clinical trial work. I did not collect or process samples during the clinical trials or generate the IFN-γ ELISpot data. Additionally, the quantification of BCG from the TB023 biopsies was performed by Stephanie Harris and Joel Meyer.

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LIST OF ABBREVIATIONS

Ad	adenovirus
Ag85A	antigen 85A
AIDS	Acquired Immunodeficiency Syndrome
APC	antigen presenting cell
ART	anti-retroviral therapies
AUC	area under the curve
BAL	bronchalveolar lavage
BCG	Bacille Calmette-Guerin
BSA	bovine serum albumin
CASP	caspase
CCR	chemokine (C-C motif) receptor
CD	cluster of differentiation
cDNA	complementary DNA
cfu	colony forming units
CTLA4	cytotoxic lymphocyte antigen 4
CXCL	C-X-C chemokine ligand
CXCR	C-X-C chemokine receptor
DAMP	danger-associated molecular pattern
DAVID	database for annotation, visualization and integrated discovery
DC	dendritic cell
DEG	differentially expressed gene
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
EIF2A	eukaryotic translation initiation fator 2a
EIF2AK4	eukaryotic translation initiation fator 2a kinase 4
ELISpot	enzyme-linked immunosorbent spot
ESAT-6	early secreted antigenic target 6
FCGR	Fc Gamma Recepotor
FITC	fluorescein isothiocyanate
Foxp3	forkhead box P3
FSC	forward scatter
GEX	gene expression

GO	gene ontology
GSEA	gene set enrichment analysis
HAMP	hepcidin anti-microbial peptide
HIV	human immunodeficiency virus
HMGB	high mobility group box
ICS	intracellular cytokine staining
ID	intradermal
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL2RA	IL-2 receptor alpha
IM	intramuscular
INDO	indoleamine 2,3-dioxygenase
IRF	interferon regulatory factor
kbp	kilo base pairs
L. monocytogenes	*Listeria monocytogenes*
LDA	linear discriminant analysis
LILR	leukocyte immunoglobulin-like receptor
LPS	lipopolysaccharide
LTR	long terminal repeat
M. smegmatis	*Mycobacterium smegmatis*
M.bovis	*Mycobacterium bovis*
M.tb	*Mycobacterium tuberculosis*
MDA	melanoma differentiation associated gene
MDR-TB	Multi-drug resistant TB
MFI	median fluorescence intensity
MHC	major histocompatibility complex
MOI	multiplicity of infection
MRC	medical research council
mRNA	messenger RNA
mTor	mechanistic target of rapamycin
MVA	modified Vaccinia virus Ankara

MVA85A	MVA expressing antigen 85A
MVAwt	MVA wild type
NF	nuclear factor
NK	natural killer
NO	Nitric oxide
NOD	nucleotide-binding oligomerization domain
PAMP	Pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PBS	phosphate buffered saline
PCA	principal component analysis
PCR	polymerase chain reaction
PE	phycoerythrin
PE-Cy5	phycoerythrin cychrome 5
PE-Cy7	phycoerythrin cychrome 7
pfu	plaque forming units
PHA	phytohemagglutinin
PKR	protein kinase R
PMA	phorbol myristate acetate
PPD	purified protein derivative
PRR	pattern recognition receptor
rBCG	recombinant BCG
RIN	RNA integrity number
RNA	ribonucleic acid
RSA	Republic of South Africa
SNP	single nucleotide polymorphism
SSC	side scatter
STAT	signal transducer and activator of transcription
Stim	stimulated
T-bet	T-box expressed in T cells
TB	Tuberculosis
Tcm	central memory T cell
TDR-TB	Totally drug resistant TB

Teff	effector T cell
Tem	effector memory T cell
Tfh	T follicular helper cell
TGF	transforming growth factor
Th	T helper cell
TICAM	Toll-like receptor adaptor molecule
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Treg	regulatory T cell
UK	United Kingdom
UK	United Kingdom
Uns	unstimulated
VSV	vesicular stomatitis virus
WHO	World Health Organisation
XDR-TB	extensively drug resistant TB
YF-17D	yellow fever strain 17D

INTRODUCTION

1 INTRODUCTION

1.1 Tuberculosis

1.1.1 The global reach of Tuberculosis

Mycobacterium tuberculosis (*M.tb*) has been found in numerous ancient skeletal remains, indicating a relationship with humans going back millennia. Phylogenetic analyses estimate the divergence of early strains of *M.tb* over 35,000 years ago and their spread with the first human migrations out of Africa [1]. By the mid-16th century, tuberculosis is thought to have caused up to 20% of deaths in England. In 1882 however, when Robert Koch identified *M.tb* as the causative agent of tuberculosis, death rates in the UK were already decreasing and this trend continued into the 20th century, showing social determinants such as poverty and overcrowding to be strong risk factors for tuberculosis disease (reviewed in [2,3]). The first vaccine, Bacille Calmette-Guerin (BCG) was introduced in 1921 and remains the only licensed vaccine against tuberculosis. Yet 90 years later in 2011, tuberculosis ranked as the second biggest cause of death from infectious disease worldwide with 1.4 million deaths and an estimated 9 million new cases [4]. Quantifying the number of latently infected people is difficult but estimates suggest up to a third of the world's population may be harbouring the bacterium, showing this ancient "white plague" retains a global reach.

In the late 1980s, historians declared tuberculosis to be a problem of the past in the industrialised world. The following decade, however, saw cases rise considerably once more, fuelled by a number of factors including the emergence of the HIV pandemic and the rise of drug resistant strains. Poverty and geography too remain important risk factors [2].

1.1.1.1 Tuberculosis and HIV: a deadly combination

Epidemiological data shows a dramatic synergy between HIV and tuberculosis exacerbating the pandemics of these two diseases over the past two decades. Fifteen percent of new cases of active tuberculosis each year occur in the HIV+ population and tuberculosis is the leading cause of death in HIV-infected people, accounting for approximately one quarter of AIDS-related deaths [4]. There is ample evidence for the role of HIV in aggravating tuberculosis disease and studies have also implicated tuberculosis in accelerating the establishment of HIV (reviewed in [5,6]). Intense research efforts in the past two decades have led to a better understanding of the pathogenesis of both HIV and tuberculosis, the latter of which is discussed in further detail below. Understanding the interactions between the two co-infections however, both in terms of pathology and therapy, presents a distinct challenge. Immunosuppression due to loss of CD4+ T cells is the primary reason for increased susceptibility to tuberculosis in HIV-infected people however, multiple mechanisms are thought to underlie the synergistic effects of each disease on the progression of the other. Both HIV and tuberculosis are chronic diseases with powerful local and systemic effects on the host's immune system. For example, granuloma formation is necessary, though not sufficient, to control and contain tuberculosis infection but increased replication of HIV has been demonstrated locally at sites of *M.tb* infection in the lung and attributed to an increase in activated T cells, which are preferentially infected by HIV, in these areas. Death of the HIV-infected T cells in the granuloma weaken the structure, enabling spread of bacteria as the granuloma breaks down (reviewed in [5]). The role of TNF is another example of how the two diseases exacerbate each other. Whilst production of TNF by alveolar macrophages in response to *M.tb* infection is

required to control bacterial growth, TNF triggers replication at the long terminal repeat (LTR) promoter of HIV and has been shown to activate HIV replication in macrophages [7,8]. Thus control of one pathogen may lead to the spread of the other. Controlling either disease alone is a challenge, particularly in the face of synergy and drug resistance, and the burden of co-infection is largely in the developing countries of Asia and Africa, placing an increasing burden on already stretched healthcare systems.

1.1.1.2 The rise of drug resistant tuberculosis

Streptomycin, isolated in 1943, was the first drug to be effective against tuberculosis and the discovery of new drugs continued for the next 20 years, until the development of rifampicin in 1963 [9]. However, success was short-lived as the introduction of each drug was rapidly followed by the emergence of resistant strains of *M.tb*. Following a series of multi-country trials led by the British Medical Research Council (MRC), a combination treatment of four drugs was developed [10,11]; reviewed in [12]. Effective treatment and a continuing rise in living standards led to a decline of tuberculosis in the developed world, prompting widespread optimism that was accompanied by a reduction in funding for research. Meanwhile anti-tuberculous policies in the developing world were slow to be developed and later largely ignored the problem of drug-resistant tuberculosis, focusing instead on cost-effective first-line treatment for all sputum-positive patients. In the last decade of the 20th century, the global nature of drug resistance had become apparent and methods were put in place to identify and treat drug-resistant tuberculosis in resource-poor settings (reviewed in [13]). However, it had been known since the 1950s that drug resistance was not always associated with lower fitness or transmissibility rates [14] and epidemics of resistant strains appeared and have since been documented in multiple countries. Drug resistance

markedly increases the cost and duration of treatment, with the highest burden often in poor countries whose health care systems are ill equipped to deal with the problem. This has been compounded by the emergence of multi-drug resistant (MDR-TB), extensively drug resistant (XDR-TB) and, more recently, totally resistant strains (TDR-TB) [15]. The prevalence and fitness of these strains varies, however the high transmissibility and virulence of some drug resistant strains remains a major cause for concern [16]. Furthermore, it has been suggested that drug resistant strains are more likely to develop in HIV/*M.tb* co-infected individuals where immune selection forces acting on the bacteria are weaker [17]. After decades of neglect, a concerted global effort is now being made to tackle the tuberculosis pandemic. Research efforts have also re-gathered momentum in the past two decades leading to a better understanding of the pathogenesis of tuberculosis and the development of new candidate vaccines and drugs. In December 2012, the United States Food and Drug Administration (US FDA) approved the use of bedaquiline; the first new anti-tuberculous drug in 40 years [18].

1.1.2 Pathogenesis of tuberculosis

M.tb is a complex organism whose relationship with humans stretches back to the start of humanity. Many of the interactions between the two species remain poorly understood and are not explored in depth here. Instead, this section highlights some of the research that has informed the design of vaccines against tuberculosis and other more recent findings, which illustrate some of the gaps in knowledge that remain and the challenges these pose for the field.

The early stages of infection with *M.tb* have been well studied. Inhalation of the bacteria leads to infection of professional phagocytes in the lung including

macrophages and neutrophils. Multiple components of *M.tb* activate a number of pattern recognition receptors (PRRs; reviewed in [19]), including Toll-like receptors (TLRs) 2 and 9, DC-sign, the mannose receptor and Mincle [20]. The resulting inflammation and influx of innate immune cells benefits the pathogen by providing new niches in which to multiply. Additionally, several bacterially encoded mechanisms delay recognition of infected cells through modulation of intracellular trafficking pathways and promote cell-to-cell spread by delaying apoptosis and promoting necrosis of infected cells [21–23]. Bacterial growth continues largely unabated until the onset of adaptive immunity, which is delayed in *M.tb* infection. This is thought to be a result of a delay in trafficking of pathogen to local lymph nodes, due in part to inhibition of apoptosis in neutrophils and infection of myeloid dendritic cells (DCs), causing them to become unresponsive to CCR7 [24–27]. Despite this, experiments in which antigen-specific CD4+ T cells adoptively transferred to mice early in infection did not adversely affect growth or survival of the bacteria during the first week of infection suggest delayed adaptive immunity is not the sole reason for early bacterial success [28,29]. In both humans and mice, the development of adaptive responses leads to control, but in many cases not elimination, of the bacteria and establishment of the latent state as bacteria are contained within granulomas in the lung. Both host and pathogen components are involved in this step of disease progression as *M.tb* encodes several genes facilitating establishment of latency, including the "dormancy" regulon, controlled by the two-component signaling system dosR-dosS [30,31]. Furthermore, a subset of bacteria continues to replicate during this time and recent evidence shows that mutations accumulate during latency [32]. This implies dormancy is not simply a period of host domination but a dynamic equilibrium between host and pathogen whose intricacies and respective benefits remain largely mysterious.

A vaccine aiming to provide sterilizing immunity must act during these early stages. Experimental evidence points to an important role for adaptive immunity in limiting the original infection even if this is in many cases not sufficient to eliminate the bacteria. Importantly, the delay in recruitment of antigen-specific cells to the site of infection suggests induction of mucosal immunity may be a crucial component of a successful vaccine. Early inflammation and the behaviour of phagocytes also plays an important role in controlling or allowing infection, and emerging evidence that vaccination can modulate innate responses through imprinting of innate cells [33,34] means this may become an important aspect of the vaccination strategy. Finally the potential effects of vaccination on the establishment of latency present intriguing but as yet unexplored questions.

Latent tuberculosis may persist for many years but reactivation occurs in around 10% of those infected. The mechanisms leading to reactivation are in general poorly understood except for in a subset of well-defined cases. Reactivation of tuberculosis is more common in those infected with HIV or on anti-TNF therapy (reviewed in [35]). These two cases are explored below, however it is noteworthy that they apply only to a minority of tuberculosis sufferers and have become relevant only recently. Moreover, as reactivation and exit from the latent state is crucial to the survival and spread of *M.tb,* it is likely that the bacteria has also evolved mechanisms to trigger a reversal of the dormant state.

The high incidence of *M.tb* infection in the HIV-infected population indicates a crucial role for CD4+ T cells in maintaining the latent state. Depletion of CD4+ T cells late in

HIV infection leads to an increase in reactivation frequency in this population, however the risk of developing active tuberculosis is also increased early in HIV infection before levels of circulating CD4+ T cells start to decrease [36]. Furthermore, patients on anti-retroviral therapy (ART) remain more susceptible to tuberculosis than uninfected individuals, despite relative immune reconstitution [37]. Multiple mechanisms are likely to contribute, for example the observation that HIV infection leads to depletion of *M.tb* antigen-specific CD4+ cells in the periphery faster than those specific for other antigens [38]. In both cases, the precise mechanism involved and the extent to which this leads to increased reactivation frequency is unclear (reviewed in [5]). The second group in whom susceptibility to active tuberculosis is increased is those on anti-TNF therapy. Several mechanisms thought to play a role include effects on macrophages and their ability to eliminate *M.tb,* an increase in regulatory T cells and the depletion of a subset of CD8+CD45A+ effector memory T cells (reviewed in [35]). Additionally, individuals with genetic defects in IL-12, IFN-γ and STAT1 are at high risk of active tuberculosis, showing a crucial role for Th1 CD4+ T cells in the control of *M.tb* and maintenance of latency [39].

The two lines of evidence mentioned above led to the theory that a weakened immune state was responsible for reactivation. Although that is undoubtedly true, it is now thought that other mechanisms are also likely to be important in otherwise healthy individuals. Possible mechanisms involved include T cell exhaustion and altered antigen expression or cell trafficking however these models have not yet been extensively tested. It has been reported that, prior to the introduction of chemotherapy, as many as 50% of active cases of tuberculosis spontaneously resolved, with a subset of these patients thought to have cleared the infection, as evidenced by a

lack of circulating effector memory cells but detection of central memory T cell responses [40]. The mechanisms by which the bacterium reactivates and subsequently overcomes the host or is controlled or even cleared remain largely unexplained. Finally it is likely the bacterium also plays a role in reactivation and that this role is more prominent than current models suggest.

A major concern in the development of novel tuberculosis vaccines was the possibility that strong T cell responses would lead to immunopathology [41–43]. As discussed above, the host-pathogen interactions in this disease are complex and *M.tb* has evolved not only to evade but also use immune responses against it. The initial inflammation and influx of innate cells provide a niche for expanding bacterial populations in early infection and T cells are also thought to promote bacterial dissemination in the later stages of disease. T cell responses can lead to the immunopathology, tissue destruction and break-up of the granuloma underlying the highly infectious cavitatory form of tuberculosis [35]. Furthermore, a genetic analysis of known T cell epitopes in *M.tb* showed them to be highly conserved and under positive selection, suggesting T cell responses are of evolutionary benefit to the pathogen [44]. Despite these concerns, a decade of tuberculosis vaccine research has not shown any evidence that immunopathology is a problem, again highlighting the need to further understand this aspect of tuberculosis disease. It is likely that spatio-temporal as well as qualitative differences in the induction of T cell responses are important in determining their role.

There are many stages of tuberculosis disease and these are reflected in the different physiological states of the bacterium. Many of the host-pathogen interactions remain poorly understood and much of the basis of protective immunity at each stage of the

disease has yet to be elucidated. This lack of knowledge has made vaccine design difficult, however much progress has been made in the past decade and studies arising from vaccine trials will generate new insights into the complex field of tuberculosis.

1.1.3 Vaccination against tuberculosis: BCG and new vaccines

Current strategies for tuberculosis control continue to face major challenges in terms of cost, implementation and growing resistance to drugs. Developing an efficacious vaccine is therefore a global priority. When developing a new vaccine, it is important to consider the characteristics of the different groups bearing the burden of tuberculosis. Whilst an ideal vaccine would prevent infection and provide sterilizing immunity, such an approach can only be taken by vaccinating an uninfected population. In high burden areas however, latent infection establishes itself early and as many as 6% of children may have already been infected by 3 months of age [43]. A vaccine aiming to provide sterilizing immunity would therefore need to induce protective responses in very young infants. A vaccine enabling better control of infection and reducing the development of active pulmonary disease in adults would be beneficial both in reducing the burden of disease and also decreasing the rates of infection as tuberculosis is primarily transmitted by adults with active pulmonary disease. As previously mentioned, a significant subset of this latter group is co-infected with HIV and such a vaccine would need to be effective in HIV-infected individuals. The immunosuppression that results from HIV infection thus presents a particular difficulty to vaccine design in this group. A vaccine targeting disease in adults would need to remain effective even as the immune system becomes compromised. Studies have shown that a robust cellular response is required for protection against tuberculosis and that IFN-γ and TNF play major roles in protection. None of these

factors however is sufficient for protection and a correlate of protection remains an elusive goal. Nonetheless, most novel vaccines aim to induce high levels of cellular immunity by either boosting or ameliorating the current BCG vaccine [45], whose protective effects are discussed below.

BCG was first tested in patients in 1921 and has since been given to over 3.5 billion people. The vaccine is a live-attenuated strain of the related *Mycobacterium bovis,* passaged 230 times over 13 years. BCG is a replicating vaccine that induces a complex immune response in humans. It has continued to evolve over time and several strains, known collectively as BCG, are currently used. The BCG vaccine provides partial immunity to tuberculosis, which varies by geography, population and vaccine strain. A meta-analysis of prospective and retrospective efficacy trials of BCG compiled by Colditz in 1994 shows a variable efficacy of BCG against pulmonary tuberculosis of 0-80% [46]. Several factors have been proposed to explain these differences [47–49]. Genetically distinct strains of BCG induce different immune responses and different populations also respond differently to BCG vaccinations. There is evidence that this could be due to prior exposure to environmental mycobacteria, which are more prevalent in settings where BCG is known to work less well. A study comparing responses to BCG in infants from Malawi and the UK showed different immune responses to BCG are already apparent a few weeks after birth in immunologically naïve infants, suggesting the reasons for variable efficacy may be more complex [50], although evidence of exposure to environmental mycobacteria has been documented in Gambian infants as young as 4 months [51]. As the vaccine protects against severe forms of childhood tuberculosis, provides some protection from leprosy and reduces pulmonary tuberculosis in some populations [52], it is still given at birth in most endemic

countries. In addition to this, several studies in West Africa have shown non-specific protective effects of BCG reducing mortality from a range of diseases in the first year of life [53]. As BCG approaches its 100th anniversary, it is clear that there is still much to be learned about the immunity induced by BCG, how this mediates its protective effects both against *M.tb* and other pathogens, and why these effects show such large geographic variation.

The idea that vaccines may have nonspecific effects on immunity, in particular following BCG, first emerged in the 1920s. Several studies have since shown evidence of nonspecific effects of live vaccines such as BCG and measles in reducing mortality from other diseases, although controlling for confounding factors in these studies is difficult [54]. However there is increasing epidemiological and molecular evidence that BCG may "train" immunity. A recent study showed innate imprinting of monocytes following BCG vaccination may be responsible for this effect. In the study, monocytes were isolated from volunteers pre- and two weeks and three months post-vaccination and stimulated with sonicated or heat-killed *M.tb*, *S. aureus* or *C. albicans*. Production of pro-inflammatory cytokines was increased in response to all three antigens following BCG vaccination. This was independent of B or T cells but dependent on activation of NOD2 and was accompanied by a significant increase in H3K4 trimethylation in circulating cells at the level of cytokine and TLR4 promoters three months after vaccination. This study suggests a mechanism for the non-specific effects of BCG vaccination, which may be particularly potent when given to young infants whose immune systems will mature in the context of BCG inoculation [34]. A second paper has shown the importance of innate imprinting in the setting of mucosal vaccination. In this study, in a prime-boost regime inducing mucosal immunity,

boosting with either an adenovirus or vesicular stomatitis virus (VSV) induced comparable T cell responses but the former provided significantly better protection. The authors found this was due to differentially imprinted immunity. Specifically, increased IL-12 in the broncho-alveolar lavage (BAL) following adenovirus led to CD11c+ phagocytes producing more nitric oxide (NO) and IFN-γ in response to *M.tb* infection [33]. These studies suggest a hitherto underappreciated role in the plasticity of innate immune cells, the ability of vaccination to influence the behaviour of these cells in the long term and the importance of this in protection from disease. It is possible that simply measuring T cell responses to candidate vaccines is insufficient, as it does not capture important differences in the innate immune compartment.

The benefits of BCG are such that it remains an important tool in the fight against tuberculosis and most novel vaccines aim to either modify BCG to improve its efficacy or be given after BCG to boost its protective effects. Twelve novel vaccines are currently in clinical trials with several more in preclinical development.

Novel tuberculosis vaccines in development fall into two broad categories; those that aim to improve and replace BCG and those that will be given as a boost following vaccination with BCG (or one of its replacements). There are several ways BCG could be modified. Firstly, it could be made to overexpress certain antigens to generate stronger immune responses to them. This was the strategy used to make the first recombinant BCG, rBCG30, which overexpresses antigen 85B [55]. This vaccine was immunogenic in pre-clinical models and safe in a Phase I safety trial but is not currently being developed further. A second recombinant BCG, VPM 1002, expresses the *Listeria monocytogenes* (*L. monocytogenes)* lysteriolysin and has a deletion in the

UreC gene. Lysteriolysin causes perforation of the phagosomal membrane, leading to improved detection and immune responses to BCG. UreC inhibits acidification of the phagosome so its deletion improves the activity of lysteriolysin, which works optimally at a pH of 5.5. Lysteriolysin also carries a PEST sequence, causing its destruction upon entry into the cytosol [56]. It is in a Phase IIa trial. A similar approach was taken to engineer Aeras-422, which expresses perfringolysin and antigens 85A, 85B and Rv3407, however a Phase I trial of this vaccine was terminated due to reactivation of latent Varicella Zoster virus infection in two of eight volunteers receiving the high dose. It is thought this may have been due to perfringolysin's ability to act in a broad range of pH levels and its lack of a PEST sequence [57].

Efforts have also been made to develop attenuated forms of *M.tb* although these are less clinically advanced. Of these, the ΔPhoPΔ*fad* recently entered Phase I clinical trials. This strain is defective in the induction of the PhoP regulon, which contains genes involved in cell wall synthesis and virulence [58,59]. A modified strain of *M. smegmatis* is the only vaccine so far to have shown a substantial improvement in protection from *M.tb* in mice compared to BCG, even showing sterilizing immunity in one experiment. The *esx-3* locus of *M. smegmatis* was knocked out and later replaced with the *M.tb esx-3* locus. The resulting hybrid, named IKEPLUS, was unexpectedly avirulent when given to mice and furthermore, provided protection from subsequent challenge with *M.tb* [60]. Although this vaccine is still in very early phases of development, it is the most protective to date in a preclinical model.

The second strategy has been to develop subunit vaccines aimed at boosting responses induced by BCG. The most advanced of these vaccines use viral vectors such as MVA

and Ad35 expressing immunodominant antigens. MVA85A is designed to augment the T cell responses induced by BCG through expansion of antigen 85A-specific T cells, and the immune response to MVA85A has been studied using the *ex-vivo* IFN-γ ELISpot in multiple populations. This work shows the majority of the antigen-specific response to MVA85A is mediated by CD4+ T cells, peaks around 7 days after vaccination and is maintained at a level above baseline for at least 6 months [61–63]. This vaccine was recently evaluated in a Phase IIb efficacy trial in South African infants, which did not show significant improvement over BCG alone [43]. Aeras 402, an Ad35 vector expressing antigens 85A, 85B and TB10.4 also induces CD8+ T cell responses and is undergoing a Phase IIa trial in African infants [64]. MVA85A and Aeras 402 are now also being tested in combination in a phase I study (ClinicalTrials.gov Identifier: NCT01683773).

Other strategies use adjuvants to induce immune responses to fusion proteins. Hybrid 1, a fusion of antigen 85B and ESAT-6 has been tested in combination with IC31, a TLR9 agonist, or CAF01 (both Phase I). HyVac 4 in IC31 (Ag85B with TB10.4) is also in Phase I trials [65,66]. Finally the vaccine developed by GlaxoSmithKline, M72, is a fusion of Rv1196 and Rv0125 and is given with their adjuvant AS01, which triggers TLR4 and induces CD8 responses [67,68]. It is currently in a Phase IIa trial.

The above vaccines are designed to prevent tuberculosis disease. Two therapeutic vaccines, designed to enhance the effects of chemotherapy, are also under development. These include an attenuated form of *M. vaccae* which recently completed a Phase III trial [69] and RUTI; detoxified *M.tb* in liposomes, which is currently in a Phase IIa trial [4].

The list of vaccines now in the clinical and pre-clinical pipeline is a testament to the efforts of the past 20 years, at the start of which no potential vaccine existed. However the recent disappointing efficacy of the MVA85A trial is a reminder that the basis of protection remains unknown, making it difficult to select which vaccine candidates to take forward. Solving this conundrum is crucial as efficacy trials are prohibitively lengthy and expensive.

1.2 Vaccinology

1.2.1 Vaccine development and design: a historical perspective

Vaccines are the most effective life-saving medical intervention and a historical milestone in health and medicine. The earliest example of a deliberate education of the adaptive immune system to confer protection against a future risk of infection is seen in the practice of variolation, already present in China and India 1000 years ago. In 1796, modern vaccination was born when Edward Jenner began the practice of inoculating children with cowpox to protect against the devastating disease of smallpox (reviewed in [70]). Since then, the most successful vaccines have been empirically developed in the form of live-attenuated vaccines. This group of vaccines, which includes yellow fever, measles, mumps and rubella, safely mimic acute infection and confer the same lifelong immunity. A second group of vaccines, which includes toxoid, subunit, carbohydrate and conjugate vaccines, makes use of adjuvants to direct a specific immune response to a small part of a pathogen or its toxins. This group of vaccines usually provides immunity through less broad mechanisms and for a shorter timespan, requiring frequent boosting. For diseases in which natural immunity is

absent or incomplete however, making a vaccine has proved more challenging. Characteristics such as persistence, high mutation rates and a strong requirement for cellular immunity for protection have hindered efforts to develop vaccines against diseases such as tuberculosis, malaria and HIV. Two decades of research have highlighted a need for a much deeper understanding of the immunological mechanisms of vaccination and protection, how these relate to each other and by what means we may be able to manipulate them (reviewed in [71]).

Designing new, efficacious vaccines depends on an understanding of each stage of the immunological process. What is the end goal of vaccination? Which cell subsets need to be induced, what antigen should be targeted and in which compartment is the immune response required? At the other end of the process, the questions to be answered focus on the antigen delivery system: where viral vectors and adjuvants are used, how do these activate innate immunity? How do these mechanisms differ between species (as most early testing is done in preclinical animal models) and populations? Where we measure specific facets of the adaptive immune response, which relates to the type of immunity we aim to induce through vaccination, how do these relate to the innate mechanisms activated at the time of vaccination? With all vaccines, there is a spectrum in the response induced in vaccinees: what are the mechanisms that determine where on the spectrum an individual lies? To what extent does this affect protection? Having a correlate of protection helps to answer many of these questions and largely dispenses with the need for large-scale efficacy trials however for HIV, tuberculosis and malaria, no correlate of protection has yet been identified. Many of the questions formulated above have unknown or incomplete answers yet informed vaccine design requires an understanding of each of these steps and the relationships between them.

Where these processes are still poorly understood, vaccine design remains largely empirical.

1.2.2 The induction of memory responses

The ability of the immune system to remember previous encounter with pathogens serves to protect from future infections, with different cell subsets providing distinct layers of defence.

Memory B cells or plasma cells in the bone marrow continue to produce antibody in the absence of antigen and can provide lifelong immunity to extracellular pathogens or toxins. In the event of infection, proliferation of plasma cells leads to more antibody-producing cells and a transient increase in antibody serum levels. In many cases, this is enough to prevent an infection becoming established and, for this reason, serum antibody levels are a good correlate of protection for many vaccines (reviewed in [72]).

By contrast, T cells only detect pathogens once they have invaded host cells as their activation depends on peptide presentation through MHC molecules of infected cells or activated antigen-presenting cells (APCs) such as DCs and monocytes. This section discusses the process of T cell activation and differentiation into different effector and memory lineages. Understanding these processes is key to being able to modulate them through vaccination to induce protective responses to different diseases.

Activation of pattern recognition receptors on APCs by pattern- or danger-associated molecular patterns (PAMPs and DAMPs) is often the first step in the induction of an immune response. The combination of receptors activated depends upon the pathogen itself, associated tissue damage which may trigger PRR activation through the

production of DAMPs, and the type of cell activated. This combinatorial signalling leads to activation, differentiation and migration of APCs as well as differential cytokine production and increased co-stimulation. Together, these factors determine the milieu in which naïve T cells will become activated and influence their fate. In particular, the differentiation of CD4+ T helper cells into different subsets is key to determining the characteristics of the immune response that follows.

The Th1/Th2 paradigm of CD4+ T cell differentiation, which was first proposed almost 25 years ago [73], has since grown to include a number of other CD4+ T helper subsets including Th17, Tregs and, more recently Th9, Th22 and Tfh cells (reviewed in [74]). Each subset is characterized by a distinct pattern of cytokine production and is associated with expression of a "master" transcriptional regulator. Th1 cells are characterized by the expression of the transcription factor T-bet [75] and the production of IFN-γ and are crucial to the control of intracellular pathogens. Th2 cells express GATA-3, produce IL-4 and are typically associated with extracellular infections and a strong concurrent B cell response [76,77]. Th17 cells, which produce IL-17, have been associated with protection from fungal diseases but also with autoimmunity [78,79]. Tregs, which can be natural or induced –the latter are antigen-specific-, produce cytokines such as IL-10 and TGF-β that dampen immune responses [80,81]. The importance of Tfh cells in B cell maturation in germinal centres is also a hot topic of research. The cytokine milieu that prevails when naïve T cells are activated in contact with APCs determines their subsequent development pathway. For example, viruses and intracellular pathogens that activate intracellular PRRs in dendritic cells induce production of IL-12, driving the differentiation of antigen-specific CD4+ cells to IFN-γ -secreting Th1 cells [82]. By contrast, Th2 cells develop in the context of IL-4 production

by epithelial and innate cells such as mast cells and basophils [83–85]**.** *M.tb,* which activates a number of receptors including TLR2, TLR4, NOD2 and Mincle appears to induce cytokine production leading to development of both Th1 and Th17 cells (reviewed in [86–88]).

Recent work has shown that phenotypic differences are maintained in T helper cells and their progeny through distinct epigenetic marks, particularly at the loci of the associated cytokines and transcriptional regulators (reviewed in [74,89]). Both these and other studies characterising cytokine secretion have shown considerably more overlap and plasticity between T helper cell subsets than was previously thought [90,91]. For example, T cells secreting both IFN-γ and IL-17 have been described in both humans and mice and it is thought these are likely to be important in protection from *M.tb* [92–94]*.* Additionally, cytokine promoters often contain repressive epigenetic marks in cell subsets in which they are not expressed (for example *Ifng* in Th2 cells), however "master regulator" loci have either permissive or "bivalent" marks, suggesting these genes are poised for expression even in different subsets. For example, in Th1 cells, the *Tbx21* locus, which encodes the master regulator T-bet, has permissive H3K4me3 marks whilst in Th17 and Th2 cells it acquires repressive H3K27me3 but also retains the H3K4me3. These bivalent epigenetic domains are thought to be part of the reason underlying plasticity and changes in cell phenotype in CD4+ T helper cells. As more details emerge about the molecular mechanisms underlying T cell phenotypes, there will also be more opportunities to influence the differentiation of these cells towards a protective phenotype.

In addition to their characteristic cytokine production, T cells may be of a naïve or memory phenotype, and this second class may be further subdivided into effector memory (Tem) or central memory (Tcm) cells. Following an original encounter with antigen, antigen-specific B and T cells clonally expand. As the infection is cleared and antigenic stimulation ends, the pool of activated cells contracts and a subset of memory cells remain. These cells persist in the absence of antigenic stimulation, sustained by IL-15 and IL-7, which are present in distinct anatomical niches and whose concentrations determine the size of the memory pool (reviewed in [72]).

These memory cells provide two lines of defence. Circulating antibodies and Tem cells are present at the site of pathogen entry, including the blood and mucosal surfaces. These will respond immediately to infection but have a limited potential for further expansion. Tcm cells and memory B cells by contrast have high proliferative potential and, upon re-encounter with antigen, can divide, differentiate into effector cells and migrate to the site of infection. However their response is slightly delayed as these cells reside in lymphoid organs and are therefore not activated until antigen is trafficked to these sites.

An ideal vaccine would induce both effector and central memory cells, however the mechanisms underlying cell fate decisions in the development of these memory cells are not fully characterized. Microarray studies have shown only a small proportion of genes (~5%) to be differentially expressed between naïve and memory T cells despite their clear functional differences [95]. Genes that are more highly expressed in memory T cells include HLA class II receptors, chemokine receptors, adhesion molecules, intracellular signaling genes and effector molecule genes. There are also genes

involved in memory T cell survival and a number of transcriptional regulators. Even fewer genes separate Tem from Tcm cells and these include cell surface receptors, interleukin receptors and cytotoxic molecules, explaining the increased effector and migratory functions of circulating Tem cells. As in T helper cells, characterization of covalent modifications to DNA have shown the changes to the epigenome to have an important role in the establishment and maintenance of these fates. The exact mechanisms by which the fate of T cells is decided however remain unclear. It is not clear whether central and effector memory T cells develop from effector T cells that were activated at the time of primary infection or whether they are induced from naïve T cells in parallel to but separately from effector cells [72,95–97]. New technologies enabling *in vivo* lineage tracing of T cell populations at the single cell level have shown these to be highly discordant between lineages, both in terms of their initial and their recall proliferative capacity [98]. The mechanisms that govern these differences are presently unknown, however some data is emerging on how memory responses may be modulated. For example, mTor is a transcription factor known to be important in CD8+ T cell memory responses and this pathway can be specifically inhibited by the drug rapamycin. Giving rapamycin to mice at different times during CD8+ memory pool formation following vaccination can change both the magnitude and quality of CD8+ memory T cell formation [99]. This study suggests future vaccine design may be able to more specifically modulate the formation of memory cell pools.

Finally, achieving a balance between systemic and mucosal immunity is an important target for vaccination. As previously mentioned, effector memory cells provide the first line of defense at the site of pathogen entry and their anatomical location is therefore of paramount importance. How the expression pattern of homing receptors is

imprinted on activated T and B cells is not well understood, however it is thought that the original site of pathogen (or vaccine) encounter may be important. Mucosal memory also appears to wane much more rapidly than systemic immunity for reasons that are not clear [72,100]. A better understanding of the factors governing homing receptor expression on both effector and memory cells would therefore be very valuable to the field of vaccinology.

In summary, there exist many steps at which the development of an adaptive immune response may be modulated. Early signals following infection or vaccination from innate cells influence the fate of naïve T and B cells as they become activated. At the same time, or from this initial expansion of cells, a pool of memory cells also develops. Much evidence is now emerging about the role of epigenetic mechanisms in maintaining these cell fates or allowing plasticity. The development of technologies able to probe single cells will do much to further our knowledge of the kinetics of immune memory development. Inducing lasting memory through vaccination has proven difficult, particularly from non-live vaccines. Understanding the mechanisms that govern cell fate decisions in the development of memory immune responses is therefore crucial to enabling the design of vaccines that induce long-lasting immunity of the right type and at the desired anatomical sites.

1.2.3 Understanding the basis of vaccine induced immunity

Recent studies have shown that systems biology can be a powerful tool in investigating vaccine-induced immune responses. Two independent studies in 2008-2009 used the yellow fever vaccine YF-17D as a model to show the potential of systems biology approaches in characterizing immune responses to vaccination. YF-17D is a live-attenuated vaccine developed through serial passaging of a clinical isolate in the

1930s, which protects up to 80-90% of vaccinees. Both groups show that YF-17D modulates a number of innate pathways including the interferon response and genes involved in virus sensing [101,102]. These changes are accompanied by an upregulation of master transcription factors such as IRF7 and STAT1 and lead to the development of antibody response and a CD8+ T cell response. Querec et al. go on to show that the magnitude of both the antibody and T cell responses show large variation that was not explained by plasma cytokine concentrations or frequencies of activation states of different PBMC subsets at 1, 3 or 7 days post-vaccination. However, using microarray data and bioinformatics techniques, they were able to identify a reproducible gene signature able to predict high and low CD8+ T cell responses to YF-17D in two independent trials. This signature included the kinase EIF2AK4 and other genes involved in the integrated stress response. EIF2AK4 activates the integrated stress response secondary to arginine depletion and the authors speculate that induction of the stress response directs the adaptive response to vaccination in this setting.

This seminal study has led to a paradigm shift in vaccinology. Firstly, it illustrates the limitations of reductionist approaches which seek to explain adaptive responses by looking at a few facets of immunity and instead shows the power of unbiased approaches in trying to determine cause and effect relationships in complex systems. High throughput experimental approaches are valuable hypothesis generating tools; in this case the authors hypothesise that the strength of the integrated stress response following vaccination, as evidenced in particular by increased gene expression of EIF2AK4 and phosphorylation of EIF2a predicts the magnitude of the antigen-specific CD8+ T cell response in the vaccinees. Experiments to test this hypothesis are now underway. Using high throughput unbiased methodologies to generate testable

hypotheses can lead to breakthroughs in understanding the mechanisms by which a vaccine induces an immune response; a complex and multifactorial process.

1.3 Poxviruses

1.3.1 Poxviruses as pathogens and vaccines

Variola virus, the causative agent of smallpox, has killed more humans in history than all other infectious diseases combined. Along with cowpox and vaccinia, variola is a member of the *Orthopoxvirus* genus of the family *Chordopoxvirinae;* large linear double-stranded DNA viruses with genome sizes between 130 and 260kbp*.* The earliest confirmed death from variola is that of the Egyptian pharaoh Ramses V who died from the disease in 1157 BC. The virus spread out of Africa into Asia and Europe around 2000 years ago; the first incontestable account of smallpox in Europe dates from 581 A.D. By the 18th century, the disease was killing an estimated 400,000 Europeans a year. Variola has a fatality rate upwards of 30% in naïve populations and its arrival with European settlers in the New World and Australia decimated indigenous populations [70,103,104]. Death due to Variola virus is thought to be caused by a cytokine storm following systemic inflammation as no obvious organ damage was ever identified, with "toxemia" ascribed as the cause of death as late as 1962. In the absence of inflammation however, poxvirus infections can be lethal and the interferon response is crucial to virus control [105]. This represents a strong evolutionary force and perhaps explains the observation that responses to poxviruses, both as infectious agents and vaccines, are both complex and diverse.

Unlike cowpox and vaccinia, variola infects only humans and has no known zoonotic host. As the virus was eradicated over 40 years ago, much of the scientific knowledge

of disease comes from historical sources and observations made in animal models of related poxviruses. Genes involved in key functions such as transcription and viral assembly are highly conserved between poxvirus strains but much of the genome is given to immunomodulatory and host range genes, which are often specific to different viruses and determine their tropism. The tropism of poxviruses is thought to be due to a number of factors discussed below and can vary from very narrow, such as Variola virus to very broad, such as vaccinia and monkeypox viruses which are able to productively infect many mammalian species (reviewed in [105,106]).

1.3.2 Immune response to poxviruses: a tug of war

Unusually, cell entry is not thought to play a part in the tropism of poxviruses, which efficiently enter a wide array of cells. Instead, permissive infection is determined by a complex interplay between host and viral factors during viral replication and assembly. Small changes in these interactions determine successful replication or viral control and both parties have multiple angles of attack. PRRs on the cell surface and within the cell activate sentinel pathways early in infection, whilst the arsenal of immunomodulatory genes encoded by poxviruses aims to subvert the immune response. The presence or absence of such factors in a given strain can be a strong determinant of viral tropism and the immune response accompanying infection [106].

Poxviruses are sensed through a multitude of PRRs, both extracellular and intracellular. In many cases the ligand remains unclear and data comes from measurements of the innate response either following infection or in knockout models or is inferred from immunomodulatory genes encoded by poxviruses. Vaccinia virus engages a number of TLRs with particular importance placed on TLR2, which has been implicated in activation of CD8+ T cells and natural killer (NK) cells and production of

Type I interferon [107]. In another study, Type I interferon was found to be independent of TLR2 [108]. Two studies found that signaling through TLR3 worsened pathology during infection with vaccinia and another shows viral DNA sensed by TLR8 is important in activation of murine plasmacytoid DCs. Vaccinia also encodes a TLR4 inhibitor, suggesting signaling through this receptor also. By contrast, studies of MVA, an attenuated strain of vaccinia, suggest it activates TLRs 2, 6 and 9 [106,109,110]. These studies show a complex interaction with multiple TLRs that may vary even between closely related strains. Establishing the ligands in these interactions, which may either be derived from the virus or DAMPs released following host cell damage, would help clarify this area.

Both vaccinia and MVA activate MDA5, a cytosolic RIG-I like receptor sensing double-stranded (ds) RNA. Other receptors that have been linked to innate sensing of vaccinia include the inflammasome component AIM2, several cytotoxicity receptors on NK cells and type I interferons and NF-κB, which are targets of a number of virally-encoded immunomodulatory proteins [106,109,111]. Detecting poxviruses is not a problem, indeed as previously discussed an excessive immune response may be responsible for much of the pathology seen in natural infection and immunomodulatory proteins encoded by the virus may be necessary to dampen down the initial immune response to ensure viral propagation. When using poxviruses as tools in vaccine design however, the importance of understanding innate sensing of the poxvirus becomes clear. Studies have shown a role for numerous pattern recognition receptors in innate sensing of poxviruses and evolution has driven poxviruses to develop strategies to combat these. Future work must focus on identifying the ligands in each interaction and

understanding the effect that activating different receptors has both on the ability of the virus, or vaccine, to persist and on shaping the developing immune response.

Much of the work investigating poxvirus infection has been done on vaccinia, which was used as a vaccine in the smallpox eradication campaign and has a broad tropism, being permissive in most mammalian cells. MVA, which is used extensively as a vector in vaccine development, is a derivative of vaccinia.

Poxviruses encode a number of proteins targeting signal transduction in the host cell. The interferon-mediated antiviral state is crucial to virus control and maintaining the species barrier [111]. The interplay between host and virus in modulating this pathway can differentiate abortive, benign, resolved or disseminated disease. Poxviruses encode scavenging interferon receptor mimics, STAT phosphatases and inhibitors of mediators of the interferon state such as protein kinase R (PKR). All poxviruses also encode an array of apoptosis inhibitors, which may act in different ways [105,106,109]. F1L for example, which is present in both vaccinia and MVA, functions both as an inhibitor of the pro-apoptotic Bcl-2 family of proteins and an inhibitor of caspase-9 [112]. Poxviruses encode so many genes that interact with the host that deletion or duplication of any number of them can change the permissive profile of the virus and the immune response it elicits in the host.

The successful eradication of variola virus means human infection with poxviruses is now rare, although outbreaks of monkeypox infection still occur. The study of related poxviruses in their animal hosts however has increased understanding of the relationship between the host immune response to infection and the pathology of

infection. Ectromelia is a poxvirus that naturally infects mice and genetic studies have identified 4 genes associated with resistance to infection, named *Rmp1-4.* These studies found that the ability of a naïve host to mount a cell-mediated immune response including a strong Th1 type cytokine response, potent cytotoxic T lymphocytes and antibody responses was crucial to resolving infection [113,114]. A recombinant ectromelia virus encoding interleukin 4 (IL-4), which drives a Th2 response, was lethal in non-susceptible mice, indicating the importance of a cellular T cell response in clearing infection [115].

Rabbits have also proven an insightful model. Two different strains of myxoma virus naturally infect rabbits in South America and California, in both cases causing a benign infection that resolves in 10-40 days and results in long-lasting immunity. Both strains however cause severe pathology and disseminated disease in European rabbits who die within 10-12 days following infection with South American myxoma virus and 7-8 days when infected with the Californian strain. The strains also cause different pathologies, the former causing death through immunosuppression and the latter probably by septic shock due to a cytokine storm, as suspected in variola infection of humans. When the South American strain of myxoma virus was introduced in Australia to control soaring rabbit numbers, it initially decimated local rabbit populations but resistance soon developed. Resistant rabbits also had less severe symptoms following infection with the Californian strain of myxoma virus although the infection was still lethal. Subsequent sequencing of the two viruses shows they differ by a single duplication event of 5 genes in the California strain. Comparing the immunological changes over the course of infection in susceptible and resistant rabbits underscored

the importance of both an initial, controlled inflammatory reaction and a later T cell response in the resolution of myxoma infection [105].

Understanding the factors that influence the host response in natural infection helps explain the actions of poxviruses when used as vaccines. The work described above highlights several salient points. Small changes in the viral genome, particularly in immunomodulatory genes can have large effects on the host immune response. Host genetics are also an important determinant of the strength of the immune response. Understanding both is important in the design of vaccines if we want them to be successful in different populations across a broad genetic spectrum.

1.3.3 MVA as an antigen delivery system in novel vaccine strategies

Vaccinia played a pivotal role in the birth of vaccinology and remains the only successful eradication of a pathogen through mass vaccination. As the search for innovative vaccines to challenging diseases continues, the answer may once again lie in the poxvirus family. Poxviruses seem to provide the ideal vector for antigen delivery in vaccination. Their large genomes can accommodate DNA encoding sizeable antigens and they stimulate strong cellular immunity lasting decades. In addition, attenuated strains of several poxviruses can be used safely to immunize humans whilst retaining the ability to induce strong immune responses [116].

Multiple vaccine trials have used poxvirus vectors, typically expressing an immunodominant antigen or an epitope string from the pathogen of interest. These trials show an induction of CD4+ and CD8+ T cells secreting a mixture of IL2, TNF and IFN-γ in response to antigenic stimulation, often in combination (so-called polyfunctional T cells). Poxviruses also elicit high IFN-γ ELISpot responses in

vaccinees. Both the quantitative and qualitative characteristics of the induced antigen specific T cell population depend on the poxvirus vector used and, where the vaccine is given as a boost, the nature of the priming vector. Completed trials have used canarypox, fowlpox, MVA and oncolytic poxviruses with the aim of preventing or treating cancer, influenza, HIV, malaria and tuberculosis [43,61,117–121]. The results of each trial are not discussed in detail but all show a large variability in immune responses between individuals and, with the exception of the cancer field, disappointing efficacy results. Some efforts have been made to explore the links between induced immunity, host factors and protection. Studies in MVA are discussed in more detail below, with particular reference to the novel tuberculosis vaccine MVA85A.

MVA is an attenuated poxvirus strain derived by serial passaging (over 570 times) of vaccinia virus (VACV) through chicken embryo fibrobast (CEF) cells and used in the final stages of the smallpox eradication campaign due to its increased safety profile. The MVA genome was sequenced in 1998 and has been compared to the vaccinia genomes of the Copenhagen and choriollantois vaccinia Ankara (CVA) strains, the latter being the parent strain of MVA [116,122,123]. MVA has acquired six major deletions totaling 31kb or 15% of the vaccinia genome. 122/195 open reading frames (ORFs) in vaccinia are thought to contain insertion, deletions or non-synonymous mutations. These are primarily found in the terminal regions containing host-range genes, immunomodulatory genes and ankyrin-like genes however mutations are also present in a number of structural genes. Targeted deletion of the 6 major deleted regions in the parent strain of CVA does not restore an MVA-like phenotype, suggesting other smaller changes are important [124]. Compared to the parent vaccinia strain, MVA shows reduced pathogenicity and an inability to productively replicate in mammalian cells.

MVA is replication-deficient in most mammalian and all human cell types, with the block occurring late in the viral replication stages, during virion morphogenesis, in most cases. An exception is DCs, in which viral replication fails during replication of early transcripts. In addition, immature monocyte-derived DCs (MoDDCs) show high levels of apoptosis although both mature and immature cells retain the ability to present viral antigen. It has been shown however that bystander cells in infected DC cultures are responsible for the majority of the inflammatory response [125,126].

Of the five host range genes present in CVA, only two remain intact in MVA: C12L, an IL-18 binding protein, and K1L. Restoring the other three genes however does not completely abrogate the host-range restriction of MVA. Whilst K1 is essential for late viral gene replication, deletion of C12L enhances the immunogenicity of MVA [127,128]. MVA also contains deletions in a number of immunomodulatory genes including soluble receptors for type I and II interferon, TNF and CC-chemokines, although it retains a soluble IL-1β receptor. Further deletion of immunomodulatory genes remaining in MVA can enhance its immunogenicity as separate studies targeting the Il-1β receptor mimic, A35 -a virulence gene which inhibits the interaction between CD4 and MHC Class II- and the anti-apoptotic gene F1L have shown [112,129,130].

The immune response to MVA in HeLa cells and PBMC from blood donors has been characterised by microarray. These results show a strong induction of the immune response including upregulation of interleukin genes such as IL-1A, IL-6, IL-7, IL-8 and IL-15, induction of the NF-κB pathway and an increase in transcription of histone family members [131,132]. The absence of many poxviral genes whose function is to suppress the immune response is thought to underlie the high immunogenicity of MVA

despite its inability to replicate, making it an attractive candidate for future vaccination strategies.

The novel tuberculosis vaccine MVA85A is considered as a specific example of an MVA-vectored vaccine under development. In human clinical trials, MVA85A has been shown to elicit modest T cell responses to antigen 85A epitopes in mycobacterially naïve volunteers and potent T cell responses in BCG-vaccinated volunteers [61]. It is designed to boost memory T cell responses induced after vaccination with BCG to improve protection, which it does in mice, cattle and non-human primate models [93,133,134]. Responses to antigen 85A peptide pools following BCG prime-MVA85A boost vaccine regimes are high and dominated by CD4+ T cells; in a recent trial, a median of 6493 spot forming cells/million PBMC (range 4896-7152) at peak was measured by IFN-γ ELISpot [135]. MVA85A has been given to adults latently infected with *M.tb* as well as HIV-infected adults and shown to be safe and immunogenic in these populations too [136,137].

T cell responses to MVA85A have been measured in African adults, adolescents and infants and, although high, are lower than those seen in UK adults [63,138,139]. A non-interference study looking at the effects of concurrent EPI vaccination on immunogenicity in Gambian infants also showed T cell responses to MVA85A were decreased when the vaccine was administered with other childhood vaccinations and it is thought this is due to the alum-based adjuvants in other childhood vaccines promoting antibody-based responses [140]. MVA85A induces polyfunctional T cells secreting a combination of IL-2, TNF and IFN-γ. Th17 cells, which peak 2 weeks post vaccination, are also induced [93]. It has been administered to human volunteers

intramuscularly, intradermally and by inhalation through a nebulizer and is safe and immunogenic in each case ([135], Satti et al., in preparation).

A study evaluating the efficacy of BCG-MVA85A compared to BCG alone in South African infants was recently completed and did not show a significant difference in preventing disease between the two groups. However frequencies of antigen-specific Th1 cells in the infants, measured by flow cytometry and *ex-vivo* IFN-γ ELISpot, were much lower than those seen in adults, and it is unclear whether the response to vaccination in this group was simply not strong enough to ensure protection in such a high-transmission setting [43,141].

These studies provide a framework for continuing research to understand the molecular mechanism by which MVA-vectored vaccines elicit an immune response. Understanding the basis of vaccine-induced immunity will enable the design of better vaccines to induce protective immunity in different populations.

1.4 Factors affecting responses to vaccination

The human immune system is a complex network of redundant and pleiotropic factors, which have evolved to recognize danger and respond in a balanced way, eliminating pathogens and cancerous cells whilst avoiding autoimmunity and toxic shock. With this in mind, it is perhaps unsurprising that manipulating the immune system through vaccination can achieve such a spectrum of effects. Many vaccines are successful by achieving sufficient immunity at the population level, with any individuals unresponsive to vaccinations protected by herd immunity [142]. Other vaccines are sufficiently potent that even low responders will acquire an immune response that is

sufficient for protection. As we continue to seek effective vaccines to more complex diseases, understanding the differences underlying variation in response to vaccination between individuals and populations becomes increasingly important.

Factors affecting immune responses to vaccination include age, genetics, epigenetic mechanisms, nutritional status, maternal factors and environmental considerations, including exposure to and prevalence of other infectious diseases (reviewed in [143]). Some examples of studies showing how these factors affect response to vaccination are discussed below.

The majority of vaccinations are given in infancy as part of the World health Organisation's (WHO) extended programme of immunization schedule (EPI). Infants under five years of age remain most at risk from infectious diseases and it is this population that many vaccines aim to protect. Early decisions on which vaccines to take forward are made on the basis of their performance in pre-clinical models and adults however the differences in the immune systems of adults and infants have not been fully characterized. Infants have immature immune systems, which undergo substantial changes in the first hours, days and months of their lives. For example, it has been shown that infants have higher numbers of circulating Tregs than adults and that the predominant homing receptor on infant Tregs is the gut homing receptor $\alpha_4\beta_7$. By 18 months of age, infant Tregs have switched to primarily expressing the homing receptor CCR4, which also predominates in adults, and have also started the switch towards a more memory-associated phenotype [144]. Infants also have much higher levels of leukocytes per volume of blood than adults and the implications of this in vaccination and immunity remain unclear. Furthermore, maternal antibodies provide

essential protection from infectious diseases in young infants however these antibodies may also attenuate responses to vaccination, if vaccines are given too early. Vaccine trials in infants have found them to make much weaker responses to vaccination; whether this is due to maternal effects or a more regulatory or immature immune system is largely unknown; it is also possible that the way responses are compared is not meaningful due to the differences in leukocyte density between adults and infants [145].

Weaker responses to vaccines have been reported between populations, notably when comparing UK and African adults. This could be due to genetics; polymorphisms in different TLR genes have been associated with altered responses to BCG and measles vaccines [146,147]. Hyporesponsiveness to a Borrelia vaccine was also associated with low cell-surface expression of TLR1 [148]. Environmental factors are also likely to play a role. Infections are known to affect immune responses; parasitic worm infections, common in much of the tropical world, can skew immune responses towards a Th2 bias whilst vaccinating the immune-compromised HIV+ population presents particular challenges. Similarly, vaccine formulations and combinations can affect the immune response to vaccination [140,149]. In the context of BCG, exposure to environmental mycobacteria may be a reason for lower efficacy of BCG in tropical countries although differences in responses from UK and Malawi infants in very early life suggest other environmental and genetic factors also play a role [50]. Influences such as nutritional status can also affect responses to infection: Vitamin D, iron, vitamin A and obesity are all known to have immunomodulatory effects. Finally, the importance of epigenetic mechanisms in immunology has been characterized in several plants and animals and evidence for it is now also emerging in humans. As previously discussed, “trained” immunity may

change the way we think about vaccination and the different roles of innate and adaptive immunity in the coming years.

The many factors affecting immune responses make it hard to ascribe cause or effect even where correlations are found. Controlling for all these factors in trials and studies is difficult and analyzing efficacy and immunogenicity across populations can mask important differences in undefined subpopulations. Even when reasons are uncovered that explain variation in response to vaccination, such as EIF2AK4 in YF-17D vaccination, the underlying cause may still take a long time to uncover. Nevertheless, understanding the interactions between these factors and the immune responses induced by different vaccine remains an important goal in vaccinology. In the future, understanding the factors linking vaccination, immunity and protection at the molecular level may lead to the ability to predict the success and failure of vaccines at an individual level, enabling more rapid design and testing of vaccines and targeted deployment of successful candidates.

1.5 Aims of the project

Previous studies looking at immune responses to BCG and MVA85A have characterised the kinetics of the adaptive response and the variability associated with the magnitude of this response within and between populations. In this project, the principal aim was to characterise the changes in gene expression during early time points following vaccination in different populations and relate these changes to differences in immunogenicity. More specifically, the following aims were established over the course of the project:

a) To characterize the immune response to MVA85A vaccination by looking for changes in the transcriptome shortly after vaccination in UK adults.

b) To investigate these changes to further our understanding of the molecular mechanisms underlying the immunogenicity of MVA85A.

c) To characterise the changes in the transcriptome following vaccination with BCG and relate changes in gene expression with ability to control mycobacterial growth in a human *in-vivo* BCG challenge model

d) To characterise the response to vaccination with BCG and MVA85A in South African infants and link these findings with results from other immunological assays

e) To compare the factors affecting the immune response to vaccination in South African infants and adults in the UK to better understand the differences between these populations.

1.6 Experimental approaches

Clinical samples are stored during all vaccine trials for subsequent exploratory immunology analysis. These provide a valuable resource for studying the development of the immune response in humans. As so much remains unknown in this field, we selected an unbiased approach to describe the early events following vaccination. DNA microarrays have therefore been used in many of the following experiments in an attempt to first describe the events that follow vaccination. In the case where a link was made between early events and antigen-specific immune responses at the time of the trial, further experiments were undertaken. These experiments used more targeted approaches including quantitative PCR (qPCR), multi-parameter flow cytometry and the use of mouse models.

1.6.1 Trials from which samples in this thesis were taken

Table 1.6.1 Table of trials from which samples discussed in this thesis were taken.

Trial	Purpose	n*	Age	Location	Dose (pfu)	Results chapter
TB009	Dose escalation	10	Adults	UK	$1x10^8$	4
TB020	Phase IIb efficacy	100	Infants	RSA	$1x10^8$	6
TB022	Route comparison	2 x 12	Adults	UK	$1x10^8$	3
TB023	BCG challenge trial	2 x 12	Adults	UK	$1x10^8$	5

* samples used in this thesis; may be a subset of the total number of volunteers enrolled in the trial.

The full title and identification number of each trial is listed below:

TB009: Effect of vaccine dose on the safety and immunogenicity of a candidate TB vaccine MVA85A in BCG-vaccinated UK adults [62]

ClinicalTrials.gov ID: NCT00465465

TB020: Safety and efficacy of MVA85A, a new tuberculosis vaccine in infants previously vaccinated with BCG: a randomized, placebo-controlled phase 2b trial [43]

ClinicalTrials.gov ID: NCT00953927

TB022: Comparing the safety and immunogenicity of a candidate TB vaccine MVA85A administered by intramuscular and intradermal delivery [135]

ClinicalTrials.gov ID: NCT01181856

TB023: Evaluation of a human BCG challenge model to assess anti-mycobacterial immunity induced by BCG and a candidate TB vaccine, MVA85A, alone and in combination (Harris et al., submitted)

ClinicalTrials.gov ID: NCT01194180

MATERIALS AND METHODS

2 MATERIALS AND METHODS

2.1 Table of Reagents

Reagent	Supplier	Product Code
4-20% Precise protein gel	Fisher Scientific UK Ltd	PN25224
50bp DNA ladder	New England Biolabs (UK) Ltd	N3236S
AF647 anti-human foxp3	Biolegend	320013
AF700 anti-human CD3	Biolegend	300324
AF700 anti-human CD4	Biolegend	317426
Agilent 6000 pico kit	Agilent	5067-1613
Alexa Fluor 700 anti-human CD3	eBioscience	56-0038-41
Alkaline Phosphatase-AffiniPure Donkey Anti-Mouse Ig	Stratech Scientific Limited	715-055-150
AlumaSeal(TM) CS Films for cold storage	Sigma Aldrich Company Ltd.	Z722642-50EA
Amicon Ultra-0.5 10kDA filters	Millipore UK	UFC501008
anti-human HMGB1 antibody	Biolegend	651402
anti-mouse TLR2 antibody Rat IgG2a	R&D Systems Europe	MAB1530
anti-tlr 1 neutralising antibody	Invivogen Europe	pab-hstlr1
anti-tlr 2 neutralising antibody	Invivogen Europe	pab-hstlr2
anti-tlr 6 neutralising antibody	Invivogen Europe	pab-hstlr6
APC anti-human CD16	Biolegend	302012
APC anti-human CD4	Biolegend	317416
APC-AF780 anti-human CD8	eBioscience	9047-0087-120
APC-Cy7 anti-human CD25	Biolegend	302614
BCG Vaccine	Statens Serum Institute	
Benzonase	Merck Biosciences Ltd.	70664-3
Blotting paper Whatman 3MM 200 x 200	Fisher Scientific UK Ltd	CJF-500-150K
Bovine Serum Albumin	Sigma Aldrich Company Ltd.	A3059
ColorPlus Prestained Protein Ladder, Broad Range (10-230 kDa)	New England Biolabs (UK) Ltd	P7711S
Compbeads	Beckton Dickinson Ltd.	552843
Cytofix/Cytoperm	Beckton Dickinson Ltd.	554714
Dimethylsulfoxide	Sigma Aldrich Company Ltd.	D2650-100ML
Dneasy Blood and Tissue kit	Qiagen	69504
Ethanol 200 proof (absolute) for Molecular Biology	Sigma Aldrich Company Ltd.	E7023-500ML
Fetal Calf Serum	Sigma Aldrich Company Ltd.	F2442
FITC anti-human CD39	Biolegend	328206

FITC anti-human CD56	Biolegend	318303
FrameStar PCR plates	4titude	4ti-0952
Illumina HumanHT-12 v4.0 Expression BeadChip Kit	Illumina Inc.	BD 103 0604
Illumina TotalPrep-96 RNA Amplification Kit	Ambion	4393543
L-Glutamine	Sigma Aldrich Company Ltd.	I0634
Live/Dead Fixable red dead cell stain	Invitrogen	L23102
Live/Dead Fixable yellow dead cell stain	Invitrogen	L34955
MinElute PCR purification kit	Qiagen	28004
Omniscript RT kit	Qiagen	205111
pAb control	Invivogen Europe	pab-sctr
Paxgene Blood RNA kit	Qiagen	762174
PB anti-human CD19	Biolegend	302224
PE anti-human CD152 (CTLA4)	Biolegend	349905
PE anti-human TLR1	eBioscience	12-9911-42
PE-Cy7 anti-human CD14	eBioscience	25-0149-42
PE-Cy7 anti-human CD28	Biolegend	302925
Penicillin/Streptomycin	Sigma Aldrich Company Ltd.	P0781
PerCP-Cy5.5 anti-human CD5	Biolegend	300619
Phosphate Buffered Saline (PBS)	Sigma Aldrich Company Ltd.	D8537
Phusion high fidelity PCR mastermix	Fisher Scientific UK Ltd	FZF-531L
PstI-HF	New England Biolabs (UK) Ltd	1741041
Purified Protein Derivative *M.tb* (PPD-T)	Statens Serum Institute	2390
QDot 605 anti-human CD3	Invitrogen Ltd.	q10054
QDot 655 anti-human CD14	Invitrogen Ltd.	q10056
Quantitect SYBR Green PCR kit	Qiagen	204143
rat anti-human tlr4	Invivogen Europe	pab-hstlr4
RAt IgG2B isotype control	R&D Systems Europe	MAB0061
RNA 6000 Nano kit	Agilent	5067-1511
RNA 6000 Nano ladder	Agilent	5067-1529
RNA 6000 Pico kit	Agilent	5067-1513
RNase-free DNase Set	Qiagen	79254
Rneasy Mini Kit	Qiagen	74106
RPMI 1640	Sigma Aldrich Company Ltd.	R0883-500ML
Sodium azide	Sigma Aldrich Company Ltd.	S2002
Trans-blot turbo Transfer Pack	Bio-Rad Laboratories Ltd	170-4156
Tween80	Beckton Dickinson Ltd.	231181

2.2 Table of Solutions

Solution	Recipe
ACK	8.29g NaCl (0.15M) + 1g $KHCO_3$ (1mM) + 37.2g Na_2EDTA in 800mL dH_2O. pH adjusted to 7.2-7.4 with 1M HCl then made up to 1L with dH_2O.
FACS Buffer	PBS + 1%FCS
Freezing mix	90% FBS + 10% DMSO
R0 Medium	RPMI 1640 + 2mM L-Glutamine + 100U/mL Penicillin + 100µg/mL Streptomycin sulphate
R10 medium	RPMI 1640 + 10% FBS + 2mM L-Glutamine + 100U/mL Penicillin + 100µg/mL Streptomycin sulphate
RLT + β-ME	RLT buffer + 1% β-mercaptoethanol

2.3 Human Samples

2.3.1 Clinical Trials

Human samples used in the experiments detailed in this thesis were taken from a number of clinical trials conducted in Oxford, UK and South Africa. Samples were stored for exploratory analysis as part of the trials. The trials were registered on the clinical trials database (ID numbers below). All trials were fully approved by the ethical and regulatory agencies (as appropriate; Oxfordshire Research Ethics Committee, the UK Medicines and Healthcare products Regulatory Agency, the University of Cape Town Faculty of Health Sciences Human Research Ethics Committee, Oxford University Tropical Research Ethics Committee, and the Medicines Control Council of South Africa). Full written consent was obtained from each volunteer or their parent or legal guardian as appropriate prior to enrollment in the trials, which were conducted according to the principles of the Declaration of Helsinki and in accordance with Good Clinical Practice (GCP). Storage of samples for exploratory immunological analyses was fully ethically approved. The trials and

associated primary papers, which include detailed methods of cell separation, cryopreservation and primary immunological assays, are listed below.

TB009: Effect of vaccine dose on the safety and immunogenicity of a candidate TB vaccine MVA85A in BCG-vaccinated UK adults [62]

ClinicalTrials.gov ID: NCT00465465

TB020: Safety and efficacy of MVA85A, a new tuberculosis vaccine in infants previously vaccinated with BCG: a randomized, placebo-controlled phase 2b trial [43]

ClinicalTrials.gov ID: NCT00953927

TB022: Comparing the safety and immunogenicity of a candidate TB vaccine MVA85A administered by intramuscular and intradermal delivery [135]

ClinicalTrials.gov ID: NCT01181856

TB023: Evaluation of a human BCG challenge model to assess anti-mycobacterial immunity induced by BCG and a candidate TB vaccine, MVA85A, alone and in combination (Harris et al., submitted)

ClinicalTrials.gov ID: NCT01194180

2.3.2 Samples from non-clinical trials

In some studies, non-clinical trial samples were used. Blood was collected from Jenner Institute personnel and PBMC isolated from whole blood using Leucosep tubes with Lymphoprep providing a density gradient for separation. These were cryopreserved in liquid nitrogen in aliquots of 5×10^6 cells in Freezing Mix.

2.4 Sample Processing

2.4.1 Ex vivo IFN-γ ELISpot assay

This assay was performed by other members of the group during clinical trials on fresh samples and is shown here for reference only as results of this assay were used in this thesis.

PBMC were isolated from whole blood using Leucosep tubes (Greiner Bio-One), with LymphoPrepTM (Axis-Shield) providing a density gradient for separation. IFN-γ ELISpot assays were per- formed on freshly isolated PBMC from all 24 subjects at screening and at 1, 2, 4, 12 and 24 weeks post-vaccination, using the Human IFN-γ ELISpot (ALP) kit (Mabtech). 3 ×105 PBMC, suspended in 80 µl media, were added to each ELISpot well. Twenty microlitres of anti- gen was added to duplicate wells to give a total volume of 100µl per well. The antigens used were a single pool of Ag85A peptides, consisting of 66 15mers, overlapping by 10 amino acids, (2µg/ml each peptide). The 66 peptides were also split into 7 pools (A–G) of 9 or 10 peptides (10µg/ml each peptide). Responses to purified pro- tein derivative (PPD) from M. tb (SSI) were also assessed at each time-point (20µg/ml). Staphylococcus enterotoxin B (SEB) and phytohemagglutinin (PHA) (Sigma) were used as positive controls (10µg/ml). Unstimulated PBMC were used as a measure of back- ground IFN-γ production. ELISpot plates were incubated for 18–20 h at 37◦C, 5% CO2, before being developed. Spots were counted on an ELISpot reader (AID Germany, software version 5.0). Results are reported as spot-forming cells (SFC) per million PBMC, calculated by subtracting the mean count of the unstimulated PBMC from the mean count of duplicate antigen wells and correcting for number of PBMC in the well. Responses

were considered positive if the count was twice (but at least 5 spots greater) than that of the unstimulated wells.

2.4.2 Cell thawing

Samples were retrieved from long-term storage in liquid nitrogen, flash-thawed at 37°C and transferred to 9mL of R10 media. Cells were washed once and rested for two hours in R10 with 2µL/mL Benzonase at 37C, 5% CO_2. Cells were resuspended in 10mL R10 and counted using a CASY counter (Schärfe System, GmbH).

2.4.3 Stimulation of PBMC

Six-hour stimulations

$2x10^6$ cells for each condition were stimulated for six hours with either R10 media alone or containing Ag85A (20 µg/mL), wild type MVA or MVA85A both at an MOI of 1 (MOI of 5 for the blocking experiments). The stimulation volume was 200µL. In the TLR blocking experiments, 20µg/mL of the following antibodies were added to the medium as indicated. All antibodies were neutralizing polyclonal rat IgG from invivoGen; TLR1 (pab-hstlr1), TLR2 (pab-hstlr2), TLR6 (pab-hstlr1) and isotype control (pab-sctr). Both MVA and MVA85A were made in-house by the Vector Core at the Jenner Institute.

Twelve-hour stimulations

1-$2x10^6$ cells for each condition were stimulated for twelve hours with either R10 media alone or containing antigen 85A 66-peptide pool (final concentration of 2ug/mL for each peptide) or 1 x 10^6cfu BCG.

2.4.4 RNA extraction

Cells were pelleted, lysed in 350µL RLT buffer containing 10µL/mL β-mercaptoethanol and stored at -20°C. The RNA was extracted using the Qiagen RNeasy mini kit according to manufacturer's instructions and stored at -80°C.

For the PAXgene samples, PAXgene tubes were removed from storage at -20C, thawed and the RNA extracted with the PreAnalytix PAXgene Blood RNA kit according to manufacturer's instructions.

For the TB020 day 1 heelprick samples, 50-500µL whole blood was dropped from the heel directly into RLT buffer and stored at -80C. The samples were thawed at room temperature and the RNA extracted with the Qiagen RNeasy Mini kit according to manufacturer's instructions with the following modifications: RNA was precipitated using 80% EtOH (rather than 70%) and an extra wash with 350µL RW1 buffer was performed.

2.4.5 Microarray

The RNA was amplified using the Illumina Totalprep RNA Amplification Kit, according to manufacturer's instruction. The quality of the RNA was checked on an Agilent bioanalyser following extraction and again following amplification using the RNA Pico (for the total RNA) or Nano (for amplified RNA) kits. Amplified RNA (750ng per array) was hybridized to the Illumina HumanHT-12 version 4 expression beadchip according to manufacturer's instructions. Arrays were scanned with an Illumina bead array reader confocal scanner.

2.4.6 RT-PCR

RNA was reverse transcribed to cDNA using oligo-dT and the Omniscript reverse transcription kit (Qiagen). cDNA was stored at -20°C until qPCR was performed, using the Roche LightCycler® 480 and Quantitect mastermix (Qiagen). Quantified, purified and diluted PCR product was used to generate internal standard curves for each primer pair (table 3). Cycle number values were converted to copy number using these curves. Cycling conditions consisted of an initial activation step of 15 minute at 95°C followed by 45 cycles of 15 seconds at 94°C, 20 seconds at 60 °C and 15 seconds at 72 °C, for each primer pair. HPRT was used as the housekeeping gene for normalization. Primer sequences are shown in Table 2.4.1:

Table 2.4.1 qPCR Primer sequences

Gene	Forward primer	Reverse primer
GAPT	CCTGCCAAACTTATCCTTCCTTCACAGC	ATGCGGGTTTGGAGTGATTCAGGA
ZMYND15	TCCTGAATCACTCCAAACCCGCAT	GTGATGTTCTAGGCATTTGGGCAG
MGST1	TTGGCCTCCTGTATTCCTTGAGTG	TGGTAGATCCGTGCTCCGACAAAT
NPL	CAACTCTCAGTCATTCATTTCACAGAT	AGGATTAGGAACCAGAGACCGAGA
CD300LF	AAGAGGCCAGAAGGTCAAAGAGGT	TTTCAATGGGCTCAAGTCAAGGCG
FPR3	TGAGTCATTCCAGGATGAGTGGCT	ACACCCACAGTGGCCTCATTATCT
TLR1	AACCCATTCCGCAGTACTCCATTC	ATGGCTGCCCTTAAGTTAGCCCAA
TICAM2	AGTACCGGGATCTGCACACATCTT	AGGCTTGACTTACTTGCATGCTCC
CD14	AACTCCCTCAATCTGTCGTTCGCT	TTCAGTCTGTTGCAGCTGAGATCG
TLR6	ATATGGCTTCATGGCAGCAAGGGA	AATATGATTCACAGGGCACTCCGAGG
CXCL2	TAGCCACACTCAAGAATGGGCAGA	ACAGCCACCAATAAGCTTCCTCCT

2.4.7 Genotyping experiment

DNA was extracted from PBMC using DNeasy Blood and Tissue Kit, according to manufacturer's instructions. The region of interest was amplified by PCR using Phusion polymerase and 10uM of forward and reverse primer. The product was purified on a Qiagen MinElute column and digested with the restriction enzyme PstI

before being run on a 1% agarose gel. Genotype was determined from the number and size of DNA bands present.

Primer sequences were taken from [150]:

Forward: CTTGATCTTCACAGCAATAAAATAAAGAGCATTCC

Reverse: GGCCATGATACACTAGAACA CACATCACT

2.4.8 Flow cytometry

Cells were sedimented at 300x*g* for 5 minutes and washed twice with phosphate buffered saline (PBS). Cells were then stained with 10µL of a 1:40 dilution of live/dead stain for 10 minutes on ice. 45µL staining cocktail was added and cells incubated on ice for 30 minutes. Cells were washed twice with 150µL FACS buffer. Cells with surface staining only were resuspended in 50µL FACS buffer and run on an LSRII flow cytometer at this point. In experiments with an intracellular FoxP3 stain, cells were incubated for 20 minutes in 150µL Fixation/Permeabilisation solution then washed twice in 150µL Perm/Wash buffer diluted 1:9 with water. The intracellular staining cocktail (45µL) was then added and cells incubated a further 30 minutes on ice. Two washes were performed with Perm/Wash as above, then cells re-suspended in 50µL FACS buffer, transferred to cluster tubes and analysed on an LSRII flow cytometer. Antibodies included in the staining mix for each experiment are shown below in Table 2.4.2. The exact antibody used is given in the reagents table in the appendix.

Table 2.4.2 Antibody cocktail mixes for individual flow experiments

TB022 (TLR1)		TB022 (Tregs)		TB020	
Marker	Colour	Marker	Colour	Marker	Colour
Live/Dead	PB	Live/Dead	PB	Live/Dead	PE-TR
CD14	QD650	CD5	PerCP-Cy5.5	CD16	AF488
CD19	PB	CD25	APC-Cy7	CTLA4	PE
CD4	APC	CD39	FITC	CD19	PE-Cy5
CD3	AF700	CTLA4	PE	CD14	PE-Cy7
CD8	APC-AF780	CD28	PE-Cy7	CD4	PB
TLR1	PE	FoxP3	AF647	CD8	QD605
		CD4	AF700	CD127	QD655
		CD3	QD605	gd-TCR	APC
		CD19	PB	CD3	AF700
		CD14	QD650	CD25	APC-Cy7

2.4.9 Western blot

Stimulations with MVAwt were stopped after 0 minutes, 30 minutes and 1, 2, 4 and 6 hours and the supernatants stored at -20 ^{o}C. They were then thawed and concentrated for 20 minutes in Amicon Ultra-0.5 10kDA filters (Millipore UK) according to the manufacturer's instructions. The supernatant was mixed 1:1 with loading dye, run 1 hour at 100 Volts on a 4-20% precise protein gel (Thermo Scientific Pierce) and transferred to a membrane (Trans-Blot Turbo Transfer Pack, Bio-Rad Laboratories Ltd) by Turbo blotting for 7 minutes. The membrane was incubated sequentially for 1 hour with PBS with 3% Bovine Serum Albumin (BSA) alone, primary antibody (mouse anti-human HMGB1, Biolegend, UK) and finally secondary antibody (Alkaline Phosphatase-AffiniPure Donkey Anti-Mouse IgG, Stratech Scientific Limited). The membrane was washed and developed. Unfortunately a good loading control for western blots using cell supernatants is unavailable [151,152].

2.5 Data Analysis

2.5.1 Microarray

Raw Illumina probe data was exported from Beadstudio and screened for quality. Gene expression data was analysed using the bioconductor platform in R [153]. Genes not expressed above background levels in any sample were removed ($p<0.05$). In limma [154,155], background correction and quantile normalization were performed using the neqc function [156]. Probes with an interquartile intensity range <0.3 (log2 transformed) across all samples were filtered using bioconductor's genefilter package.

The following R and bioconductor packages were used in the analysis:

Table 2.5.1 Table of R and Bioconductor packages used in the microarray analyses

Package	**Use**	**Reference**
Bioconductor		
Limma	Background correction, normalisation, quality control, differential expression analysis	154–157
arrayQualityMetrics	Quality control	158
Betr	Differential expression analysis	159
Heatplus	Heatmap drawing	
Genefilter	Filtering of genes	
R		
pamR	Identification of classifier genes	160
Cluster	Cluster analysis	
MASS	Linear discriminant analysis	
glmnet	Linear models with elastic net	161

Quality control was performed using the bioconductor package arrayQuality Metrics. This package produces a report to compare metrics between arrays within an experiment. The report enables comparison between arrays using plots of distances between arrays and principal component analysis. Measures of array intensity

distribution are also plotted as boxplots. Outliers are identified for each measure, aiding the identification of samples that should be excluded. In addition, I used dendrograms and the function for multi dimensional scaling plots in the limma package to visualize the underlying structure of the data and identify potential technical artefacts or grouping of the data. Where appropriate, these plots are shown in the results chapters.

Identification of differentially expressed genes was performed using the package limma, which uses linear models and an empirical Bayes approach to identify differentially expressed genes between groups of samples. These sets of genes were analysed using a number of tools describe below. The package betr, which I used to analyse the TB023 data presented in chapter 5, uses a similar approach applied to time course data.

Pathway analysis was performed using two web-based tools; DAVID (Database for Annotation, Visualisation and Integrated Discovery) and GSEA (Gene Set Enrichment Analysis) [162,163]. These two methods analyse upregulated genes by grouping them into pathways and transcriptional modules, which allows greater analytical power and biological insight. Whilst DAVID relies on published pathways and gene ontology databases to generate results, GSEA uses published gene sets. In GSEA, lists of differentially expressed genes were ranked by fold-change in expression and used in the preranked analysis. I systematically tested gene sets from the Molecular signature Database (MsigDB, http://www.broad.mit.edu/gsea/msigdb) C2 collection collected from various sources such as online pathway databases and publications in PubMed.

In order to identify associations between gene expression and different outcome measures, the packages pamR and glmnet were used. In the TB022 analysis, pamR was used to identify genes that could classify volunteers as high or low responders based on their *ex-vivo* IFN-γ ELISpot response measured during the trial. In the TB020 pilot study hierarchical clustering methods were used to group genes with similar patterns of expression. The expression values for each gene were then standardized across the volunteers and the average calculated for each cluster. I then used the package glmnet to look for associations between clusters and response variables, for examples IFN-γ ELISpot responses to Ag85A at day 28, similar to a previously described method [164,165].

2.5.2 RT-PCR

The mean *C*t value of duplicate capillaries was converted to copy number using internal standard curves generated from purified and quantified PCR product. Expression levels for each gene were normalised by dividing copy number of gene by copy number of the housekeeping gene HPRT.

2.5.3 Other statistical analyses

GraphPad Prism was used to perform statistical analyses including the Student's t-test, area under the curve analyses, Spearman rank correlations and Mann-Witney tests as detailed in the Results chapters.

2.6 Mouse experiments

All experiments were performed using 6- to 8-wk-old female BALB/c mice (Harlan Orlac). All procedures were carried out under the terms of the UK Animals (Scientific Procedures) Act Home Office Project License (UK Home Office PPL 30/2412) and were

approved by the University of Oxford Animal Care and Ethical Review Committee. Mice were vaccinated intradermally (ID) in each ear (25 µl/ear). Groups of 5 mice were vaccinated with 5×10^5 plaque forming units (pfu) of MVA85A in PBS alone or also containing 20µg of anti-TLR2 antibody (MAB 1530, R&D Systems) or an isotype control. Mice were culled 7 days later and ammonium chloride lysis buffer (ACK)-treated splenocytes used in an *ex-vivo* IFN-γ ELISpot assay. The cells were stimulated with either media alone, PHA and PMA or antigen 85A 66-peptide pool as previously described [166].

RESULTS

3 CHARACTERISING THE EARLY IMMUNE RESPONSE TO MVA85A VACCINATION

Aims:

To characterize the immune response to MVA85A vaccination by looking for changes in the transcriptome in the blood shortly after vaccination.

To utilize and investigate these changes in gene transcription pattern between time points and volunteer groups to further our understanding of the mechanisms underlying the cellular immunogenicity of MVA85A.

3.1 Introduction

The immune system is a complex network of interconnected genes, which are amongst the most highly selected in the human genome. It is perhaps unsurprising therefore that the immune response to most vaccines is so spectacularly variable, both in terms of initial innate reaction and number of antigen-specific cells or antibody titres induced by vaccination. Responses to MVA85A show great variation both within and between populations, as shown in Figure 3.1.1a where the immune responses of 24 volunteers from a recent UK trial are plotted in order of decreasing magnitude. Investigating the underlying factors that determine this range was one of the main aims of this thesis. In these first experiments, I used an unbiased high-throughput approach to characterize the innate immune response to MVA85A and look for associations between the early events following vaccination and the antigen-specific responses measured throughout the trial.

MVA85A is designed to augment the T cell responses induced by BCG through expansion of Ag85A-specific T cells. Previous work has characterized aspects of the immune response to vaccination in the 14 days following vaccination (Figure 3.1.1b, David Porter, unpublished). These show the antigen-specific immune response, as measured by IFN-γ ELISpot to Ag85A peptides, is first detected four days post vaccination and peaks 7 days post-vaccination. Plasma IFN-γ and erythema and swelling at the site of vaccination also show a peak at this time. These last 3 measures show an additional peak two days post vaccination, suggesting this is the peak of the innate immune response to the vaccine.

In the experiments presented here, I performed DNA microarrays on unstimulated PBMC collected during a recent Phase I safety and immunogenicity trial conducted in Oxford in which 24 volunteers with a prior history of BCG were vaccinated with MVA85A. Further details of the trial and experiments are given below.

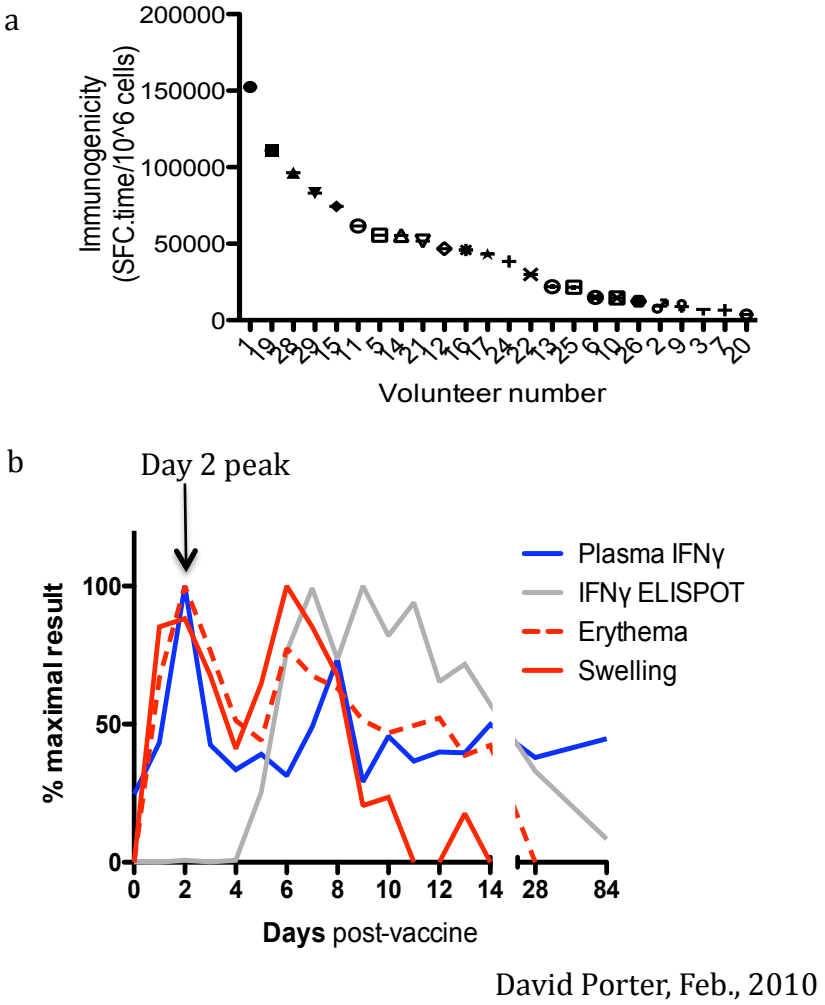


Figure 3.1.1 The immune response to MVA85A. a. IFN-γ ELISpot responses (area under the curve over 24 weeks) for individual TB022 volunteers ranked by magnitude of response. b. Summary of trial looking at the kinetics of the immune response following vaccination with MVA85A. Volunteers were seen daily for 2 weeks post-vaccination (clinicalTrials.gov Identifier: NCT00548444).

3.2 Gene expression in vivo post-MVA85A

Samples used in this study were taken from a trial of 24 BCG-primed healthy adults from the UK, vaccinated either intradermally (ID) or intramuscularly (IM) with 1x 10^8 pfu MVA85A. Full details of the trial have been published and a summary of ELISpot responses is included in the appendix [135]. PBMC from volunteers were cryopreserved on day of vaccination and at the following time points: day 2 and weeks 1, 2, 4, 12 and 24 post-vaccination. IFN-γ ELISpots to Ag85A peptide pools were done on fresh PBMC

at each time point except day 2. In this study, unstimulated PBMC from day of vaccination (day 0) and two and seven days later (day 2, day 7) were thawed and lysed for gene expression analysis on Illumina microarrays.

The median IFN-γ ELISpot responses to Ag85A peptides were not significantly different between the IM and ID groups at any time point and, from a filtered list of 22,000 genes, no genes were differentially expressed between the two groups at any time point. Both groups were therefore combined for all subsequent analyses. Quality control plots are given in Figure 3.2.1.

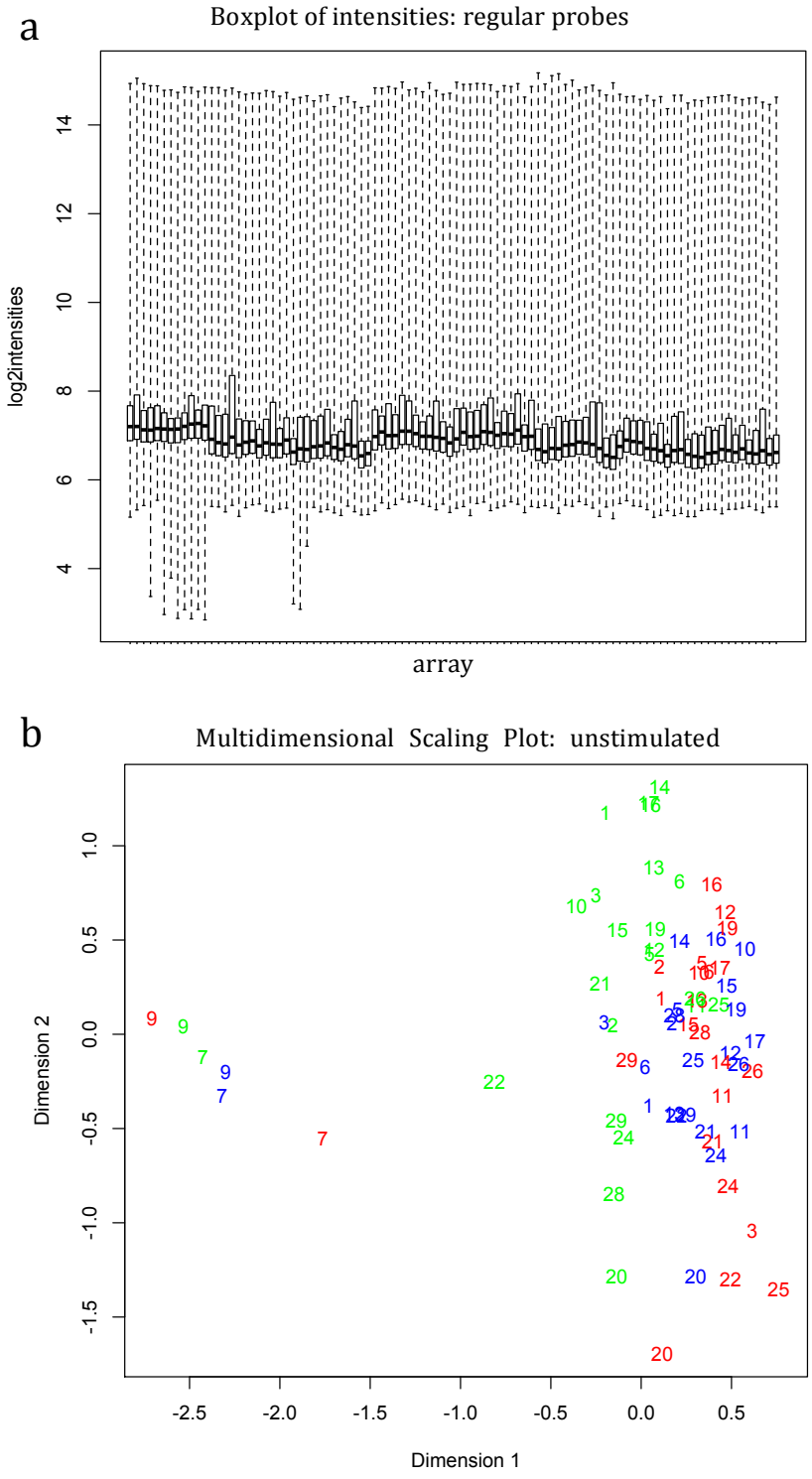


Figure 3.2.1 Quality control plots. a. Boxplot showing the median intensities, IQR and 5th and 95th percentiles pre-normalisation on a log2 scale for all samples in the experiment. b. Multidimensional scaling plot drawn in limma for the 72 unstimulated PBMC samples using the top 200 genes to calculate pairwise distance. Colours indicate sample day (blue=day 0, green=day 2, red= day 7). Samples were randomly distributed across arrays (volunteers 9 and 7 were not on the same chip).

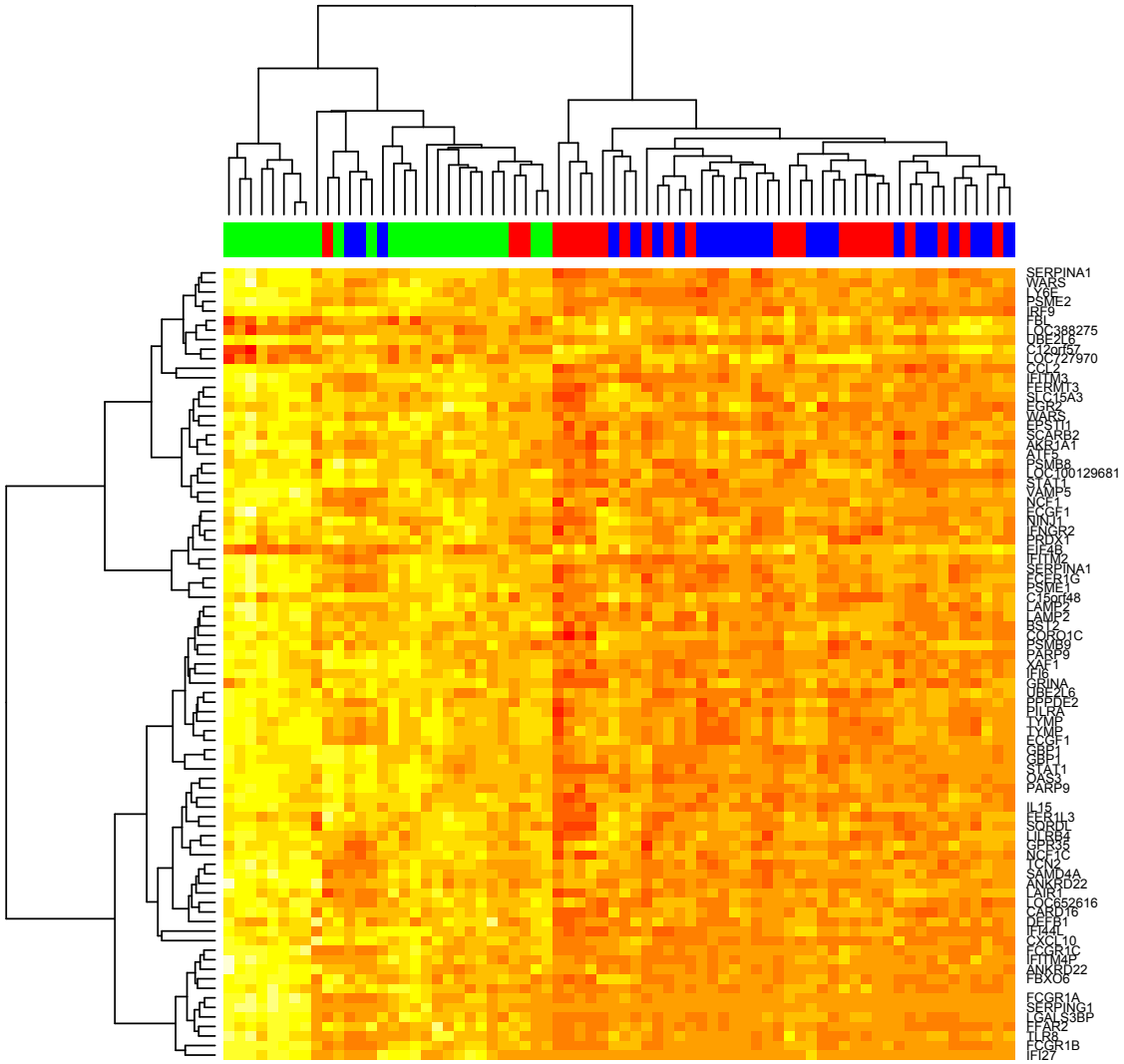


Figure 3.2.2 Heatmap of individual samples using a list of differentially expressed genes between days 0 and 2 (generated in limma and with an fdr cut-off of 0.0005). Function uses complete linkage clustering of euclidean distance. Colour banner shows day of sample collection: blue=day 0, green= day 2, red= day 7. Upregulated genes are shown in yellow.

Comparing two days after vaccination to baseline, 1100 genes were differentially expressed (false discovery rate <0.05). Cluster analysis using this subset of genes shows clear separation of day 2 samples from day 0 and day 7 in almost all cases (Figure 3.2.2), showing a strong proinflammatory signature visible in unstimulated PBMC from volunteers 2 days after vaccination. Analysis of these genes using Gene Set

Enrichment Analysis (GSEA) and the Database for Annotation, Visualisation and Integrated Discovery (DAVID) showed an enrichment for gene ontology (GO) terms and gene sets relating to an inflammatory response, positive regulation of the immune response, lysosome and apoptosis (Table 3.2.1). By day 7, gene expression had returned almost completely to baseline (Figure 3.2.3). The greatest change in gene expression was seen in the interferon-induced chemokine *CXCL10* with an average fold increase in expression of 8.5 on day 2 compared to day 0.

Table 3.2.1 Genes upregulated 2 days post vaccination with MVA85A compared to baseline in healthy volunteers.

Cluster (enrichment score)	Annotation term[a]	Adjusted p-value
Defense Response (9)	Defense response	3.00E-10
	Inflammatory response	9.30E-06
Lysosome (6.52)	Lysosome	3.70E-06
NF-κB Signalling (3.27)	Positive regulation of I-κB kinase/NF-κB cascade	8.90E-03
Immunoglobulin Binding (3.25)	IgG binding	4.30E-04
	Immunoglobulin binding	4.40E-04
Immune Response (3.24)	Positive regulation of immune response	3.00E-05
	Lymphocyte mediated immunity	7.50E-04
	Innate immunity	8.40E-04
Apoptosis (3.19)	Regulation of programmed cell death	1.30E-02
	Regulation of apoptosis	1.60E-02
Chemotaxis (2.58)	Chemotaxis	2.40E-03

a. Representative annotation terms and their p-value for each annotation cluster

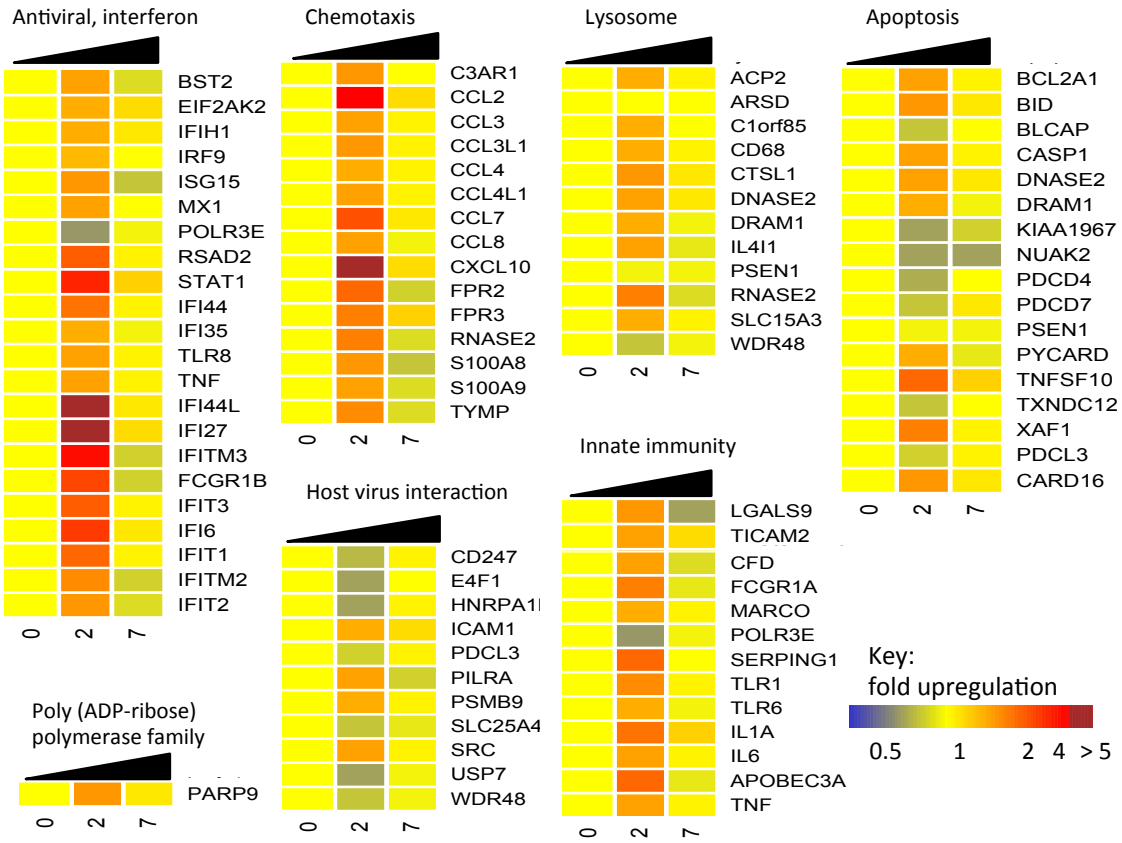


Figure 3.2.3 Transcriptional response to MVA85A. Heat map showing changes in gene expression of common genes 2 and 7 days following MVA85A vaccination. List of differentially expressed genes and fold changes obtained in limma. The genes are sorted into categories based on DAVID Bioinformatics Database gene descriptions. The heat map colours show the average expression across the 24 volunteers at each time point (given in days at the bottom of each column). All genes contributing to enrichment of a given category in DAVID are included however not all gene categories have been shown.

3.3 Gene expression in PBMC stimulated in vitro with MVA85A

As a control, excess PBMC from day 0 (pre-vaccination) from four volunteers were stimulated *in vitro* for 6 hours with either media alone, Ag85A, MVA85A or wild type MVA (MVAwt). These were then lysed and processed with the other 72 samples. Changes in gene expression were analysed between the four conditions and also compared between *in vitro* MVA85A stimulated PBMC and PBMC from volunteers 2 days after vaccination. The volunteers were chosen to include one low and one high responders to MVA85A from each route (*im* and *id*) to give a total of four volunteers

(based on previously performed ELISpot assays). A multidimensional scaling plot is shown in

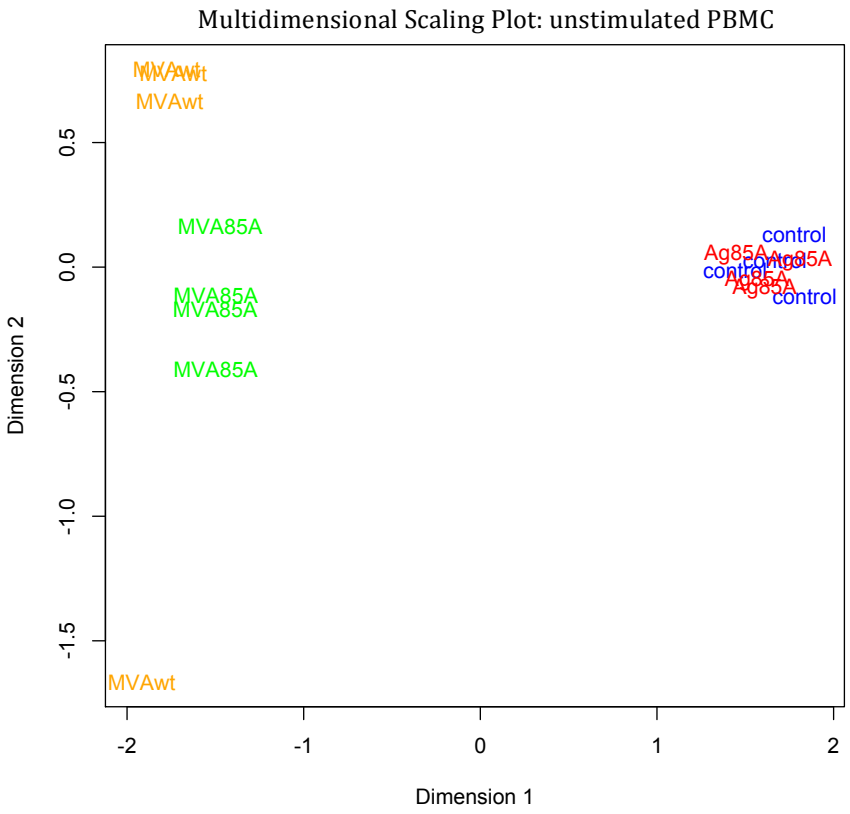


Figure 3.3.1 Multidimensional scaling plot of the 16 *in vitro* control PBMC samples used in this experiment. The top 500 genes were used to calculate pairwise distances. Samples were randomly distributed across arrays. Colours and labels indicate stimulation condition.

Of the 1100 genes differentially expressed on day 2 in the volunteers, approximately 50% were also differentially expressed in the cells stimulated *in vitro*. These genes were enriched for GO terms including phagocytosis, chemotaxis and the inflammatory response (Table 3.3.1). The 500 genes upregulated *in vivo* but not *in vitro* were enriched for genes involved in the innate immune response. A further 4000 genes were differentially expressed in MVA85A stimulated PBMC compared to the controls,

including genes involved in the lysosome, inflammation, apoptosis, cytokine-cytokine interactions and leukocyte migration.

Table 3.3.1 Table of GO categories: DEGs shared between D2-D0 (top half) and specific to MVA85A and control *in vitro* stimulations

Up in D2-D0 and MVA85A- control	Benjamini P-value
Lysosome	5.60E-08
TLR Signaling Pathway	9.90E-07
Chemokine Signaling Pathway	8.70E-03
NOD-like R Signaling Pathway	1.30E-02
Apoptosis	1.80E-02
Cytokine-Cytokine R Interaction	1.90E-02
FcγR-mediated phagocytosis	2.50E-02
RIG-I-like R Signaling Pathway	2.50E-02
Cytosolic DNA Sensing pathway	2.80E-02
Up in MVA85A-control only	
SLE	9.70E-06
RIG-I-like R Signaling Pathway	9.60E-06
Cytosolic DNA Sensing Pathway	2.70E-05
Jak_STAT Signaling Pathway	5.70E-05
NK cell mediated Cytotoxicity	3.90E-04
Cytokine-Cytokine R Interaction	2.30E-03
TLR Signaling Pathway	2.20E-03
Antigen Processing and Presentation	2.20E-03
T cell Receptor Signaling Pathway	3.50E-02

Comparison of the 4 *in vitro* conditions (Figure 3.3.2) shows few changes in gene expression following stimulation with Ag85A alone although around 30 genes are upregulated by all three of Ag85A, MVA wild type (MVAwt) and MVA85A compared to media alone (Table 3.3.2). Infection with either MVA85A or MVAwt leads to differential expression of over 5000 genes compared to the control; of these, 3770 were modulated by both MVAs. 170 of these genes show significant differential expression following stimulation with MVA85A compared to MVAwt. These results

suggest that the majority of the transcriptional changes are in response to the MVA however Ag85A also has an effect, albeit a small one, on gene expression.

Table 3.3.2 Tables of DEGs relative to the control stimulations with MVAwt, MVA85A or Ag85A.

PROBE_ID	SYMBOL
ILMN_1763000	ADAP2
ILMN_1774447	AMPD3
ILMN_2218856	CCL3L1
ILMN_1747355	CCL3L1
ILMN_1773245	CCL3L1
ILMN_1722622	CD163
ILMN_2379599	CD163
ILMN_1784863	CD36
ILMN_1796094	CD36
ILMN_1695423	CD9
ILMN_1733998	DHRS9
ILMN_2384181	DHRS9
ILMN_1811616	EEPD1
ILMN_1740213	ELOVL7
ILMN_2146761	FABP5
ILMN_1752728	FUCA1
ILMN_1730816	GPR162
ILMN_1669317	GPR77
ILMN_1699651	IL6
ILMN_1813704	KIAA1199
ILMN_1661631	LILRA3
ILMN_3294222	LOC100132673
ILMN_3237878	LOC100133875
ILMN_3274711	LOC392501
ILMN_1656938	LOC731486
ILMN_1770610	MERTK
ILMN_2138589	MERTK
ILMN_1686664	MT2A
ILMN_2149494	NPL
ILMN_1684982	PDK4
ILMN_1800225	PPARG
ILMN_1771688	RAB7B
ILMN_1795183	RNASE1
ILMN_2333670	RNASE1
ILMN_2338452	SERPINA1
ILMN_1732923	SIPA1L2
ILMN_2100357	SMPDL3A
ILMN_1655987	STAB1
ILMN_1707591	TNIP3

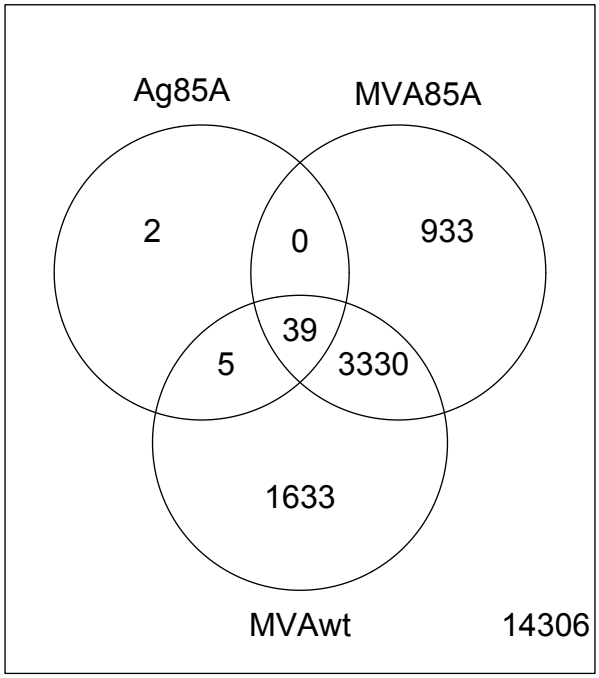


Figure 3.3.2 Differentially expressed probes relative to control in 3 *in vitro* stimulations. DEGs identified in limma, fdr<0.05. Diagram shows overlap in identified probes between conditions.

3.4 Differences in gene expression between high and low responders

In order to gain an insight into the mechanisms associated with differences in ELIspot responses between volunteers, I divided the 24 volunteers into "high" or "low" responders based on an area under the curve analysis for IFN-γ ELISpot responses to 66 overlapping peptides covering Ag85A (AUC). AUC correlated with immunogenicity at each time point post-vaccination ($r>0.77$, $p<0.0001$ for all comparisons) and was used to avoid problems associated with multiple testing. Volunteers with a response greater than the median AUC were classified as high responders. Additionally, pre-vaccination responses to purified protein derivative from *M.tb* (PPD-T) or to Ag85A do not correlate with post-vaccination ELISpot responses to 85A peptides ($r=0.16$, $p=0.43$ to PPD-T; $r=0.22$, $p=0.31$ to 85A peptide pools), none of the volunteers had been previously vaccinated with a recombinant MVA and almost all were too young to have been vaccinated against smallpox which ceased to be part of the routine vaccination schedule in the UK in the early 1970s (age range 18-55 years, median 27.5 years).

Two days after vaccination, 176 genes were differentially expressed between high and low responders. This list of genes was enriched for GO terms associated with regulation of T cell activation and co-stimulation signal (DAVID, fdr<0.05). The genes contributing to the enrichment of these clusters, which are more highly expressed in low responders, are shown in Table 3.4.1. Although the fold change is low, the enrichment of genes associated with regulation of T cells suggests this change is of biological relevance. Furthermore, mRNA of several genes correlated directly with the IFN-γ ELISpot responses to vaccination (Table 3.4.2).

Table 3.4.1 DEGs between high and low responders, two days post-vaccination

SYMBOL	Description	Fold Change	adj. p-value
CTLA4	Cytotoxic T lymphocyte antigen 4	1.77	0.037
CD5	T cell surface antigen	1.62	0.032
IL2RA (CD25)	IL2 receptor, α subunit	1.58	0.043
TRAT1	TCR associated adaptor molecule	1.48	0.03
STAT5B	T cell transcription factor	1.48	0.043
CD28	co-stimulatory molecule	1.43	0.038
CD3D	T-cell receptor, δ subunit	1.41	0.025
ITK	IL-2 inducible T cell kinase	1.4	0.048
CD2	T cell surface antigen	1.34	0.047
DNAJA3	DnaJ (Hsp40) homologue	1.3	0.034

Table 3.4.2 Table of correlations between ELISpot values (AUC) and Treg markers (mRNA)

Marker	Day	Measure	Spearman's r	p value
IL2RA	0	mRNA	-0.54	0.007
STAT5B	0	mRNA	-0.44	0.03
CTLA4	2	mRNA	-0.6	0.002

On the day of vaccination (day 0), 400 genes were differentially expressed between high and low responders however these were not enriched for any GO terms. I analysed these genes using pamR (prediction analysis for microarray) and identified nine genes that could classify the 24 volunteers as high or low responders with a cross validation accuracy of 87% (Figure 3.5.1a; ***GAPT***: GRB2-binding adaptor protein, transmembrane; ***ZMYND15***: zinc finger, MYND-type containing 15; ***MGST1***: microsomal glutathione S-transferase 1; ***CD300LF***: CD300 molecule-like family member f; ***NPL***: N-acetylneuraminate pyruvate lyase; ***FPR3***: formyl peptide receptor 3; ***TLR1***: toll-like receptor 1 and ***TICAM2***: toll-like receptor adaptor molecule 2).

To determine the reproducibility of this finding, I used 10 samples from a previous trial in which the volunteers were vaccinated ID with an equivalent dose of MVA85A. We measured the expression of the nine genes by qPCR in both unstimulated PBMC and PBMC stimulated for 6 hours with PPD-T. Volunteers were classified as high or low responders based on the median AUC value for their ELISpot responses (in both trials, the medians were 41000 spots /million PBMC). I then performed a univariate logistic regression for each gene and, as the sample size was small, retained all genes with moderate discriminatory power ($p \leq 0.2$). Three genes were retained from the unstimulated data set however none of the genes correlated with immunogenicity following stimulation with PPD.

3.5 Classifying high and low responders based on differences in gene expression

The three genes retained were: *TLR1*, *CD14* and *TICAM2*. While *CD14* expression correlated with AUC ($p=0.006$, $r=0.3$), monocyte levels (as determined by full blood counts) did not ($p=0.9$, $r=0$) and therefore *CD14* expression was not simply a reflection of the number of circulating monocytes. Using linear discriminant analysis (LDA), the mRNA levels of *TLR1*, *TICAM2* and *CD14* on day of vaccination could classify individuals as high or low responders with a cross-validation accuracy of 80%. Doing the same LDA analysis on the original 24 samples, using microarray values for the expression levels of the same three genes, the cross validation accuracy was 79% (Figure 3.5.1b)

Although no discriminatory gene set was found based on gene expression 2 days after vaccination, adding *CTLA4* and *IL2RA* (*CD25*) to the LDA model developed with the

three innate sensor genes refined the model and increased its cross validation accuracy to 88% (Figure 3.5.1c,d). Unfortunately I was not able to validate this finding on the second trial, as day 2 samples were not available.

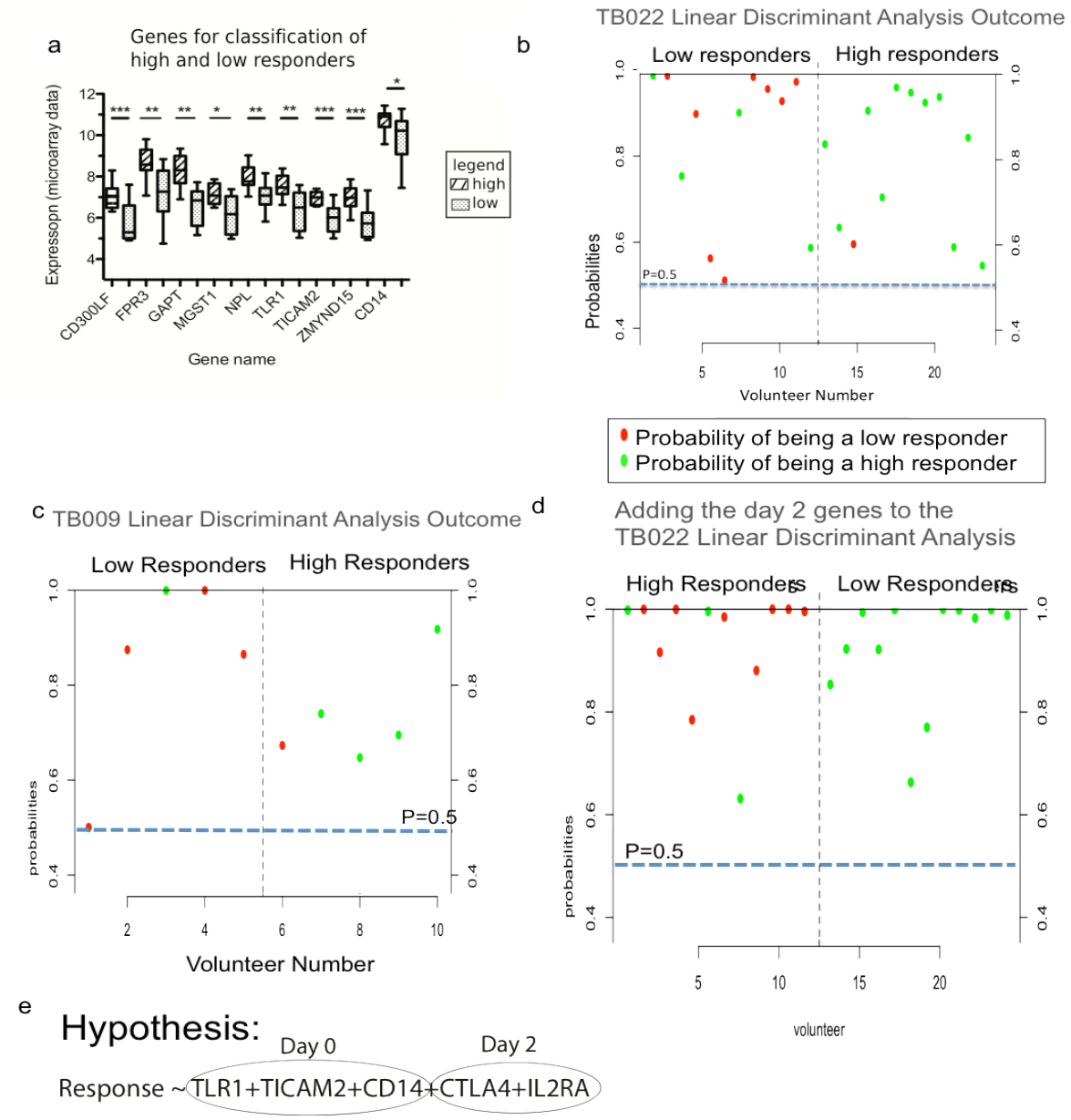


Figure 3.5.1 Expression of genes on day of vaccination and 2 days later can classify high and low responders Cohorts: TB022: 24 volunteers used in the original microarray analysis. TB009: 10 volunteers from a previous trial of MVA85A who received an equivalent dose used in the qPCR validation. a. Exploratory list of 9 genes obtained from the prediction analysis done on the microarray data from TB022 (stars indicate level of significance * $p<0.05$, **$p<0.01$, *** $p<0.005$, student's t-test). b,c. Probability plots of linear discriminant analysis classification outcomes (cross-validation probability) of high and low responders in trials TB022 (b) and TB009 (c) from expression of *TLR1, TICAM2* and *CD14* on day of vaccination. d. Probability plot of LDA classification outcome of TB022 volunteers when *CTLA4* and *IL2RA* expression on day 2 are added to the model (Other genes, eg *CD5* and *STAT5B* were removed as they did not add predictive value to the model due to co-linearity with other genes). e. Hypothesis resulting from LDA analyses.

3.6 Discussion

The primary aim of this experiment was to characterize the changes in gene expression following vaccination with MVA85A. The results show an upregulation of multiple inflammatory genes two days post vaccination and separation of time points based on expression of these genes for almost all 24 volunteers. This result shows MVA to be a particularly potent inducer of inflammation as infection of just a few cells with the virus, which is unable to replicate in mammalian cells causes a change in gene expression visible in unstimulated circulating PBMC. Most notably, *CXCL10* expression in these cells is on average 8 fold higher two days post vaccination than at baseline. This is consistent with the mild but frequent systemic adverse events observed following vaccination with MVA85A. However, although there is a gradient in the strength of the inflammatory response induced in different volunteers, magnitude of expression of these inflammatory genes does not separate high and low responders, suggesting the magnitude of the adaptive response is not directly dependent on the magnitude of inflammation as measured by the increase in expression of interferon-induced genes.

The peak adaptive response to vaccination with MVA85A is relatively early at 7 days and, consistent with this, the changes in gene expression following vaccination with MVA85A are both early and transient. This is in contrast to data published on the yellow fever vaccine, which shows a peak in expression of innate genes 7 days post-vaccination. This difference likely reflects the non-replicating nature of the MVA vector. The much earlier peak in innate signaling is likely to impact the development of the adaptive immune response.

In addition to this, I was able to examine the effects of route of vaccine administration on the ensuing immune response and changes in PBMC gene expression. It has been reported that route of vaccination is an important factor in the development of the immune response in mice [167] however we had not found significant differences in the magnitude of antigen specific responses following ID or IM vaccination. I did not observe any qualitative differences in the systemic immune response either although, due to the nature of the assay, it is difficult to exclude small differences in the magnitude of pathway activation.

Differences between differentially expressed sets of genes in the *in vivo* and *in vitro* experiments may have multiple causes. Timing will account for some differences. Additionally, contrary to circulating PBMC, most cells in the *in vitro* experiment will have been either infected with MVA or in close contact with infected cells. Changes in processes including the lysosome, apoptosis, and phagocytosis, all of which are known to be important during infection with MVA, are therefore visible. A large overlap in genes modulated by both MVAwt and MVA85A in stimulated PBMC suggests the findings of this study will be applicable to other uses of MVA as a boosting vector.

The identification of differentially expressed genes between high and low responders was an exciting result with the potential to yield insights into the mechanisms by which MVA induces an immune response and into the more fundamental processes that govern the generation of antigen-specific immune responses.

A number of genes were differentially expressed between high and low responders two days after vaccination and these were significantly enriched for genes involved in

the regulation of T cell activation, notably *CTLA4, IL2RA* and *STAT5B* which were more highly expressed in low responders. *CTLA4* is found on the surface of T cells and mediates inhibitory signals through binding of CD80 and CD86 on antigen presenting cells (APCs), IL2RA is a surface marker of Tregs and activated T cells and STAT5B regulates transcription of a number of Treg associated genes. This suggests that regulatory T cells may be an important factor in determining the magnitude of the adaptive response. Whether these differences are causative and whether they are already present prior to vaccination and have simply been amplified is not possible to tell.

Expression of three genes on day 0 -*TLR1, TICAM2* and *CD14*- was able to classify volunteers from both the current trial and a previous trial into high and low responders. Together these results, coming from two independent trials provide a good indication that expression levels of these or related genes are involved in determining the magnitude of the antigen-specific adaptive immune response. This was seen as a good opportunity to generate more specific hypotheses relating to the interaction of those genes with MVA. Several hypotheses may be considered. Firstly that *TLR1, TICAM2* and or *CD14* are directly involved in recognition of MVA and that increased expression of these receptors increases sensing and the magnitude of signaling following infection with MVA. This may directly influence the magnitude of the developing adaptive response. Secondly, that it is not these genes but co-regulated genes that are involved. These may not show up in a microarray screen, for example if the range of expression is narrower, giving less power to discriminate between higher and lower responders. This result may indicate that different innate pathways have unequal effects on the developing immune response. TLR1, TICAM2 and CD14 are all

early components in the NF-κB pathway and this may be important in the generation of adaptive responses. The ubiquitous effects of NF-κB on immune genes, and the overlap with interferon-induced genes, make it difficult to establish this. However these results form a strong basis for further experiments.

The immune system is a complex network of redundant and pleiotropic components, making it difficult to identify cause and effect relationships in heterogeneous populations. DNA microarrays offer an attractive tool to describe the events following vaccination and find associations to enable the generation of hypotheses. A description of the effects of MVA on the immune system is a helpful tool in the development of this vector as an effective vaccine to different diseases. Additionally, this experiment provided an opportunity to generate hypotheses to explain the variability in the magnitude of the response to vaccination, as measured by the number of IFN-γ secreting cells specific to the antigenic insert, seen between individuals.

4 FACTORS AFFECTING THE IMMUNE RESPONSE TO MVA85A

Aims

To use different assays and models, both *in vitro* and *in vivo*, to further investigate the relationship of Tregs and TLR receptors with immune responses to MVA85A.

4.1 Introduction

This chapter details experiments that were conceived on the basis of the microarray data presented in the previous chapter. In particular, Tregs and three genes involved in innate immune sensing *(TLR1, TICAM2* and *CD14*) were associated with the magnitude of the immune response and I was interested in characterising these associations further.

Microarray analysis provides a snapshot of gene expression in a population of cells so I employed different techniques to acquire more information on these observations. To further characterise the volunteers' cell populations, I used multiparameter flow cytometry, with a particular emphasis on Treg markers. The association of *TLR1* expression with magnitude of the immune response suggested this pathway also plays a role. To study the role of this receptor in initiating the response to MVA85A, I used neutralizing antibodies both *in vitro* in stimulations of human PBMC and *in vivo* using the mouse as a model.

4.2 Regulatory T cells

Results of the microarray analysis show differential expression of around 180 genes between high and low responders two days post vaccination and this gene list is enriched for cells involved in the regulation of T cell activation. Furthermore, expression of three of these genes directly correlates with the ELISpot response. To investigate this association further, I examined by flow cytometry whether increased

expression of genes associated with a regulatory response 2 days after vaccination is reflected in the regulatory T cell (Treg) compartment (gating strategy for flow experiments is shown in Figure 4.2.1). The number of Tregs (CD4+CD25+Foxp3+) in unstimulated PBMC from day of vaccination and 7 days later were quantified. In high responders, the number of Tregs (as a percentage of CD4+ cells) remained constant pre and post vaccination, however in low responders it increased between day 0 and day 7 (Figure 4.2.2a, p=0.009). Additionally, the expression of *STAT5B* mRNA on day 0 correlated with the percentage of Tregs seen in PBMC on day 7 (p=0.41, r=0.047). There was also a weak association between *CTLA4* mRNA on day 2 and percentage of Tregs seen in PBMC on day 7 although the correlation did not reach significance (Figure 4.2.2b, r_s=0.39 p=0.058).

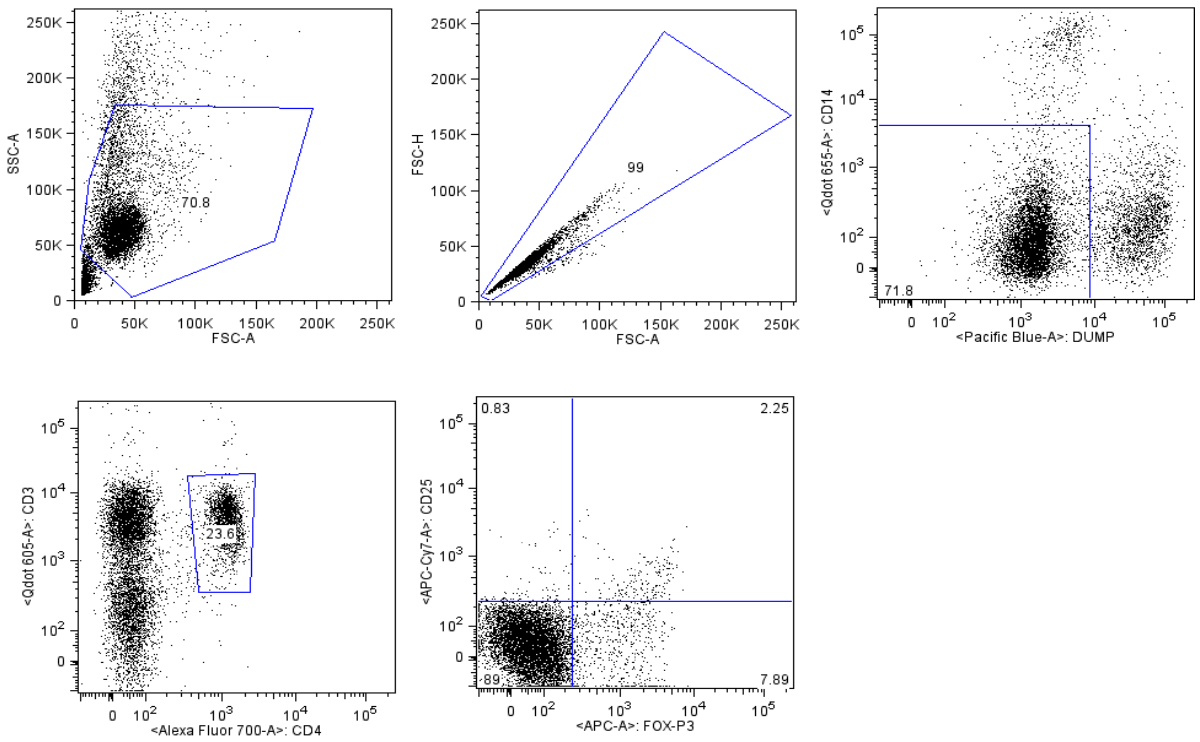


Figure 4.2.1 Gating strategy for flow cytometry analysis. Samples for flow cytometry analysis of Tregs or TLR1 were gated as shown above for steps 1-3: cells (FSC-A, SSC-A), singlets (FSC-A, FSC-H). Dump: ViVid and CD19 on Pacific blue. For Treg analysis cells were selected as shown: CD14-, CD3+, CD4+ and CD25+Foxp3+. For analysis of TLR1 expression, MFI of TLR1-PE was calculated after gating out of ViVid dead and CD19+ cells.

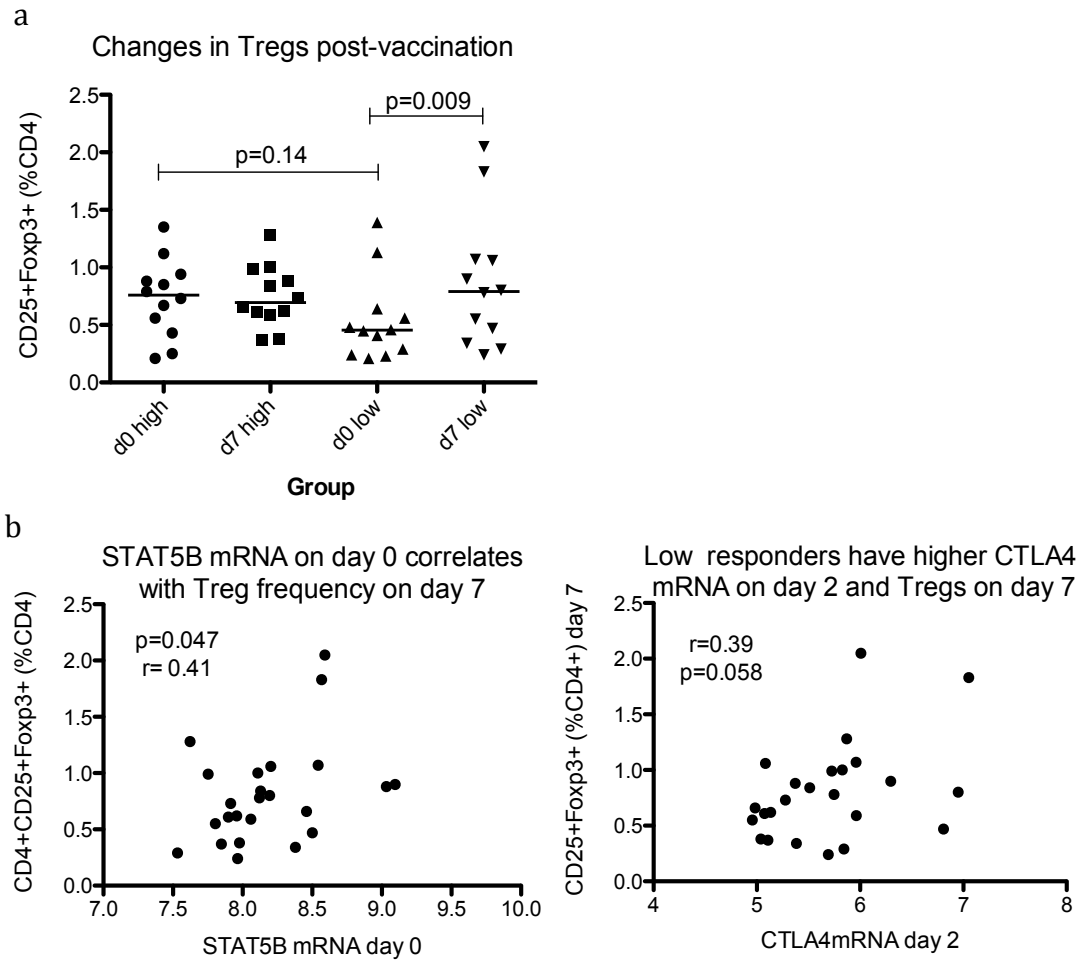


Figure 4.2.2 Regulatory T cell populations pre- and post-vaccination with MVA85A. Analysis done by flow cytometry of unstimulated PBMC from the TB022 volunteers. a. Circulating numbers of Tregs (as percentage of CD4+ cells) increase in low but not high responders post-vaccination (Wilcoxon rank-sum test, paired for samples from the same individual at different timepoints). b. Correlations of mRNA levels of STAT5B (day 0) and CTLA4 (day 2) with Treg populations (Spearman's rank correlation).

4.3 Toll-like Receptors

Using mRNA levels of three genes, *TLR1, TICAM2* and *CD14* on day of vaccination, I was able to classify high and low responders from two trials with 80% accuracy. For each of the three genes, I also performed linear regression analysis with ELISpot AUC values for individual volunteers. This analysis is not possible with a high number of genes but could be informative when thinking of the mechanisms linking vaccine sensing and immunogenicity. The levels of *TLR1* mRNA in both trials correlated with AUC (Figure

4.3.1a; TB022 p=0.005, Spearman's r=0.55; TB009 p=0.03, Spearman's r=0.70 respectively).

I examined the original 24 samples by flow cytometry, gating on live cells, and looked at mean fluorescence intensity of TLR1. TLR1 is ubiquitously expressed and, as the original microarray analysis was done on PBMC, I did not narrow down the population further using other markers. There was a correlation between the level of *TLR1* expression on day of vaccination and AUC, although the relationship was weaker at the protein level compared to the mRNA (Figure 4.3.1b, c; $p<0.05$, Spearman's r=0.45).

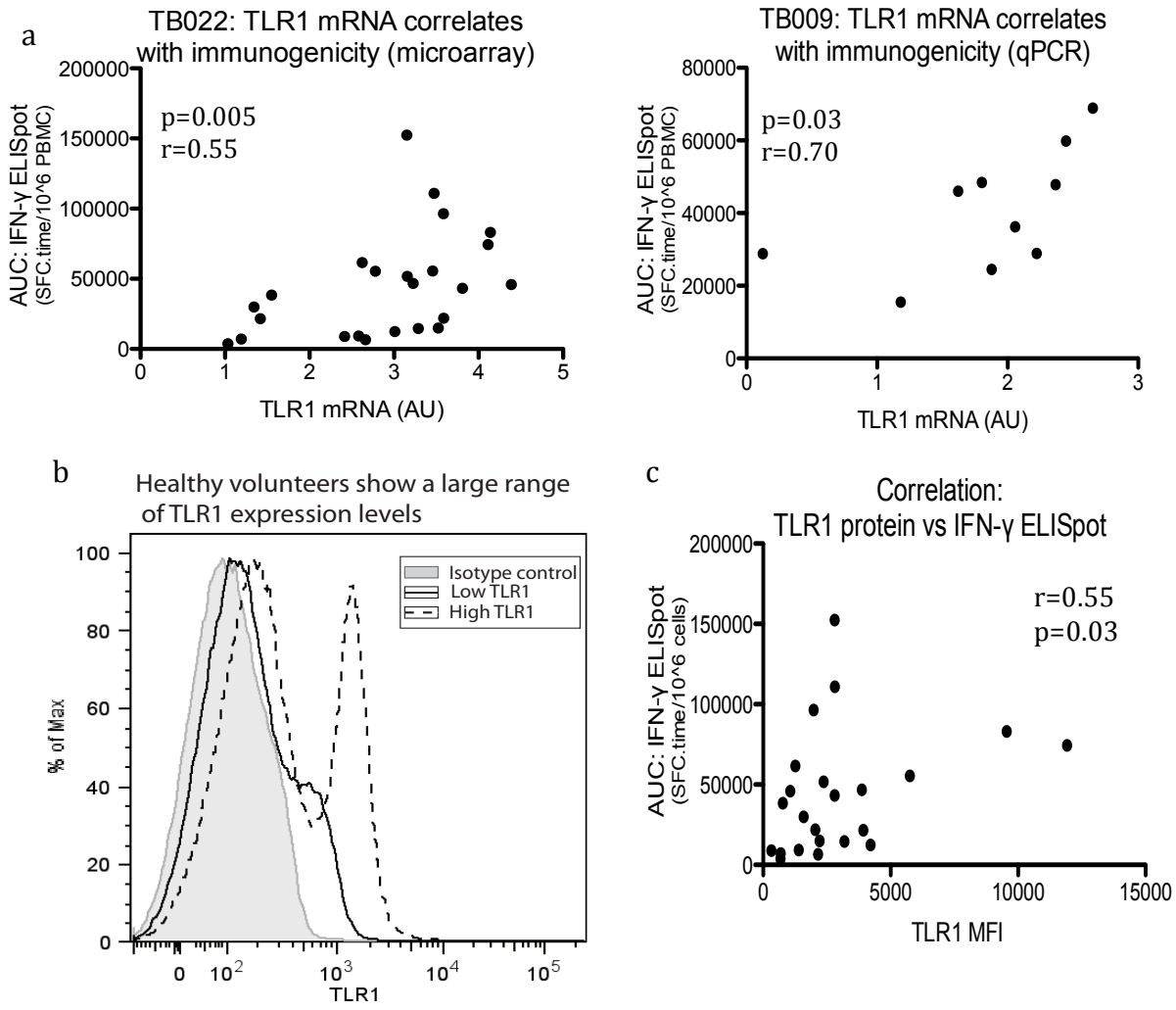


Figure 4.3.1 TLR1 mRNA levels on day of vaccination correlate with immunogenicity a. Levels of TLR1 mRNA correlated with long term immunogenicity to MVA85A in TB022 (measured by microarray) and TB009(measured by qPCR) (Spearman's rank correlation). b. Surface expression of TLR1 varies greatly between individuals (flow cytometry of unstimulated PBMC). c. Median fluorescence intensity (MFI) of surface TLR1 on all live cells in unstimulated day 0 samples correlates with immunogenicity (TB022) (Spearman's rank correlation).

4.3.1 TLR1 genotype

TLR1 genotype has been reported as a factor affecting vaccine immunogenicity. In particular, the 1805 G/T single nucleotide polymorphism (SNP) has been associated with altered cytokine responses in BCG-vaccinated infants and diminished Th1 responses [146,168]. In this case, it was unlikely that this genotype was causing the association as it has been reported that the SNP affects protein trafficking and hence

cell surface expression [150,169] however, in this experiment the association is seen at the mRNA level. Moreover, seeing an association between genotype and immunogenicity with only 24 volunteers is extremely unlikely. Nevertheless I wished to exclude this possibility and so genotyped the 24 TB022 volunteers for this SNP. The results, which show no significant difference (Wilcoxon rank sum test), are shown in Figure 4.3.2.

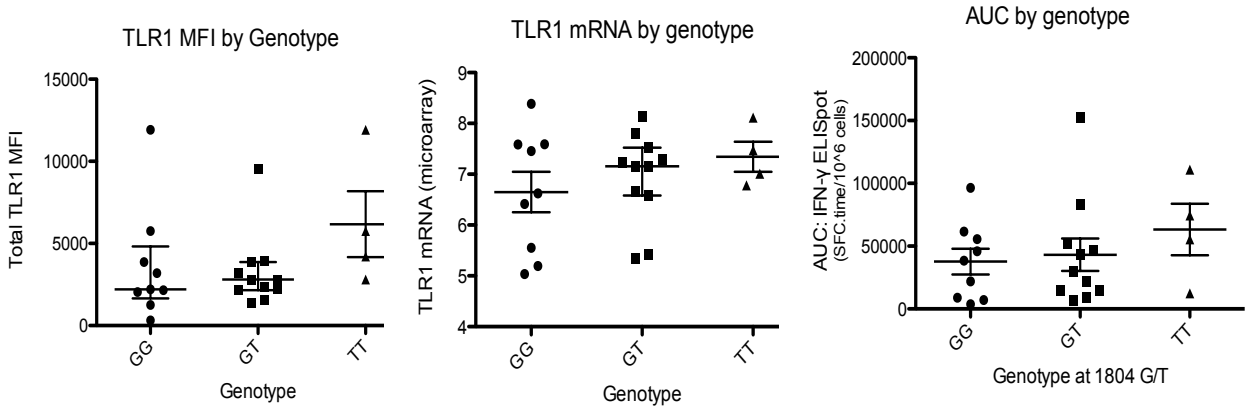


Figure 4.3.2 Genotype of TB022 volunteers at the 1805 G/T SNP in TLR1 was determined by qPCR and restriction digest of DNA from frozen PBMC. Previously determined measures for TLR1 MFI, TLR1 mRNA levels and ELISpot AUC do not show evidence of a difference between genotypes (Wilcoxon rank-sum test).

4.3.2 Effect of TLR blocking antibodies on responses to MVA85A

Following analysis of the microarray data, I sought to examine the relationship between the innate response to MVA85A and TLR1 *in vitro. TLR1* and *TLR6* are located in tandem on chromosome 4 and evolved following a gene duplication event. The two receptors share extensive homology and both signal as a heterodimer with TLR2 [170]. Contrary to TLR1, TLR6 is not ubiquitously expressed [171] and this may make it less likely to be identified in a microarray analysis of circulating PBMC. Due to this, and previous results showing that MVA signals through TLR2-6 rather than TLR1-2 [110], I

investigated all three receptors in the TLR2 family. Blocking signaling through all three TLRs during i*n vitro* stimulation of PBMC with MVA85A reduces expression of *CXCL2* in response to the virus (Figure 4.3.3a). MVA leads to activation of multiple innate signaling pathways. This data is consistent with previous results showing that activation of the NF-κB pathway in response to MVA stimulation (in this case MVA85A) is dependent on signaling through TLR2. In contrast to previous data, I did not find a difference between blocking signaling through TLR1 or TLR6, however I found the effect of TLR1 blocking to be donor dependent (i.e. in some donors, TLR1 neutralising antibodies consistently decreased *CXCL2* expression in response to MVA85A stimulation whereas with other donors there was no effect).

To look at the effect of TLR neutralization on the development of the immune response to MVA85A *in vivo,* mice were vaccinated with 5 x 10^5 pfu MVA85A with or without a TLR2 neutralising antibody or an isotype control. Mice were culled seven days later and responses to the antigen 85A insert determined by IFN-γ ELISpot on splenocyte responses to a 66 peptide pool. The results show a decrease in high responses to antigen 85A. The mean responses are slightly lower than in the control and isotype groups however the main difference seems to be in the spread of the distribution, particularly in the lack of high responses in the group receiving anti-TLR2 antibodies with the vaccine (Figure 4.3.3b; all three groups are normally distributed by the D'Agostino and Pearson normality test, F test for differences in variance: p=0.04 between MVA85A and anti-TLR2, p=0.02 between anti-TLR2 and isotype groups).

4.3.3 A role for HMGB1

The nuclear protein HMGB1 is released under some conditions of cell death and can signal as a danger-associated molecular pattern (DAMP) through TLR2 [172]. It has been

reported to be important in signaling following poxvirus infection [173,174] so I investigated whether it could be playing a role in this setting. HMGB1 was not one of the genes identified in the microarray however its mechanism of action in this setting is due to a change in cellular location (from the nucleus of dying cells to the extracellular space where its presence acts as a danger signal) rather than an increase in transcription. HMGB1 is detectable by western blot in the supernatant of infected cells 4 and 6 hours post infection but not in the supernatant of uninfected cells after 6 hours. Furthermore, anti-HMGB1 antibodies also reduced the production of *CXCL2* in cells stimulated with MVA85A (Figure 4.3.3c, d).

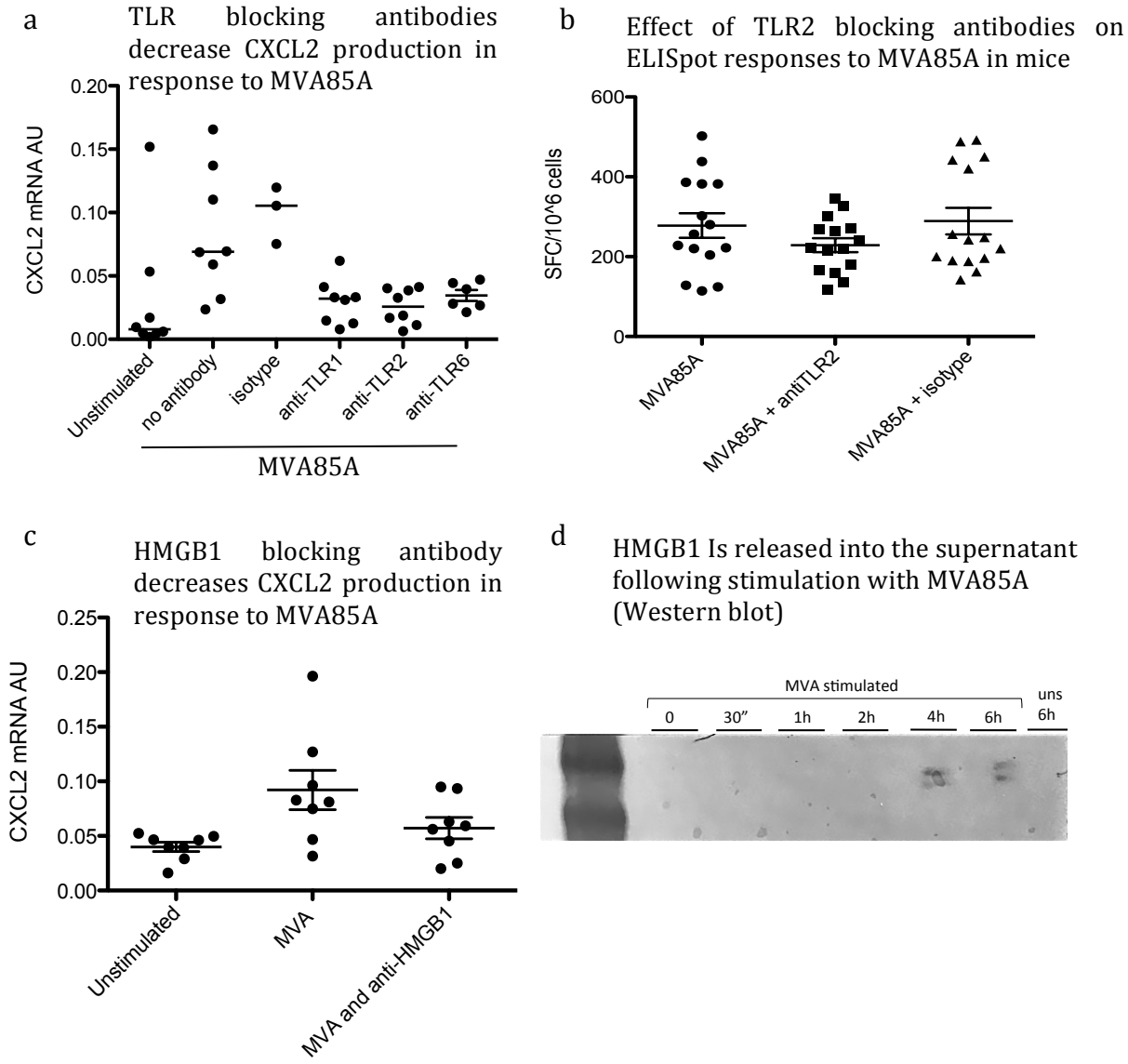


Figure 4.3.3 Toll-like receptors and HMGB1 in the innate response to MVA85A. a. Incubating PBMC with TLR blocking antibodies decreases *CXCL2* production in response to MVA85A (Wilcoxon test for all 4 comparisons $0.01<p<0.05$). b. Anti-HMGB1 also reduces *CXCL2* production in response to MVA85A stimulation (Wilcoxon paired test, $p<0.05$). c. Mixing anti-TLR2 to MVA85A when vaccinating mice reduces the spread of responses (F-test, $p<0.05$) but not the mean (student's t-test, $p=0.18$ MVA vs. anti-TLR2, $p=0.12$ MVA85A vs. isotype control). d. Western blot showing HMGB1 is released into the supernatant of MVA85A stimulated but not unstimulated cells.

4.4 Discussion

The experiments reported in this section were designed to further probe results of the microarray study described in the previous chapter.

Several genes associated with regulation of T cell responses were more highly expressed in low responders two days post-vaccination with MVA85A. Characterising these markers by flow cytometry in frozen PBMC from volunteers on day of vaccination and one week later provides information on the composition of the CD4 population that it is not possible to gain from the microarray data.

The early changes in gene expression reflected a difference in the expansion of Tregs, which was seen in low but not high volunteers. Low expression of *STAT5B* on day 0 and *CTLA4* on day 2 both showed a weak correlation with Treg numbers on day 7. These associations suggest that Tregs are an important contributor in determining the magnitude of the IFN-γ ELISpot response. Although drawing firm conclusions from small data sets is difficult, these preliminary data warrant further investigation into the role of Tregs and Treg markers in response to vaccination with MVA vectored vaccines. This increase in regulatory activity in low responders is consistent with previous work showing that high levels of TGF-β in the serum on day of vaccination with MVA85A resulted in lower IFN-γ ELISpot responses post-vaccination [175]. The role of Tregs on responses to vaccination has not been extensively studied outside the cancer field. However it is known that in cancer patients and particularly within the environment of the tumour, regulatory T cells decrease effector T cell responses to vaccination. This effect can be countered by administering vaccines with cyclophosphamide, which at low concentrations selectively inhibits Tregs [176]. It is also

known that patients with deficiencies in the STAT5B pathway have fewer circulating Tregs and high expression of this gene may therefore be linked to increases in Tregs [177]. Tregs are likely to play an important role in determining responses to vaccination; a greater understanding of the mechanisms involved may help predict and mitigate differences in responses to vaccination in different populations. Numbers of Tregs and responses to vaccination both vary by age and geographical location- it is possible that these may be linked. Although I saw a difference in the Treg population, I do not know what factors are causing a stronger regulatory response to develop in some vaccinees but not others, or whether vaccination is simply accentuating differences in the Treg compartment that were already present in volunteers. Both genetic and environmental factors are likely to influence the regulatory response to MVA85A, which may be specific to the vaccine or a reflection of the immune status of an individual at the time of vaccination. Additionally, I cannot exclude the possibility that expression of TLRs and regulatory receptors may be linked.

On the day of vaccination, *TLR1* expression also correlated with immunogenicity. This led to the hypothesis that MVA signaling through the TLR1-2-6 axis is important in the development of the adaptive immune response. *In vitro* stimulations show that innate signaling through NF-κB is decreased following stimulation with MVA85A in the presence of neutralizing antibodies to TLR1, 2 or 6. Furthermore, blocking signaling through these receptors by co-administering a TLR2 antibody with MVA85A to mice also appeared to diminish high ELISpot responses, although the mean response to vaccination is not significantly lower. This suggests that the strength of the signal through TLR2 is affecting the magnitude of the adaptive response that develops but it is neither the only innate mechanism by which MVA is sensed nor is it the sole

determinant of antigen specific immunity. Poxviruses have been infecting human populations for millennia and elicit a plethora of innate responses. The strength and versatility of the innate response to MVA is evident from the array data and has been shown by other groups [110,132]; although it is dominated by interferon responses, different aspects of the innate response may be playing different roles in directing the adaptive response to vaccination.

Although MVA and its parent strain vaccinia have previously been shown to signal through TLR2, no viral ligand for this interaction has been proposed and TLR2 is better known for binding to molecules of bacterial and fungal origin [178]. HMGB1 has been suggested as a ligand for TLR2 in signaling following infection with different poxviruses as it is released during cell death and many attenuated strains of vaccinia virus cause widespread cell death, having lost molecules which inhibit apoptosis in the parent strain [106,174]. I have shown that HMGB1 is released from cells following MVA85A stimulation and that antibodies to HMGB1 can decrease *CXCL2* expression in these stimulations. These data suggest that response to cell death following vaccination with MVA85A, in particular through the release of HMGB1 and its interaction with TLR2, is a strong determinant of the magnitude of the adaptive immune response that develops. Consistent with this, a recent study by Perdiguero and colleagues has shown that deletion of the anti-apoptotic gene F1L from the candidate HIV vaccine MVA-C enhances its immunogenicity in mice [112]. In another study, in dendritic cell cultures infected with MVA, the majority of the functional response and maturation was observed in non-infected bystander cells [126]. This leads to the model that vaccination with MVA85A causes death of infected cells and the release of HMGB1, which then signals through TLR2 and either TLR1 or TLR6. The magnitude of this signal, which in

humans may be determined by the considerable range of TLR expression seen on the surface of PBMC from healthy adults, is one of the factors determining the magnitude of the developing adaptive response to the vaccine. HMGB1 can under some conditions also activate TLR4, which signals through the adaptor protein TICAM2 [172]. Therefore it is possible that the two pathways have different relative contributions in different individuals and thus including both can refine classification models. Recently, Querec et al. demonstrated that a gene signature including the transcription initiation factor EIF2AK4 could predict antigen-specific CD8+ T cell responses to the yellow fever vaccine YF-17D. This kinase can activate the integrated stress response secondary to arginine depletion and the authors speculate that induction of the stress response directs the adaptive response to vaccination in this setting [179]. This work suggests that, as with the case of the yellow fever vaccine, a danger-associated signal resulting from cellular stress or damage appears to be a strong determinant of the magnitude of the adaptive response to MVA85A.

In summary, low responses to vaccination with MVA85A are associated with lower expression of *TLR1* on day of vaccination and a regulatory response leading to expansion of Tregs over the seven days following vaccination. This study highlights features of the immune response that are important in determining the number of antigen-specific IFN-γ secreting cells in response to vaccination with MVA85A boosting BCG-induced responses. It is possible that the relative influence of different innate mechanisms on the development of the adaptive response may change when MVA is used with a different priming vaccine or if another measure of immunogenicity is investigated. These experiments further the understanding of the relationship between early innate responses to vaccination and how these relate to the facets of the

adaptive immune response that are being measured. Generating hypotheses from a human trial ensures the mechanisms being studied are relevant to the target species for vaccination, although further work is needed to establish whether these results are applicable to other populations. Determining the importance of these mechanisms in the development of the vaccine-induced immune response in infants and HIV-infected adults in countries where tuberculosis is endemic is of particular importance.

5 THE INNATE IMMUNE RESPONSE TO BCG

Aims:

To investigate and characterise the changes in the transcriptome in the blood, in response to BCG stimulation, following vaccination with BCG.

To relate differences in gene expression with differences in ability to control mycobacterial growth in a human *in-vivo* BCG challenge model.

5.1 Introduction

In these experiments I sought to characterise the changes in the transcriptome following BCG vaccination in the same way that I had done following MVA85A vaccination. As both PAXgene tubes and PBMC were stored as part of the trial, this also gave me an opportunity to compare the two methods.

The development of new vaccines against tuberculosis is hampered by the difficulty in evaluating efficacy. The incidence of *M.tb* is very low even in high burden settings, making efficacy trials long and expensive and, although animal models exist, none exhibit all stages of human disease and it is not clear to what extent they accurately predict protection in humans. Due to the tissue damage caused by tuberculosis and the considerable difficulty in ensuring complete clearance of infection, human challenge with *M.tb* is not ethically possible. The clinical trial from which samples in this chapter were taken was conducted as part of an effort to create a human model of mycobacterial growth, and its control, using BCG as a challenge organism. Following different vaccination regimes, volunteers were inoculated intradermally with a standard dose of BCG. A punch biopsy was taken from the site of vaccination two

weeks later and the BCG quantified by both culture and qPCR. In the experiments presented in this chapter, I aimed to characterise the response to BCG vaccination and the associated variability by looking at changes in the transcriptome and also to look for relationships between gene expression and control of BCG growth, with the goal of identifying pathways important in the control of mycobacterial growth in human volunteers.

5.2 TB023: a BCG challenge model to assess anti-mycobacterial immunity

The publication associated with this study has been submitted and relevant figures are included for reference in the appendix. I studied samples from two groups in this study, neither of which received an MVA85A vaccination. Group A had no previous history of BCG vaccination or mycobacterial exposure whereas Group C had previously been vaccinated with BCG, not less than three months prior to study enrolment date. Both groups were given a standard BCG vaccination and a punch biopsy was taken from the injection site two weeks later. Blood was taken on day of BCG "challenge" and 2, 7 and 14 days later and PBMC separated and frozen down. PAXgene tubes for RNA analysis were also taken at the same time points. IFN-γ ELISpots to Ag85A peptide pools and PPD-T were performed on day of challenge and day of biopsy (day 14). A schematic of the trial design is shown in Figure 5.2.1.



C: day of BCG "challenge": 2-8 x10^5 cfu in 100µL
PAXgene and PBMC collected and cryopreserved at the other time points
C+14: punch biopsy taken from vaccination site

Figure 5.2.1 Trial design: TB023, a human BCG challenge model to assess anti-mycobacterial immunity

Group C had significantly lower amounts of BCG remaining at the site of vaccination two weeks post-challenge compared to Group A when measured by qPCR but not by culture of the homogenized tissue. The PPD-T ELISpot response on day 14 showed an inverse correlation with BCG growth for both groups. (Harris et al., submitted).

The initial microarray was performed on samples from Group C only, looking at the 4 time points in 3 conditions. The conditions were: PAXgene, unstimulated PBMC and PBMC stimulated for 12 hours with BCG at a multiplicity of infection (MOI) of 0.1-0.5. The 4 time points were day of challenge (C) and 2, 7 and 14 days later. The aim of this was to identify the most informative time points and condition before proceeding with samples from the other groups. The samples were of good quality (a subset were analysed using an Agilent Bioanalyser; RNA Integrity Number (RIN) 7.8-9.6) for both PAXgene and PBMC.

5.3 BCG-BCG group: PAXgene samples

Quality checks and gene filtering were performed in R as described in the methods and all 48 samples were included in the analysis. Multidimensional scaling analysis showed the samples to cluster by volunteer (Figure 5.3.1). Gene expression analysis done in

limma showed no differentially expressed genes between any time points. The package betr identifies differentially expressed genes in time course data and this method is more sensitive than comparing time points to each other in isolation [159]. Use of this package identified 32 differentially expressed probes over the 2 weeks following BCG challenge, mostly related to the STAT1-IFN pathway, which are listed in Table 5.3.1. Expression of four of these genes by individual volunteers is shown in Figure 5.3.2, showing an increase in inflammation that is most pronounced two days post-BCG. In many volunteers, this is accompanied by changes in the expression of *CD14*, *CD8A* and *CD4*, although the direction of these changes varies between volunteers (Figure 5.3.3).

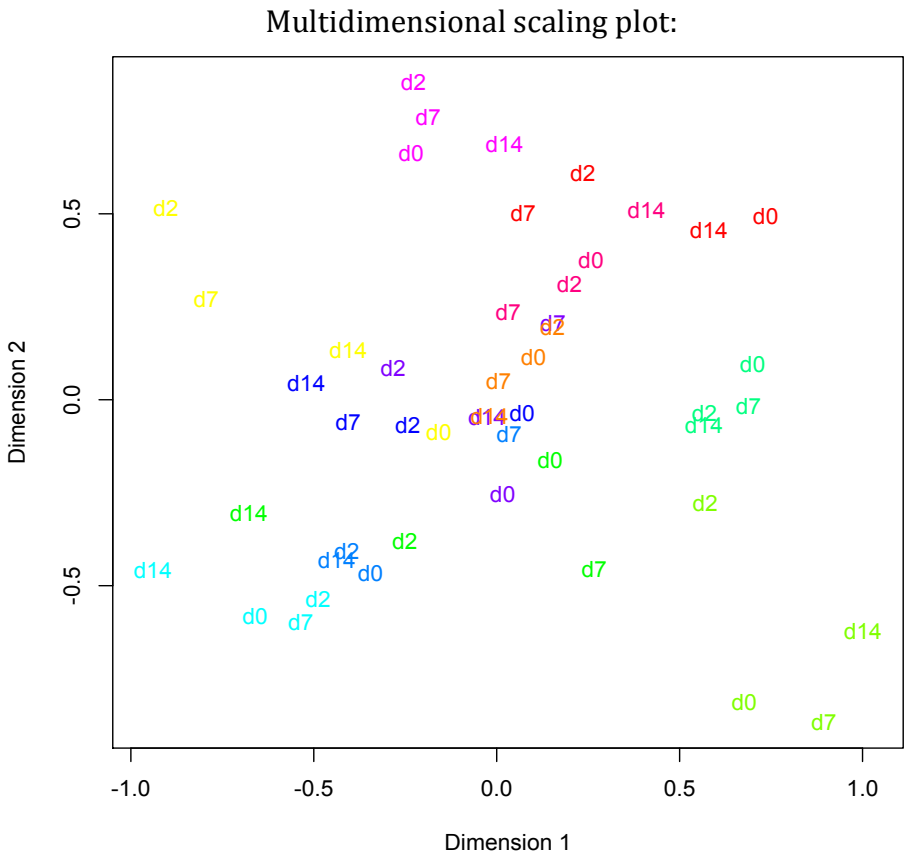


Figure 5.3.1 Multidimensional scaling plot of PAXgene samples from TB023 group C made in limma using the top 500 genes to calculate pairwise distances. Samples are coloured according to volunteer number.

Table 5.3.1 Table of DEGs during the 14 day challenge period (PAXgene)

PROBE ID	Symbol	p value
ILMN_1681301	AIM2	0.026
ILMN_1722158	CASP5	0.048
ILMN_2396020	DUSP6	0.036
ILMN_1737965	ELOVL4	0.032
ILMN_2109197	EPB41L3	0.031
ILMN_2176063	FCGR1A	0.000
ILMN_2261600	FCGR1B	0.002
ILMN_2148785	GBP1	0.040
ILMN_1788160	KIF5B	0.019
ILMN_1765132	LACTB	0.001
ILMN_1670693	LIPE	0.018
ILMN_3225471	LOC728650	0.000
ILMN_1654389	LOC728744	0.015
ILMN_3302919	MYOF	0.000
ILMN_2342835	P2RY14	0.005
ILMN_1691731	PARP14	0.048
ILMN_2053527	PARP9	0.022
ILMN_1783231	PLEKHB1	0.011
ILMN_1786612	PSME2	0.029
ILMN_1709815	PURG	0.024
ILMN_1729019	SEP7	0.017
ILMN_1726114	SLC45A3	0.050
ILMN_2170949	SNX10	0.017
ILMN_1690105	STAT1	0.020
ILMN_1777325	STAT1	0.025
ILMN_1750785	SYTL1	0.043
ILMN_1677827	TLR7	0.013
ILMN_1728106	TNF	0.042
ILMN_1727271	WARS	0.008
ILMN_2337655	WARS	0.028
ILMN_2140999	ZFYVE16	0.046

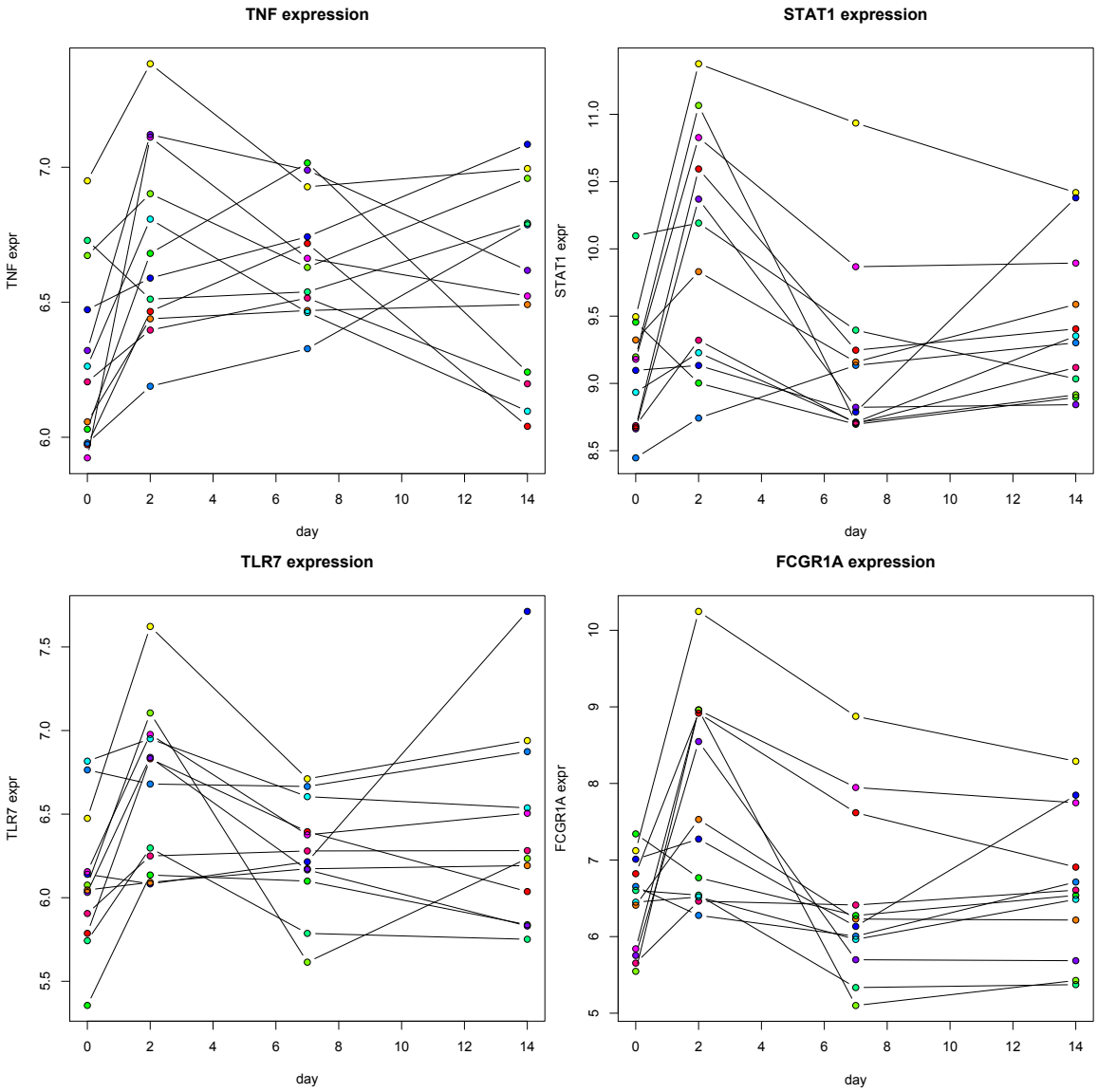


Figure 5.3.2 Changes in gene expression by volunteer across the 14 day challenge period (BCG-BCG group, PAXgene). Genes shown are *TNF, STAT1, TLR7* and *FCGR1A*; colours indicate volunteer number.

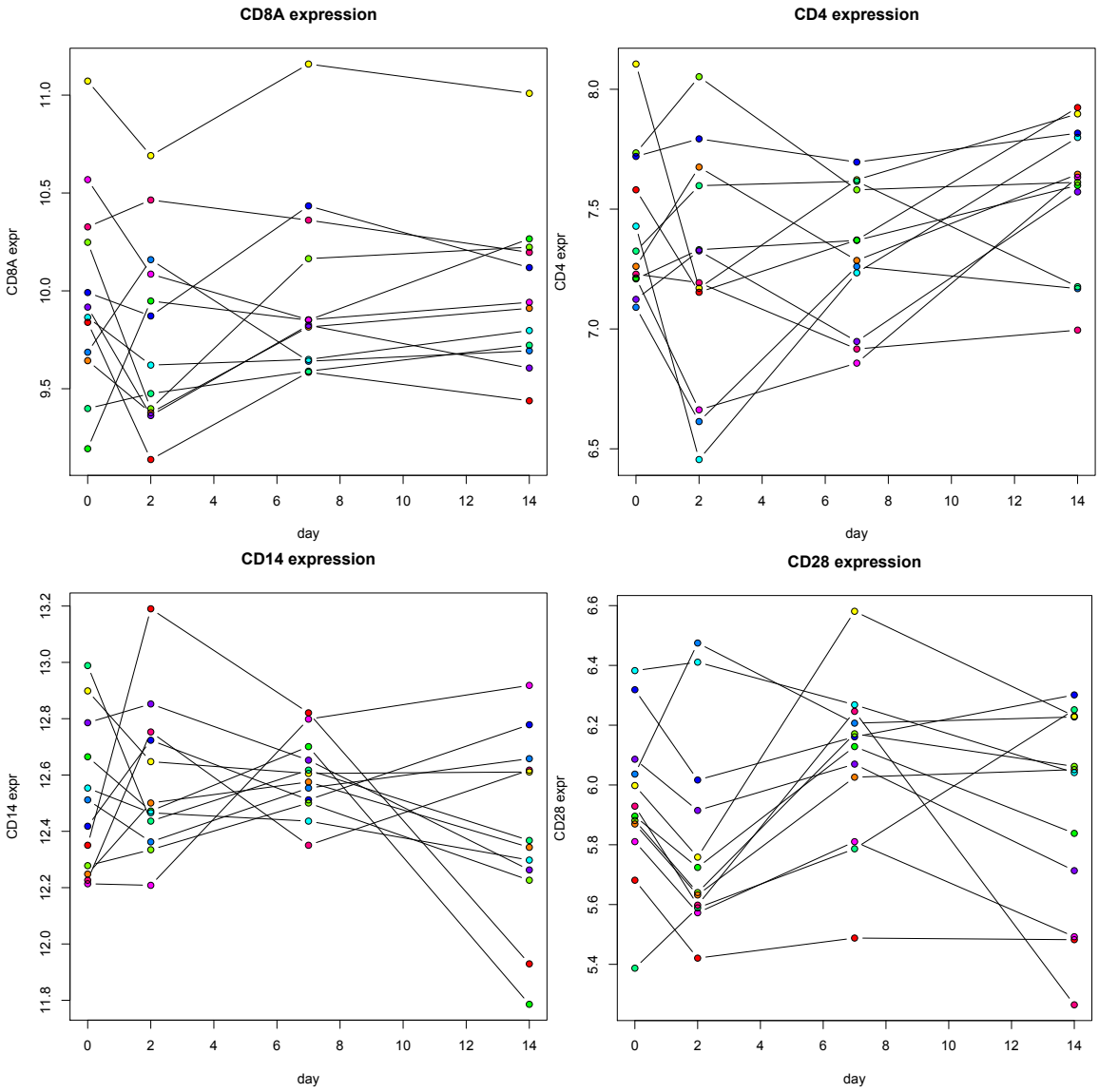


Figure 5.3.3 Changes in genes associated with different cell populations across the 14 day challenge period (BCG-BCG group, PAXgene). Genes shown are *CD8A, CD4, CD14* and *CD28*; colours indicate volunteer number.

In addition to this, I compared the gene list to the gene lists published by Berry et al. in their microarray analysis of PAXgene samples taken from patients with active or latent tuberculosis and healthy controls. Using a less stringent p-value in betr ($p \leq 0.2$) produces a list of 77 probes. Of these, 30 were present in the 393 gene signature identified by Berry et al. and a further 5 were present in the 86 gene signature. This significant overlap may be due to gene modulation due to mycobacterial infection in

both cases. Alternatively, it may be indicative of a core subset of genes that are more highly expressed in whole blood and therefore more likely to be identified in gene lists generated from whole blood samples.

5.4 BCG-BCG group: stimulated and unstimulated PBMC

Hierarchical clustering of the PBMC samples showed them to cluster first by stimulation (BCG or media alone) and then largely by volunteer (Figure 5.4.1). Compared to unstimulated, the BCG-stimulated samples showed differential expression of several thousand genes at each time point, some of which are shown in Table 5.5.1 for days 0 and 14. The two subsets were analysed separately.

5.4.1 Unstimulated PBMC

As in the PAXgene samples, both the limma and betr packages were used to find differentially expressed genes. When the volunteer is included as a factor in the model, limma identifies approximately 80% of the genes identified by betr, which identified 1500 differentially expressed genes over the four time points in Group C. The gene list was enriched for GO terms including leukocyte activation, glycolysis and apotosis, as shown in Table 5.4.1. As seen from the PAXgene samples, the greatest changes in gene expression occurred two days post-vaccination and are shown for four representative genes in Figure 5.5.1B.

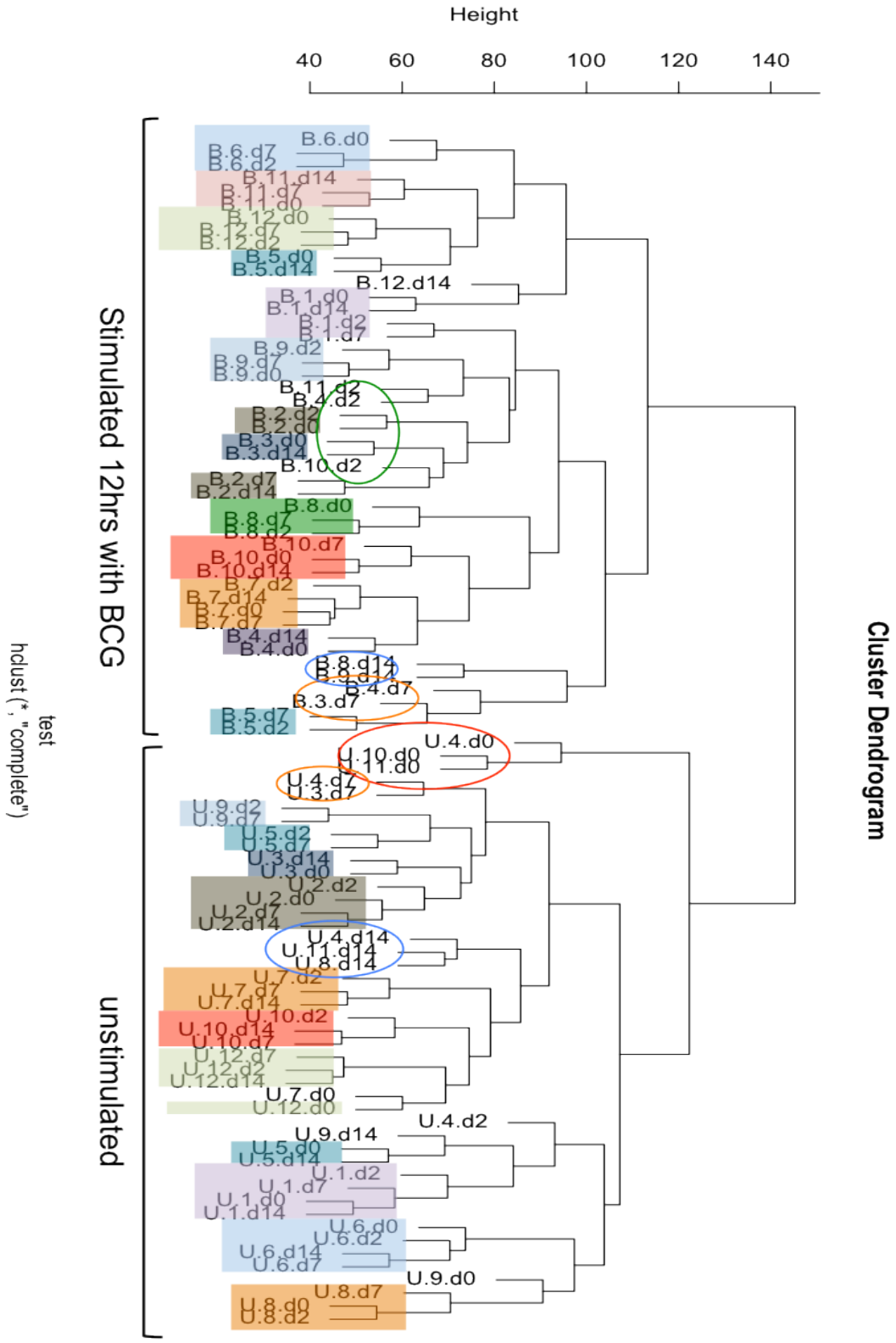


Figure 5.4.1 Dendrogram of PBMC samples from group C. BCG stimulated samples cluster to the left of the dendrogram. Filled coloured blocks indicate the same volunteer, clear coloured circles the same time point. Dendrogram drawn for 18,000 genes using Euclidean distance and complete agglomerative clustering.

Table 5.4.1 Table of GO terms enriched in Group C unstimulated PBMC. In the BCG-BCG group, the differentially expressed genes during the 14 day challenge period in the unstimulated PBMC were enriched for the following gene ontology terms

Term	Count	%	P-Value	Benjamini
establishment of protein localization	84	7	8.70E-07	5.70E-04
cell activation	42	3.5	7.40E-07	6.10E-04
glycolysis	15	1.3	7.00E-07	7.60E-04
leukocyte activation	38	3.2	4.80E-07	1.60E-03
intracellular transport	72	6	5.30E-06	2.50E-03
T cell activation	23	1.9	1.20E-05	4.40E-03
lymphocyte activation	30	2.5	2.10E-05	6.20E-03
hexose catabolic process	16	1.3	2.10E-05	6.70E-03
glucose metabolic process	25	2.1	3.20E-05	7.90E-03
monosaccharide catabolic process	16	1.3	3.00E-05	8.00E-03
positive regulation of apoptosis	49	4.1	8.30E-05	1.90E-02
immune system development	35	2.9	1.40E-04	2.40E-02
induction of apoptosis	39	3.3	1.30E-04	2.50E-02
T cell differentiation	14	1.2	1.80E-04	2.50E-02
hexose metabolic process	27	2.3	1.90E-04	2.50E-02
generation of precursor metabolites and energy	38	3.2	1.70E-04	2.60E-02
phosphate metabolic process	90	7.5	2.20E-04	2.90E-02

5.4.2 Stimulated PBMC

Differential gene expression in this set of samples was seen primarily between baseline (on days 0 and 14) and day 2 post-challenge. As above, the genes were involved in leukocyte activation, metabolic processes and cell death (Table 5.4.2). More genes were differentially expressed between time points in this set of samples (2520 identified by betr) including the cytokines *IL17F* and *IL22*. Changes in expression of representative genes in the stimulated PBMC are shown in Figure 5.5.2A.

Table 5.4.2 Table of GO terms enriched in Group C BCG-stimulated PBMC. In the BCG-BCG group, the differentially expressed genes during the 14 day challenge period in the BCG-stimulated PBMC were enriched for the following gene ontology terms

Term	Count	%	P-Value	Benjamini
regulation of cell death	103	7.1	1.80E-06	7.90E-04
response to hypoxia	29	2	1.60E-06	8.30E-04
response to cytokine stimulus	21	1.4	2.30E-06	9.00E-04
leukocyte activation	42	2.9	2.60E-06	9.10E-04
response to molecule of bacterial origin	23	1.6	5.90E-07	1.00E-03
immune response	93	6.4	3.80E-07	1.30E-03
regulation of cytokine production	34	2.3	4.90E-06	1.60E-03
response to lipopolysaccharide	20	1.4	6.10E-06	1.80E-03
cell death	90	6.2	1.30E-05	3.20E-03
cell activation	45	3.1	1.60E-05	3.80E-03
negative regulation of apoptosis	52	3.6	2.00E-05	4.50E-03
immune response-activating signal transduction	15	1	3.50E-05	5.70E-03
glycolysis	14	1	4.90E-05	7.60E-03
positive regulation of NF-κB transcription factor activity	13	0.9	5.20E-05	7.60E-03
regulation of cell proliferation	93	6.4	8.40E-05	1.10E-02
immune response-regulating signal transduction	15	1	8.60E-05	1.10E-02
lymphocyte activation	33	2.3	9.00E-05	1.10E-02
protein localization	102	7	8.40E-05	1.20E-02
hexose metabolic process	32	2.2	1.10E-04	1.30E-02
glucose catabolic process	15	1	1.30E-04	1.50E-02
monosaccharide metabolic process	35	2.4	1.50E-04	1.70E-02

5.5 The effect of a prior BCG on the response to BCG challenge

Based on these results, we decided to process the PBMC samples from Group A to see whether prior mycobacterial exposure was affecting the response to BCG. The PAXgene samples were not processed, as these seemed to be less sensitive and less informative, but all four time points were retained.

BCG stimulation of samples from Group A showed upregulation of very similar genes to those seen previously. However the fold changes were lower and this also resulted in fewer DEGs being identified. On day of challenge, Group C samples have much bigger

fold changes than Group A in response to BCG stimulation. Two weeks later, this difference between the two groups, although still visible, has decreased. Comparisons of fold changes for the two groups for the most highly differentially expressed genes following BCG stimulation are shown in Table 5.5.1.

Table 5.5.1 Comparison of fold changes of differentially expressed genes in BCG-stimulated v unstimulated PBMC in groups A (BCG naïve) and C (BCG-BCG)

		Challenge				Day 14			
		Group A		Group C		Group A		Group C	
PROBE_ID	SYMBOL	adj.P.Val	FC	adj.P.Val	FC	adj.P.Val	FC	adj.P.Val	FC
ILMN_1726448	MMP1	9.90E-23	31.86	3.87E-34	145.70	3.36E-19	24.30	4.13E-27	60.08
ILMN_1699651	IL6	5.44E-13	42.12	2.03E-25	138.98	3.10E-13	25.03	2.16E-18	35.90
ILMN_2158713	IL1F9	1.89E-31	99.69	1.57E-47	130.36	7.15E-30	112.53	6.32E-41	63.45
ILMN_1663866	TGFBI	1.05E-31	-71.21	1.32E-43	-128.73	8.92E-31	-89.07	1.84E-41	-120.62
ILMN_2207291	IFNG	3.63E-21	46.31	4.40E-39	127.96	6.78E-24	118.38	6.26E-40	188.75
ILMN_1668063	FCN1	1.55E-26	-74.84	1.30E-46	-127.53	6.71E-25	-81.87	1.10E-41	-81.58
ILMN_1773245	CCL3L1	6.58E-18	39.48	7.79E-33	103.31	9.39E-17	44.33	2.42E-28	28.85
ILMN_1661861	CSF2	2.03E-22	54.93	6.57E-48	100.97	1.20E-21	69.61	1.47E-43	70.41
ILMN_1658483	IL1A	5.26E-11	29.16	2.15E-23	85.06	5.96E-10	16.83	7.75E-17	32.09
ILMN_1657234	CCL20	5.74E-11	18.08	2.87E-24	83.86	1.04E-10	18.74	1.48E-16	31.41
ILMN_2203271	FPR3	2.85E-38	-48.24	1.55E-52	-83.48	9.00E-36	-47.24	9.44E-52	-92.77
ILMN_1774685	IL24	1.82E-11	21.50	9.31E-20	48.26	1.74E-15	10.32	9.82E-16	9.80
ILMN_1686623	CSF1R	4.44E-23	-15.60	1.25E-34	-46.77	1.49E-24	-24.18	9.56E-32	-39.03
ILMN_1674167	IL10	3.33E-18	37.33	6.57E-16	31.76	4.09E-20	39.57	1.86E-09	13.59
ILMN_1797009	F3	2.07E-13	5.70	8.03E-32	36.83	1.35E-14	7.72	3.69E-26	13.33
ILMN_1815205	LYZ	3.87E-22	-20.81	1.84E-29	-34.57	1.46E-20	-21.98	1.19E-24	-22.82
ILMN_1703538	AIF1	2.49E-19	-9.61	1.54E-44	-29.73	4.01E-18	-10.35	1.20E-36	-14.82
ILMN_1671509	CCL3	1.95E-17	22.48	5.86E-32	29.60	6.49E-16	23.22	4.06E-25	16.24
ILMN_1653766	CCL24	2.87E-14	-14.44	3.26E-26	-28.07	2.61E-15	-22.36	4.22E-26	-33.30
ILMN_1735910	VMO1	9.90E-23	-16.89	9.60E-31	-25.46	2.19E-19	-13.95	1.56E-27	-20.95
ILMN_1730816	GPR162	3.87E-22	-14.88	2.09E-40	-22.33	2.52E-21	-17.42	1.96E-38	-21.49
ILMN_1780533	RNASE6	1.76E-19	-11.65	6.18E-32	-22.15	1.93E-18	-12.90	8.09E-29	-18.72
ILMN_1728106	TNF	4.94E-19	8.29	9.26E-37	18.69	3.36E-19	10.40	3.89E-33	15.27

Table 5.5.3) samples. In contrast to Group C, there is no enrichment of terms associated with leukocyte activation, perhaps reflecting the lack of adaptive immunity to BCG in these volunteers at the time of challenge. Representative plots of the changes in gene expression over time are shown in Figure 5.5.1B (unstimulated) and Figure

5.5.2B (stimulated). Some interesting differences are seen between Groups A and C in the stimulated samples. Whereas Group A volunteers have a relatively low and constant expression of *IL17F* and *IL22* following BCG stimulation across the 14 days, volunteers in Group C upregulate these cytokines and for most volunteers the expression in response to BCG stimulation increases in the 7 days following challenge. The kinetics of *IFNG* expression also differ between groups- all Group A volunteers have increased expression of *IFNG* in the stimulated samples 2 days post-challenge compared to baseline whereas in Group C a subset of volunteers already have a higher expression of *IFNG* at baseline and this is simply maintained. These differences likely reflect the presence of an adaptive immune response in Group C volunteers but not Group A. In the unstimulated samples, the most visible difference between the two groups is the number of differentially expressed genes (DEGs), perhaps reflecting a greater magnitude in the response to BCG in previously exposed volunteers.

Table 5.5.2 Table of GO terms enriched in in Group A unstimulated PBMC. In the BCG naive group, the differentially expressed genes during the 14 day challenge period in the unstimulated PBMC were enriched for the following gene ontology terms

Term	Count	%	P-Value	Benjamini
immune response	33	10.9	4.50E-08	8.10E-05
regulation of cytokine production	14	4.6	6.40E-06	5.70E-03
defense response	26	8.6	1.50E-05	9.30E-03
chemical homeostasis	23	7.6	2.20E-05	1.00E-02
homeostatic process	27	8.9	1.50E-04	5.40E-02

Table 5.5.3 Table of GO terms enriched in in Group A BCG-stimulated PBMC. In the BCG naive group, the differentially expressed genes during the 14 day challenge period in the BCG stimulated PBMC were enriched for the following gene ontology terms

Term	Count	%	P-Value	Benjamini
immune response	25	12.1	7.30E-07	1.30E-03
defense response	23	11.2	1.40E-06	1.30E-03
response to wounding	21	10.2	2.00E-06	1.20E-03
inflammatory response	16	7.8	3.80E-06	1.70E-03
phagocytosis	7	3.4	1.50E-05	5.50E-03
positive regulation of multicellular organismal process	12	5.8	9.70E-05	2.90E-02
response to organic substance	21	10.2	1.60E-04	4.20E-02
regulation of cell proliferation	22	10.7	1.90E-04	4.20E-02
monosaccharide metabolic process	11	5.3	2.00E-04	4.00E-02
negative regulation of cell proliferation	14	6.8	2.20E-04	3.90E-02
hexose metabolic process	10	4.9	3.20E-04	5.10E-02

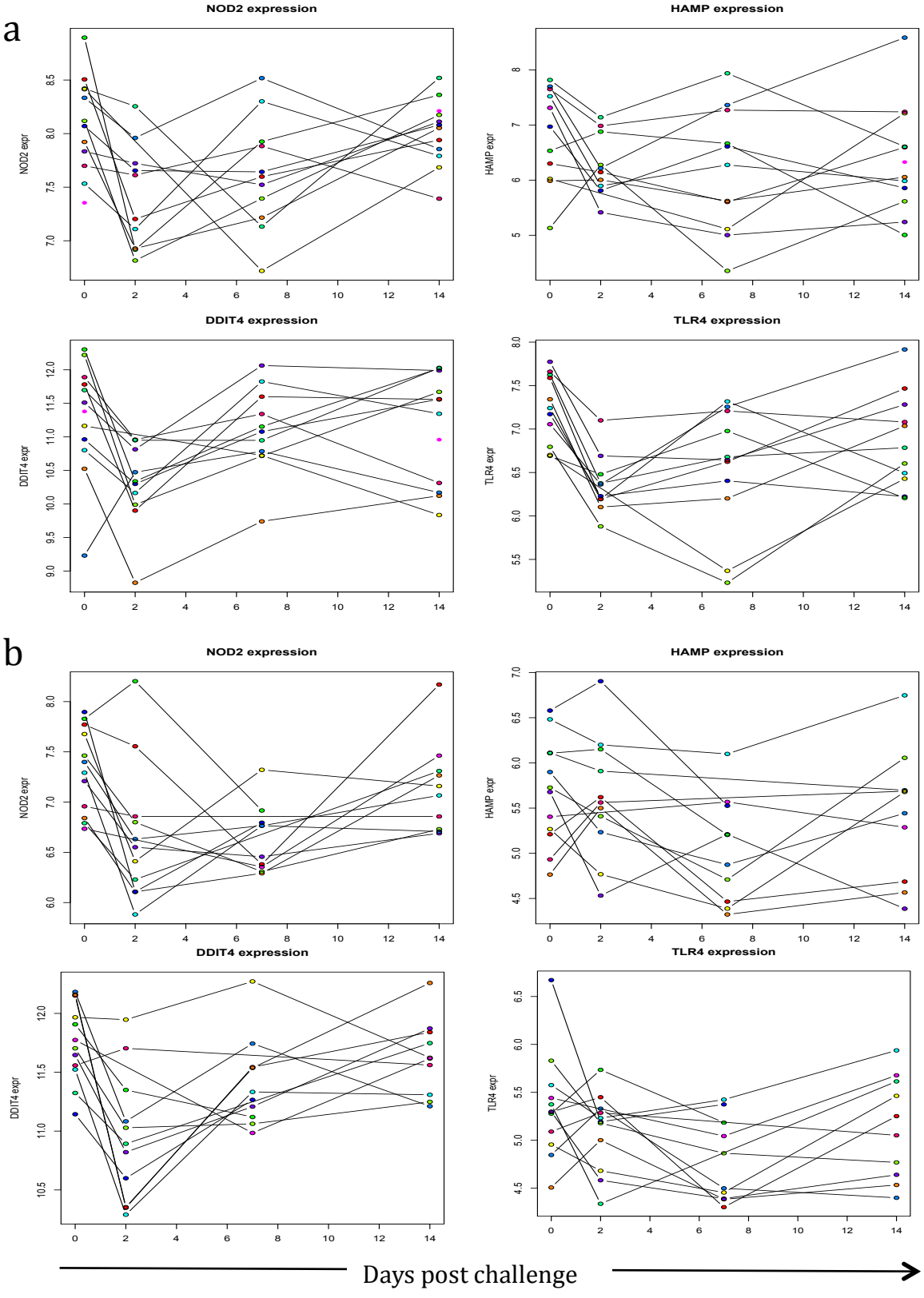


Figure 5.5.1 Change in gene expression over the 14 day challenge in unstimulated PBMC shown for individual volunteers in the BCG-BCG group (A) and the BCG naïve group (B). The four representative genes shown are *NOD2, HAMP, DDIT4* and *TLR4*; colours indicate volunteer number.

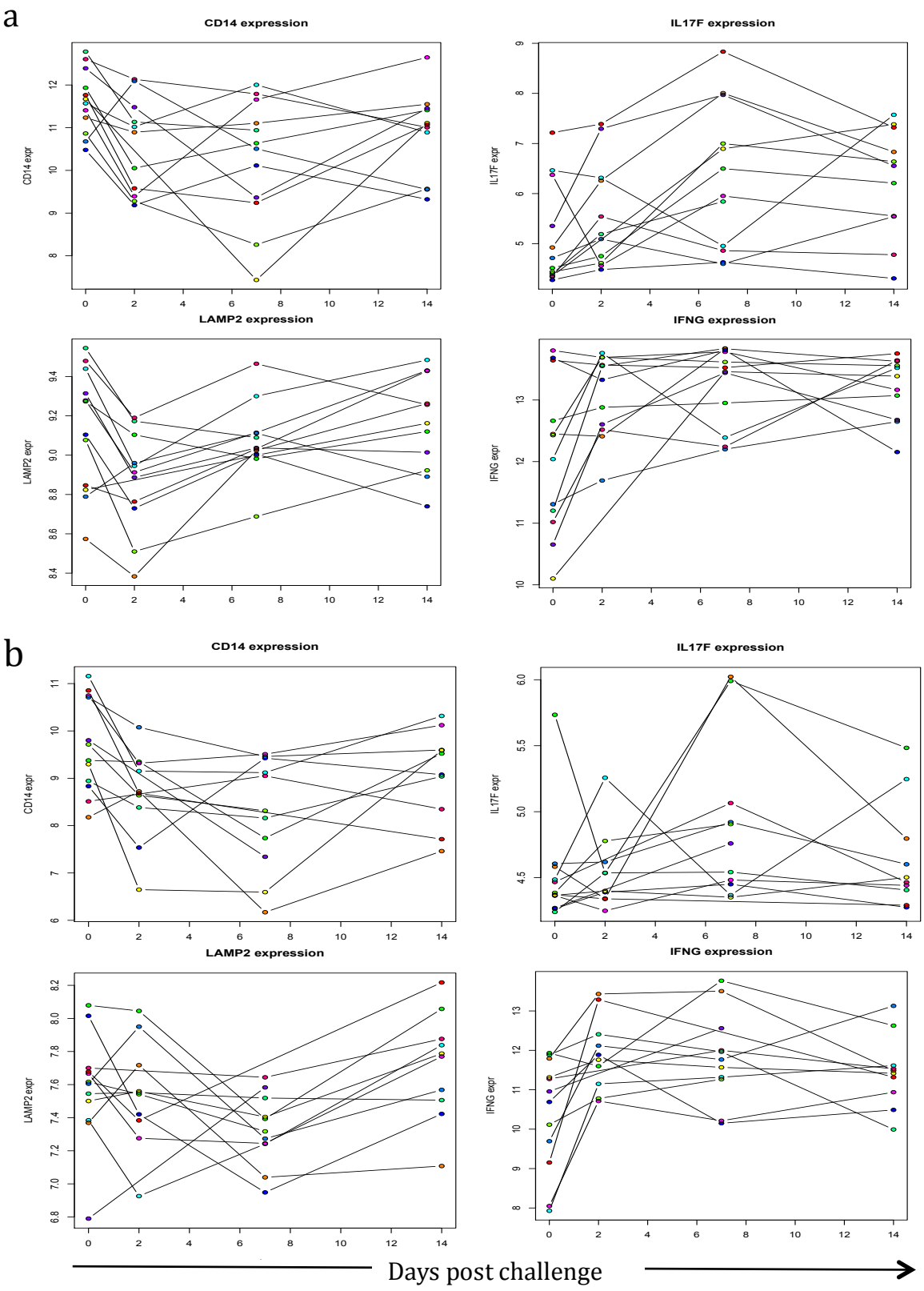


Figure 5.5.2 Change in gene expression over the 14 day challenge in BCG-stimulated PBMC shown for individual volunteers in the BCG-BCG group (A) and the BCG naïve group (B). The four representative genes shown are *CD14, IL17F, LAMP2* and *IFNG*; colours indicate volunteer number.

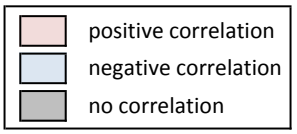
5.6 Linking gene expression and BCG growth

Due to separate processing of samples from the two groups, there were some technical differences between the two sets, which had to be addressed in the analysis. The two sets of samples were combined for pre-processing and fold change between stimulated and unstimulated samples determined. This ratio was used for the subsequent analyses.

BCG growth was quantified by qPCR as part of the trial for each volunteer, from a biopsy taken at the site of vaccination two weeks post-challenge. Dividing the volunteers into groups of "good" and "bad" controllers did not yield any informative results, likely due to smaller group sizes and decreased sensitivity. As most "good controllers" were in Group C and most "bad controllers" in Group A, there may also have been noise due to technical factors. I reduced the gene list to 85 genes which were differentially expressed between Groups A and C at different time points and which fell within the gene ontology lists relating to the immune system or the lysosome. I then looked for associations between gene expression at different time points and BCG growth using linear regression analysis. The genes shown in Table 5.6.1 correlated with BCG growth on the days shown. These genes did not show different expression in unstimulated PBMC but did show differences following BCG stimulation; associations are shown for representative genes in Figure 5.6.1, Figure 5.6.2 and Figure 5.6.3

Table 5.6.1 Table of correlations between gene expression and BCG growth. Gene expression is the ratio of expression in BCG-stimulated/unstimulated samples. BCG growth was measured by qPCR of homogenized biopsy tissue.

	D0		D2		D7		D14	
gene	r	p	r	p	r	p	r	p
NOD2	0.629	0.001					0.514	0.024
CLEC4A	0.633	0.001						
LILRA5	0.489	0.015			0.474	0.035	0.551	0.015
LILRA6	0.531	0.008					0.596	0.007
LILRB2	0.522	0.009			0.541	0.014	0.561	0.012
CXCL3	-0.588	0.003						
IL17F	-0.509	0.011			-0.657	0.002	-0.770	0.000
F3	-0.422	0.040	-0.549	0.015			-0.495	0.031
FAS	-0.478	0.018			-0.496	0.026		
IL5					-0.541	0.014	-0.618	0.005
IFNG					-0.632	0.003	-0.495	0.031
IL2			-0.568	0.011	-0.504	0.024	-0.637	0.003
IL22			-0.486	0.035	-0.626	0.003	-0.646	0.003
NDFIP2					-0.629	0.003	-0.563	0.012
FCGR1B					0.493	0.027	0.572	0.011



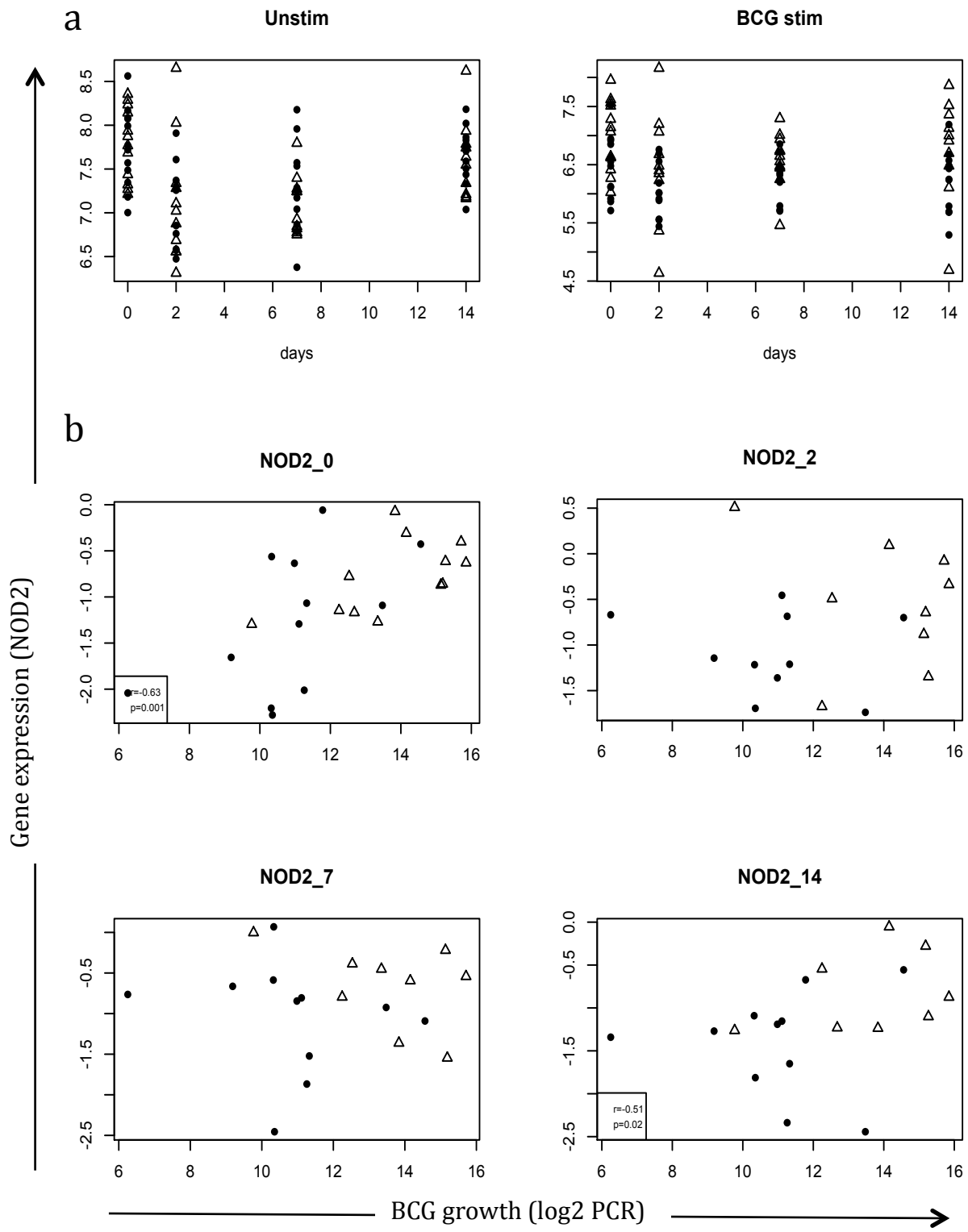


Figure 5.6.1 *NOD2* fold change in expression following BCG stimulation correlates with BCG growth. A. Gene expression in unstimulated and stimulated PBMC. B. Correlations with BCG growth measured by PCR from biopsy samples. Graphs show both Group A (BCG naïve, open triangles) and Group C (BCG-BCG, filled circles).

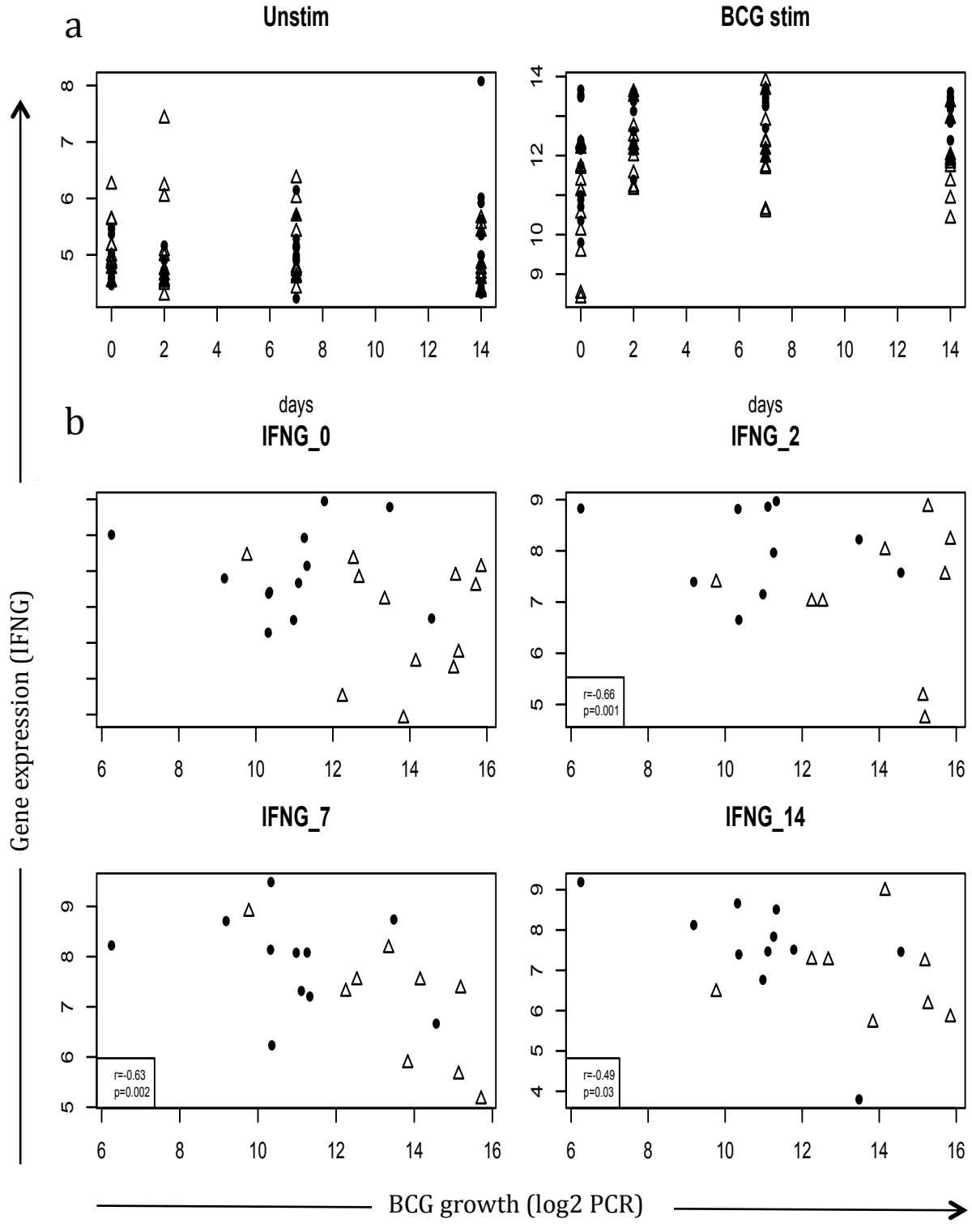


Figure 5.6.2 *IFNG* fold change in expression following BCG stimulation correlates with BCG growth. A. Gene expression in unstimulated and stimulated PBMC. B. Correlations with BCG growth measured by PCR from biopsy samples. Graphs show both Group A (BCG naïve, open triangles) and Group C (BCG-BCG, filled circles).

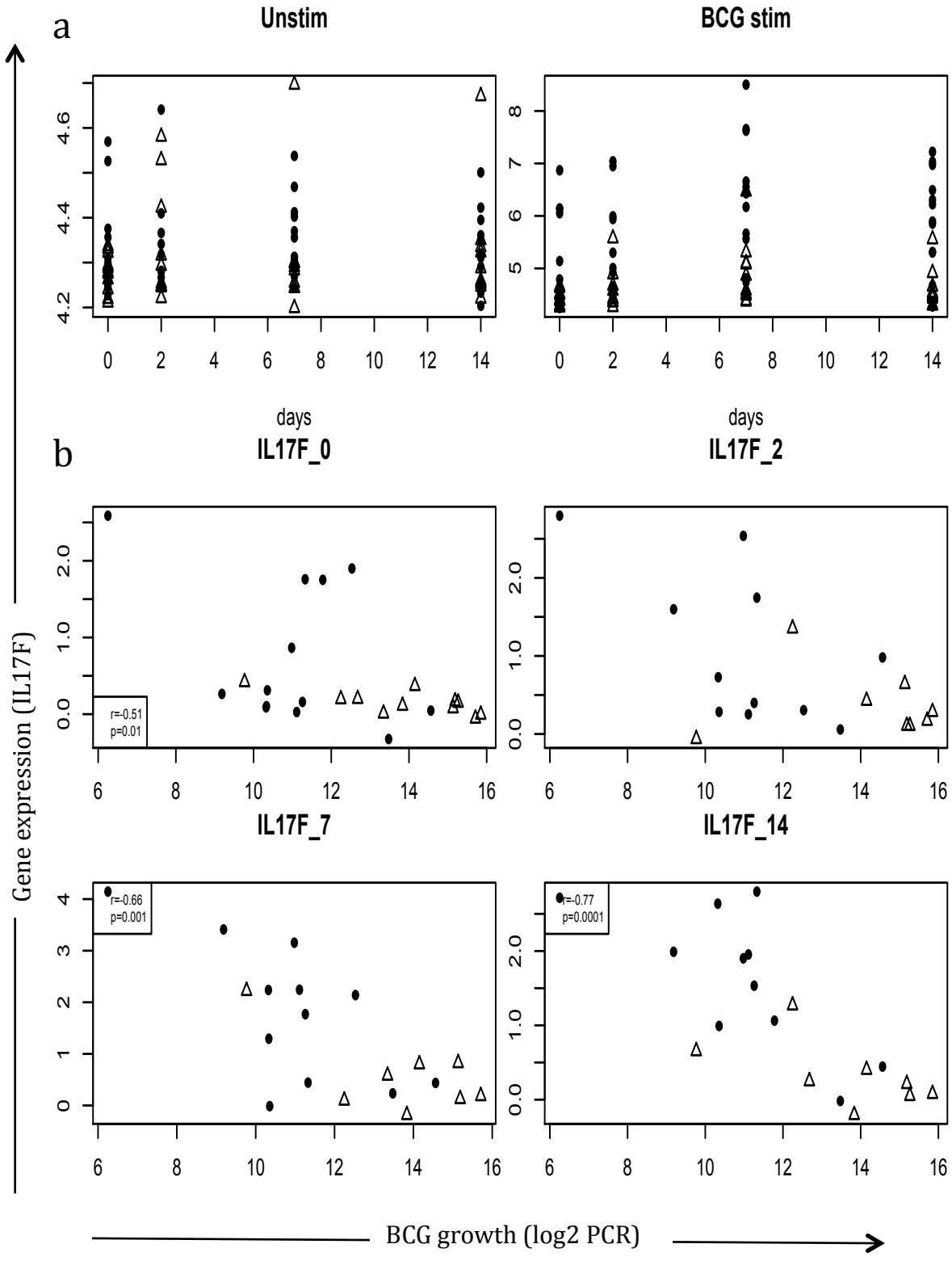


Figure 5.6.3 *IL17F* fold change in expression following BCG stimulation correlates with BCG growth. A. Gene expression in unstimulated and stimulated PBMC. B. Correlations with BCG growth measured by PCR from biopsy samples. Graphs show both Group A (BCG naïve, open triangles) and Group C (BCG-BCG, filled circles).

Photos of the BCG vaccination site taken during the 14 days of challenge showed a large variation in the severity of the local response to BCG including the extent of redness, swelling and ulceration. Volunteers for whom photos were available on day of biopsy were ranked in order of increasing reactogenicity (Figure 8.1.3 in the appendix). This rank correlated inversely with BCG growth (volunteers with more ulceration had lower levels of BCG recovered from the biopsy). Scar rank on day of biopsy also correlated with *IL17F* expression (stimulated/unstimulated) on days 0, 7 and 14 as shown in Figure 5.6.4 and less strongly with the following genes: *IFNG* (day 0) and LILRA6 (days 0 and 14) FCGR1B (day 7) ($0.01<p<0.05$ for all).

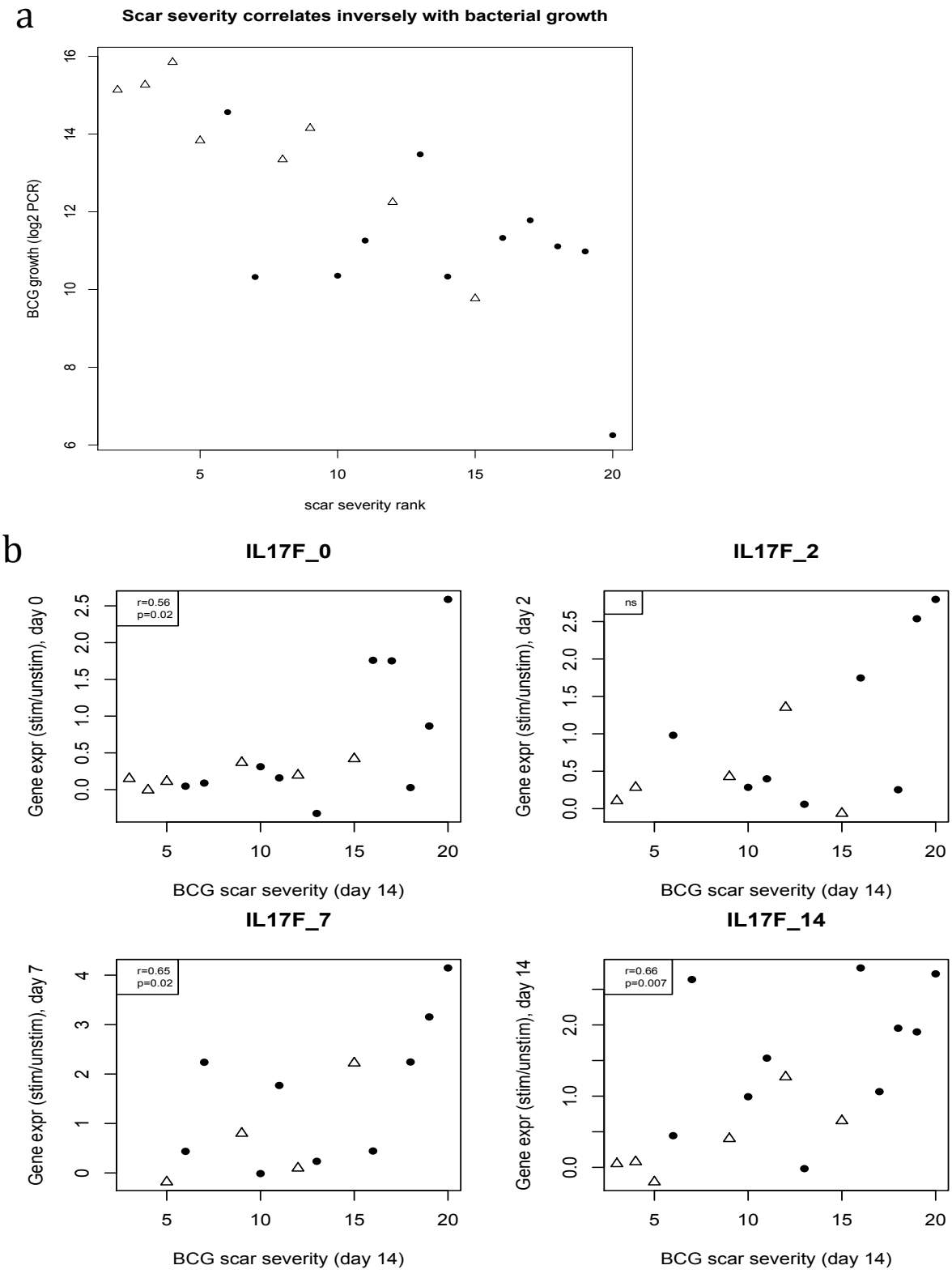


Figure 5.6.4 Scar severity correlates with control of mycobacterial growth and fold upregulation of inflammatory genes, including *IL17F*. A. Scar severity correlates inversely with recovered mycobacteria. B. *IL17F* fold upregulation in response to BCG stimulation on days 0,7 and 14 correlates with scar severity rank (1=least severe, 20= most severe). Graphs show both Group A (BCG naïve, open triangles) and Group C (BCG-BCG, filled circles).

5.7 Discussion

BCG has been administered to more than 3 billion people since its introduction over 90 years ago. During this time, the prevalence of tuberculosis has fallen dramatically in some countries but in others it is higher than ever. Many factors are responsible for this, including the variable efficacy of BCG, which in many parts of the world fails to protect against pulmonary tuberculosis in particular. The samples used in this experiment were taken from a trial conducted in UK adults to evaluate the use of BCG in a human challenge model to assess anti-mycobacterial immunity (Harris et al., submitted). BCG is known to be highly effective in this population, giving around 80% protection [180]. The aims of the experiments in this chapter were to characterise the kinetics of the immune response to BCG in naïve and previously vaccinated volunteers and to look for correlations between these immune parameters and the number of mycobacteria recovered from the site of injection at the end of the challenge period.

Unlike MVA, BCG is a live, slowly replicating organism able to persist at the site of infection for a month or more [181]. Therefore it was surprising that the peak change in gene expression was two days post-vaccination, with a return to baseline by day of biopsy two weeks later. It is likely that continuing adaptive immune responses to the BCG were too small to be detected from microarray analysis of circulating whole blood or PBMC. Far fewer genes were seen in the PAXgene sample than the PBMC, which is likely due to the masking of differences by noise from the globin mRNA signal. If this experiment were to be repeated, it would likely be beneficial to perform globin clear prior to mRNA amplification as RNA quantity was not rate limiting. Of the genes identified in the PAXgene samples however, the biggest changes were also seen 2 days post-vaccination, in agreement with the PBMC results. In the stimulated PBMC,

changes in expression also peaked two days post vaccination for many genes. In both the stimulated and unstimulated samples, these changes may reflect a change in the relative proportions of circulating cells as well as an expansion of effector T cells, particularly in the BCG-BCG group. Although full blood counts were only done prior to the start of the trial, stimulated and unstimulated whole blood was fixed and frozen for flow cytometry analysis on day of challenge, day 2 and day 14 (day of biopsy). Once these samples have been processed, it will be possible to see whether these changes are attributable to fluctuations in populations of circulating cells.

The lists of DEGs between different time points were highly concordant between the two groups, showing a high degree of innate immunity to BCG. In the previously vaccinated Group C however, the fold changes were much higher indicating that prior exposure to BCG increases the magnitude of the immune response. In the unstimulated samples, a larger number of DEGs were identified in Group C compared to Group A, likely due in part to increased fold changes. These observations are consistent with previous microarray studies done in naïve and BCG-vaccinated mice challenged with BCG [182]. Although the two groups showed very similar profiles of DEGs (both unstimulated and BCG-stimulated), fold changes were much higher in the vaccinated mice. Additionally, similar pathways were modulated in both human volunteers and mice, with upregulation of *IFNG*, *TNF* and *CSF2* and downregulation of the lysosome pathway, implying BCG induces similar responses in both mice and adults in the UK.

The BCG challenge model allows associations to be made between different immune parameters and BCG growth. In this trial, previously BCG-vaccinated volunteers had significantly lower amounts of BCG recovered from the injection site than previously

naïve volunteers, consistent with the protective effect of BCG in this population. This was therefore a good opportunity to identify potential correlates of mycobacterial control. Genes whose change in expression in the stimulated compared to unstimulated samples showed an association with BCG growth included *IFNG* and *IL17F* and other genes associated with these two cytokines such as *NOD2, IL22, IL23A* and *FCGR1B*. Several recent studies have reported important roles for these cytokines in protection from *M.tb*. IFN-γ is known to be necessary, though not sufficient, for protection and recent studies also suggest an important role for Th17 cells and the IL-23/IL-17 pathway. The latter can provide partial protection from *M.tb* challenge and have been shown to be necessary drivers of Th1 immunity and IFN-γ responses in the face of IL-10 production during infection [183–185]. In cattle, IL-22 and IFN-γ production by PPD stimulated PBMC were identified as the primary predictors of vaccine-induced protection in an *M. bovis* challenge [186]. Additionally, a recent paper identified NOD2 as a crucial component of epigenetic reprogramming of monocytes following BCG vaccination, which led to nonspecific protective effects [34]. Interestingly, in this model, *IL17F* and *IL22* expression in the BCG-stimulated PBMC peaked 7 days post-challenge in the BCG vaccinated group but showed almost no upregulation in the BCG-naïve group, suggesting these cytokines were part of an adaptive not an innate response to BGC.

The increase in local adverse events following a second BCG vaccination is well documented and was also seen in this study [181,187,188]. The severity of local inflammation correlated with the immune response (*IL17F, IFNG, FCGR1B*) and increased control of mycobacterial growth. The balance between bacterial killing and excessive inflammation is at the core of the relationship between humans and *M.tb.*

These data show too little inflammation fails to control mycobacterial growth whilst a protective immune response comes at the cost of collateral damage. As previously mentioned, BCG is effective in protecting this population from tuberculosis, suggesting the spectrum seen here in Group C may show an optimal balance. In Malawi, where BCG is not protective, vaccinated infants develop smaller scars, weaker Th1 responses and stronger Th2 and regulatory responses in response to PPD-T stimulation than their UK counterparts [50]. Likewise, laboratory mice in Brazil and Mexico show less susceptibility to infection with *M.tb* and also less protection from BCG [189]. Though the data shown here and recent studies suggest a role for the IL-17 pathway in the development of a protective response, excessive activation of this pathway has also been associated with detrimental tissue damage in this and other diseases [94,183,190], and how much of a role it can have in vaccine induced immunity is presently unknown. The human BCG challenge model may become a powerful tool in future vaccine research. Using this model and characterising the response to BCG in populations where BCG is not effective may also give important clues as to why these differences arise and how they relate to control of mycobacterial growth.

6 THE IMMUNE RESPONSE TO MVA85A AND BCG IN INFANTS FROM SOUTH AFRICA

Aims:

To characterise the innate immune response to vaccination with MVA85A and BCG in South African infants and link these findings with results from other immunological assays performed on cells collected pre- and post-vaccination.

To compare the factors affecting the immune response to vaccination in South African infants with previous data from adults in the UK to better understand the differences between these populations.

6.1 Introduction

The samples analysed in this chapter are taken from the recently completed Phase IIb placebo-controlled trial of the safety and efficacy of MVA85A in South African infants previously vaccinated with BCG [43]. The availability of matched data from three timepoints, with multiple assays being performed at two timepoints, allowed me to investigate the immune response to vaccination in this population in much greater depth. I was then able to compare findings with previous work done in vaccinated UK adults to compare the two populations.

Many early phase vaccine trials are conducted in UK adults, although infants in developing countries are often the target population. A decrease in vaccine immunogenicity between adults in the UK and in African countries has been documented [191] and responses are further reduced in African children [138,192]. Understanding the immunological basis for these differences, and whether these are

relevant to protection, is a key question in vaccine development. In the efficacy trial from which these samples were taken, ELISpot responses to Ag85A were low and it has been suggested that this could be a reason for the lack of efficacy observed in the trial [141]. Future work on samples from this trial to identify correlates of disease risk and protection may help address this question. In this chapter, the aim was to look for associations between gene expression and other immune parameters and individual responses to vaccination. I looked at responses to MVA85A and also BCG, which all infants received within 7 days of birth. Comparing these data to previous studies done in adults may start to give some insight as to why many vaccines are less immunogenic in the developing world and in children. A summary of samples used in this chapter and when these were taken is shown in Figure 6.1.1.

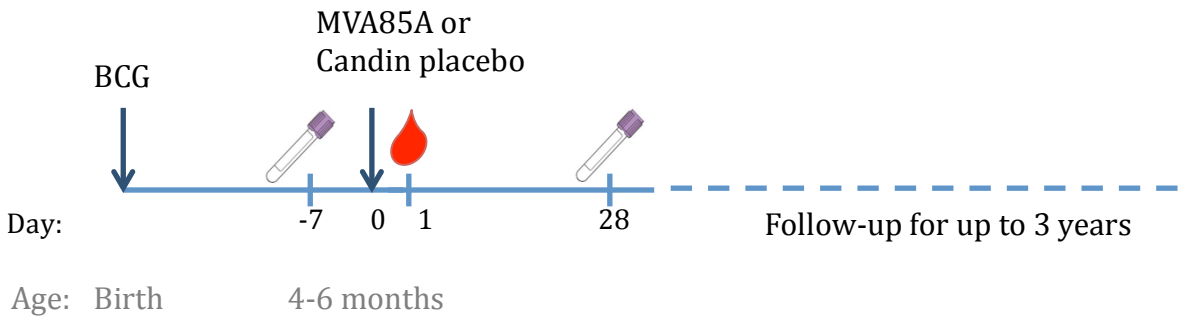


Figure 6.1.1 TB020 sample collection. All infants had PBMC collected in CPT tubes and cryopreserved 7 days pre- and 28 days post-vaccination. 100 infants had an additional heelstick whole blood sample taken 1 day post vaccination for gene expression analysis. All infants received BCG within 7 days of birth, were enrolled at 4-6 months of age and were randomized to receive either MVA85A or a candin placebo.

6.2 TB020: whole blood heelstick samples 1 day post-vaccination

A small volume of whole blood was collected from 100 infants 1 day post-vaccination, with either MVA85A or a candin placebo, and stored for gene expression analysis. The volume of whole blood collected varied between infants but was approximately 50-300μL. The RNA was extracted and amplified as described in the methods and hybridized to Illumina HT-12v4 beadchips. Quality was assessed using the bioconductor arrayQualityMetrics package and 83 samples of good quality were retained for analysis.

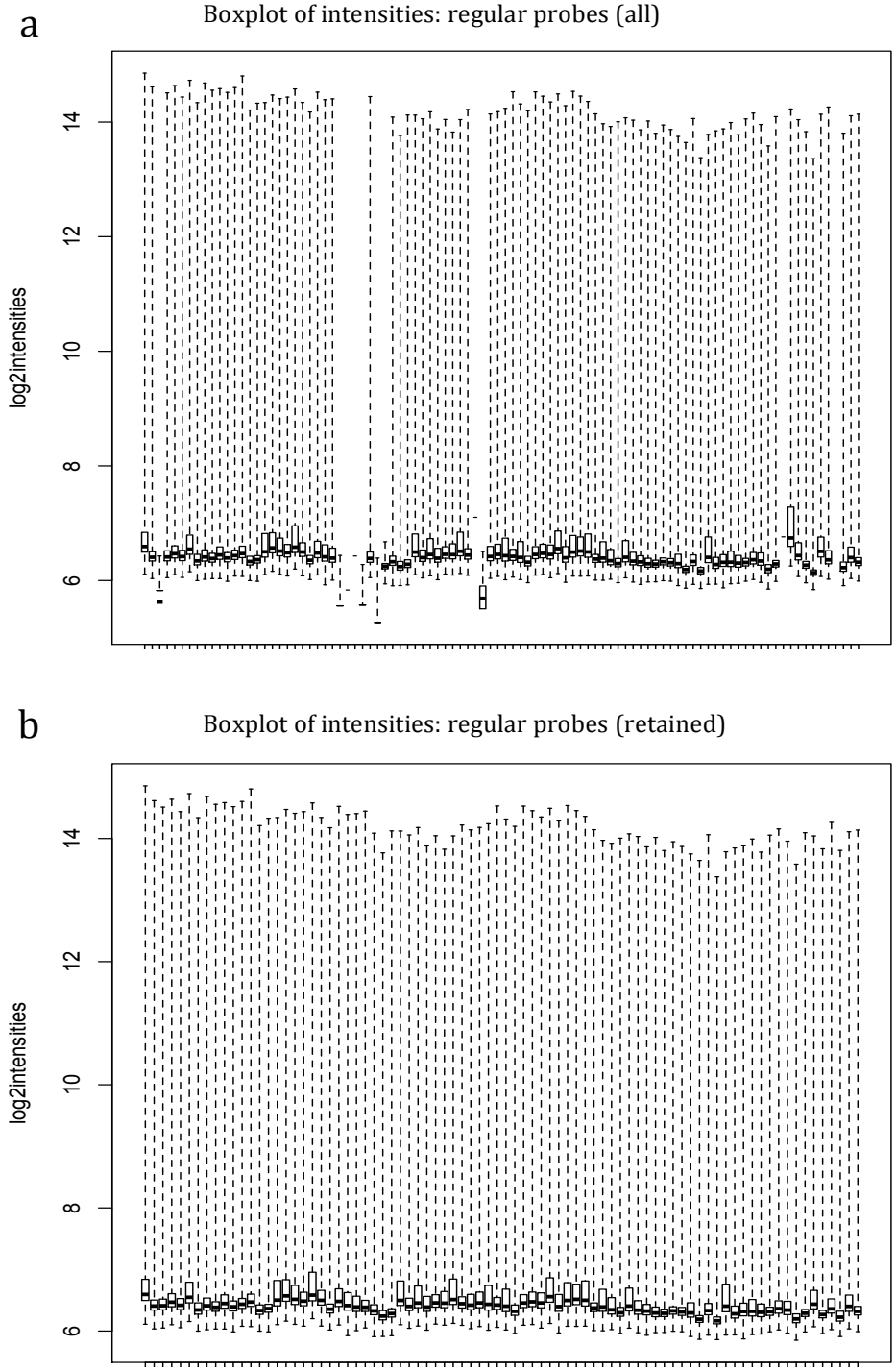


Figure 6.2.1 Boxplots showing median, interquartile range and 5th and 95th percentiles of intensity for each array prior to background correction, normalisation and filtering on a log 2 scale. a. all 96 samples. b. 83 samples retained for analysis

Sample processing and preliminary analysis were carried out prior to unblinding of the trial. Using principal component analysis, the samples were classified into two

groups and a list of differentially expressed genes determined with the aim of seeing whether this could correctly classify vaccinated infants from those in the placebo group. The results of this are shown as a heatmap in Figure 6.2.2. Once the samples were unblinded, it became clear that the principal component analysis had identified two clusters of infants showing different ranges of inflammatory responses but that these ranges overlapped between the vaccinated and placebo groups. Differential expression analysis between vaccinated and placebo samples identified a much smaller list of 32 genes (Table 6.2.1). Using these genes to create a heatmap segregates the samples into three clusters: one of vaccinated infants, one of placebo infants and one mixed (Figure 6.2.3a). As an example, Figure 6.2.3b shows the expression of *CXCL10* 1 day post vaccination in all infants. Vaccinated infants exhibit a more inflammatory gene expression profile than those in the placebo group however the range is large in both groups and there is a big overlap.

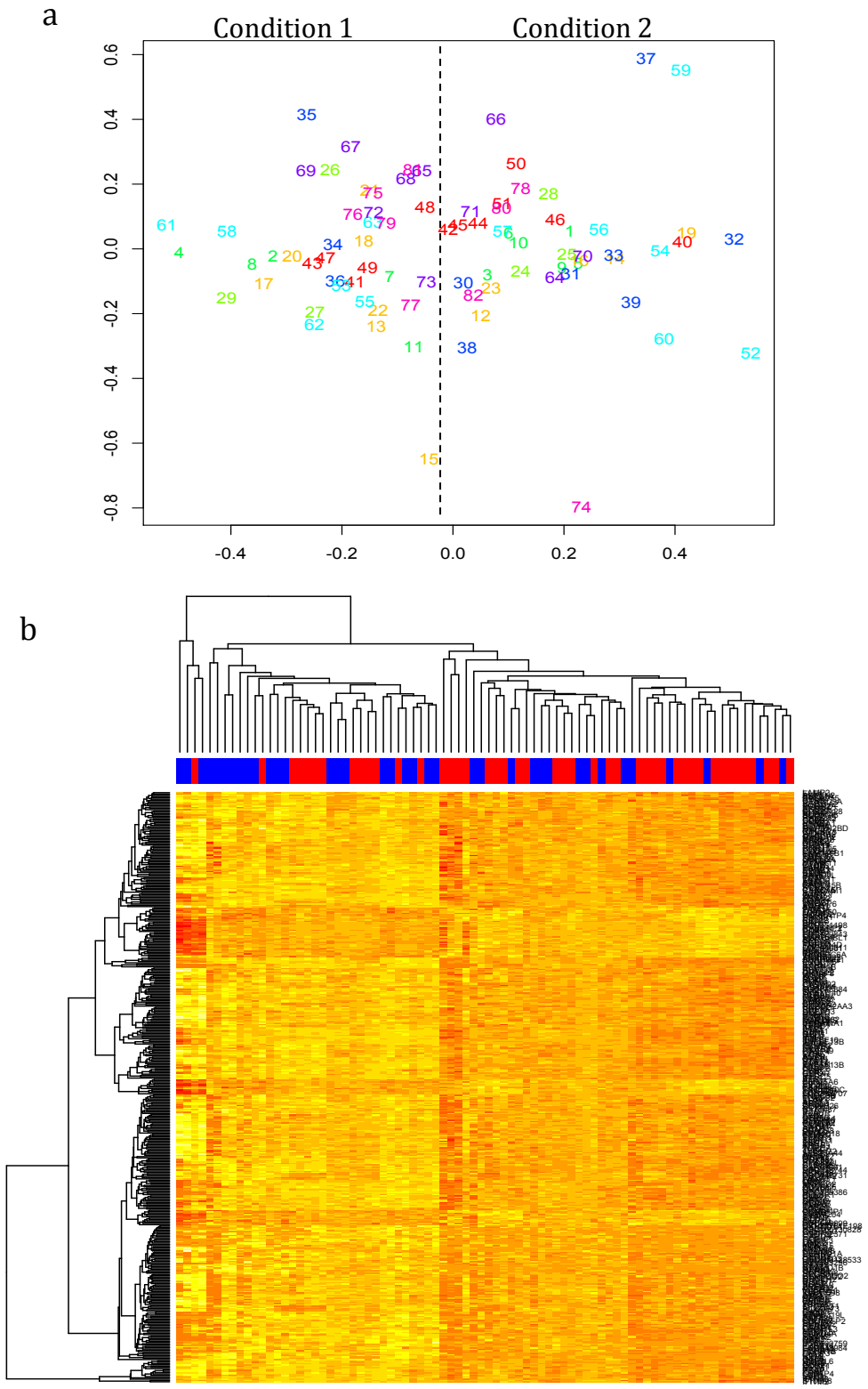
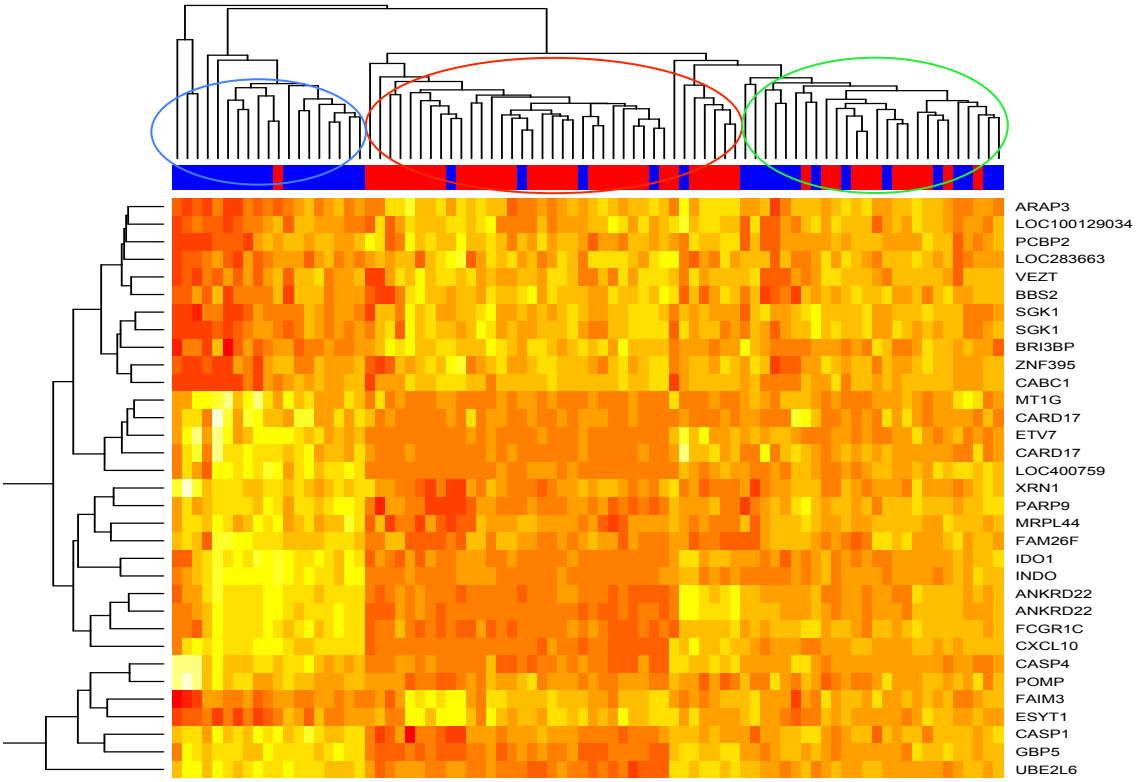


Figure 6.2.2 a. The 83 infants can be split into two groups based on multidimensional scaling (colouring shows chip). b. Heatmap drawn using the genes differentially expressed between the two groups in a (identified in limma, fdr set at 0.05). These genes are mostly associated with inflammation, separating infants into those with more or less "inflammatory" phenotype. The more inflammatory group has more infants vaccinated with MVA85A (blue banner) than placebo (red banner) however the clusters are mixed. Yellow shows upregulation.

Table 6.2.1 Table of DEGs between vaccinated and placebo infants 1 day post-vaccination.

PROBE_ID	SYMBOL	Fold change	AveExpr	adj.P.Val
ILMN_1791759	CXCL10	3.20	7.18	0.04
ILMN_1799848	ANKRD22	2.61	6.65	0.04
ILMN_1656310	INDO	2.53	5.68	0.04
ILMN_2114568	GBP5	2.52	8.70	0.02
ILMN_2132599	ANKRD22	2.46	7.09	0.04
ILMN_3239965	IDO1	2.35	6.17	0.04
ILMN_3247506	FCGR1C	2.13	6.59	0.04
ILMN_1782487	LOC400759	1.96	5.38	0.01
ILMN_2066849	FAM26F	1.89	5.99	0.04
ILMN_1809086	XRN1	1.74	6.83	0.04
ILMN_2053527	PARP9	1.67	6.81	0.04
ILMN_1769520	UBE2L6	1.61	11.07	0.04
ILMN_1707979	CARD17	1.57	5.07	0.04
ILMN_2326509	CASP1	1.57	8.86	0.04
ILMN_1671452	MRPL44	1.50	6.05	0.04
ILMN_1700671	ETV7	1.49	4.83	0.04
ILMN_1678454	CASP4	1.48	9.83	0.04
ILMN_1715401	MT1G	1.43	4.78	0.01
ILMN_3238525	CARD17	1.35	4.60	0.04
ILMN_1693287	POMP	1.32	9.68	0.04
ILMN_1761159	ESYT1	-1.34	8.78	0.05
ILMN_1693410	BRI3BP	-1.37	6.56	0.04
ILMN_1774828	VEZT	-1.40	5.72	0.04
ILMN_3236036	LOC283663	-1.41	5.39	0.04
ILMN_3256478	LOC100129034	-1.41	5.38	0.04
ILMN_3240997	ARAP3	-1.43	5.70	0.04
ILMN_1767612	BBS2	-1.44	5.60	0.04
ILMN_1775542	FAIM3	-1.45	9.66	0.04
ILMN_3251155	PCBP2	-1.48	5.49	0.01
ILMN_1772876	ZNF395	-1.50	6.21	0.04
ILMN_3305938	SGK1	-1.56	6.59	0.04
ILMN_1731064	CABC1	-1.59	6.10	0.02
ILMN_3229324	SGK1	-1.60	6.30	0.04



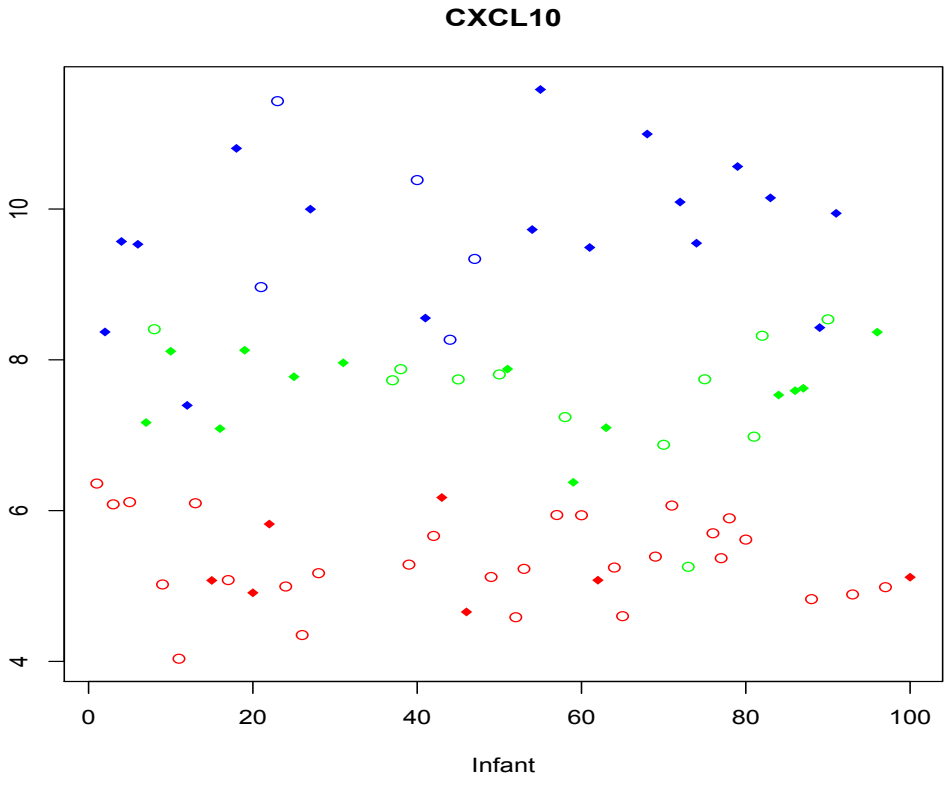


Figure 6.2.3 The range of "inflammatory" gene expression overlaps between vaccinated and unvaccinated infants. Heatmap of 33 DEGs between vaccinated and placebo infants (limma, fdr<0.05) 1 day post-vaccination. The infants fall into 3 clusters: placebo, vaccinated and mixed. CXCL10 expression: coloured by cluster as shown on the heatmap. Open circles: placebo, closed diamonds: MVA85A vaccinated.

6.3 TB020 PBMC: pre- and post-vaccination

All infants in the TB020 trial had PBMC collected and cryopreserved 7 days pre- and 28 days post-vaccination. As part of a pilot study to select assays for a future study on correlates of tuberculosis disease risk in infants from the trial, pre- and post-vaccination samples were processed for 60 infants and a number of assays performed on the cells. The samples were taken from the group of infants who had also had day 1 samples collected so that the results from the different assays could also be compared to the gene expression data from this timepoint. Cell recovery was variable between samples (range: 2-40 million PBMC, median 15 million) so not all assays were performed on each sample. The table below summarises the assays performed in this pilot study.

Table 6.3.1 Table of samples processed for each assay in the pilot study.
Maximum: 60 infants with two timepoints each.

Assay	Samples processed
Gene expression: unstimulated, BCG stimulated	60
Gene expression: Ag85A 66 peptide pool stimulation	20
IFN-γ ELIspot (unstim, PHA, 85A, PPD, BCG)	99
Cell surface flow cytometry	110
MGIT assay (mycobacterial growth inhibition) (BCG)	105
Ki67 assay	85

6.4 Dissecting the response to Ag85A peptide stimulation in vaccinated infants

Studies in infants are limited by the availability of cells on which to perform immunological assays and one of the aims of this pilot study was to investigate any correlations between assays such that cell use could later be maximized through the use of assays providing non-redundant information. We found a significant correlation

between assays using Ag85A peptide pool to stimulate PBMC, namely the *ex-vivo* IFN-γ ELISpot, gene expression and the Ki67 proliferation assay.

6.4.1 Inter-assay correlations: Ag85A 66 peptide pool

Table 6.4.1.1 shows the differentially expressed genes following Ag85A peptide stimulation when comparing either vaccinated infants pre and post vaccination or placebo with vaccinated infants 28 days post vaccination. Figure 6.4.1 shows a correlation matrix of these genes in all Ag85A stimulated samples showing their expression is highly correlated. Furthermore, expression of these genes separates those infants making a response to Ag85A as measured by ELISpot from the non-responders on ELISpot as shown in Figure 6.4.2 for unstimulated and Ag85A stimulated samples. By contrast, the relationship between *STAT1* expression and IFN-γ ELISpot following stimulation with BCG is less clear. Additionally, the Ki67 assay correlated strongly with the IFN-γ ELISpot results (r=0.83, p=0.0002, Spearman's correlation day 28 MVA85A group).

Table 6.4.1 Table of DEGs in Ag85A stimulated samples: vaccinated vs unvaccinated

Vaccinated infants: day 28- day-7

SYMBOL	logFC	Fold change	adj.P.Val
LOC400759	2.04	4.11	0.0047
GBP5	2.16	4.46	0.0079
LOC730249	1.93	3.82	0.0079
WARS	1.37	2.58	0.0079
ANKRD22	1.67	3.19	0.0180
GBP4	1.40	2.63	0.0180
WARS	1.25	2.38	0.0180
TAP1	0.79	1.73	0.0234
AIM2	1.05	2.07	0.0251
PSME2	0.72	1.65	0.0251
CXCL9	1.63	3.09	0.0259
GBP1	1.90	3.72	0.0349
STAT1	1.15	2.22	0.0354
STAT1	1.32	2.49	0.0476
STAT1	*0.92*	*1.89*	*0.0746*
GBP1	*1.87*	*3.66*	*0.0911*
CD38	*0.80*	*1.74*	*0.0925*
CXCL10	*2.30*	*4.92*	*0.1295*
LAP3	*1.13*	*2.19*	*0.1295*
CEACAM1	*0.96*	*1.95*	*0.1412*
PARP14	*0.71*	*1.63*	*0.1412*
IFNG	*1.67*	*3.19*	*0.1599*

Day 28 samples: vaccinated- placebo

SYMBOL	logFC	Fold change	adj.P.Val
LOC400759	2.71	6.55	0.012
STAT1	2.69	6.46	0.012
STAT1	2.31	4.96	0.012
CXCL10	3.41	10.64	0.015
CCL8	4.02	16.21	0.027
GBP4	2.19	4.55	0.027
CXCL9	1.95	3.86	0.027
STAT1	1.78	3.44	0.027
WARS	1.69	3.23	0.027
IFI35	1.49	2.80	0.027
WARS	1.73	3.31	0.030
GBP1	2.86	7.28	0.031
PARP9	1.56	2.94	0.031
FBXO6	1.84	3.57	0.037
PARP9	*1.46*	*2.75*	*0.051*
PARP14	*1.24*	*2.36*	*0.051*
PSME2	*1.12*	*2.17*	*0.051*
GBP5	*2.65*	*6.30*	*0.058*
EPSTI1	*1.51*	*2.85*	*0.058*
GBP1	*3.01*	*8.05*	*0.062*
IFNG	*2.17*	*4.51*	*0.062*
P2RX7	*1.77*	*3.42*	*0.062*
SAMD9L	*1.37*	*2.58*	*0.062*
PTER	*1.18*	*2.27*	*0.062*
UBE2L6	*1.14*	*2.21*	*0.076*
IFIT3	*2.35*	*5.09*	*0.080*
SNHG5	*-1.17*	*-2.25*	*0.080*
LOC730249	*2.32*	*5.00*	*0.082*
PLA2G4C	*1.39*	*2.61*	*0.082*

Figure 6.4.1 DEGs in response to Ag85A stimulation are highly correlated. Correlation matrix of DEGs between vaccinated and unvaccinated samples stimulated with Ag85A: Pearson's correlation. Upper table shows r coefficient (highlighted in pink >|0.5|), lower table shows p-value (highlighted in green <0.05).

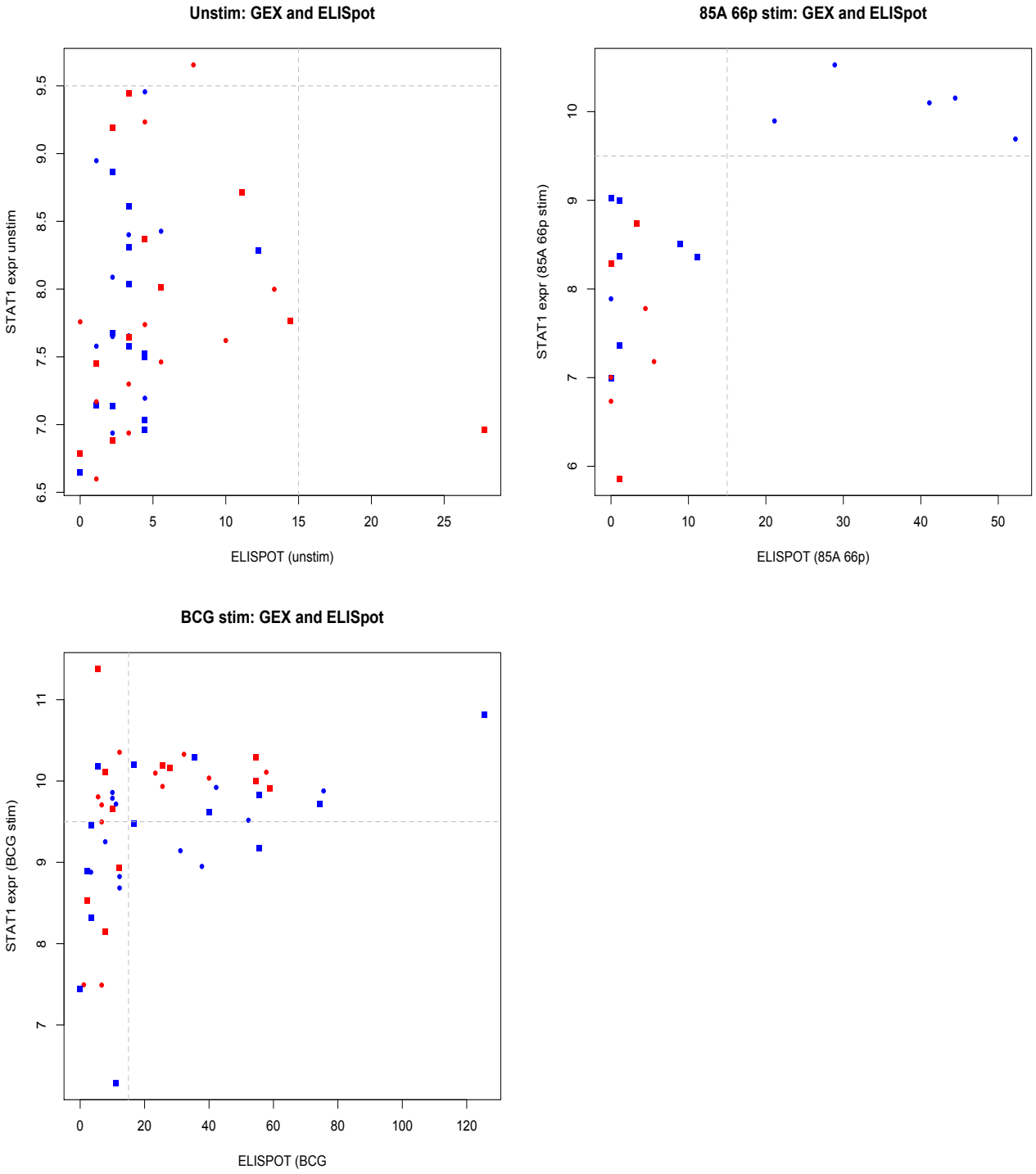
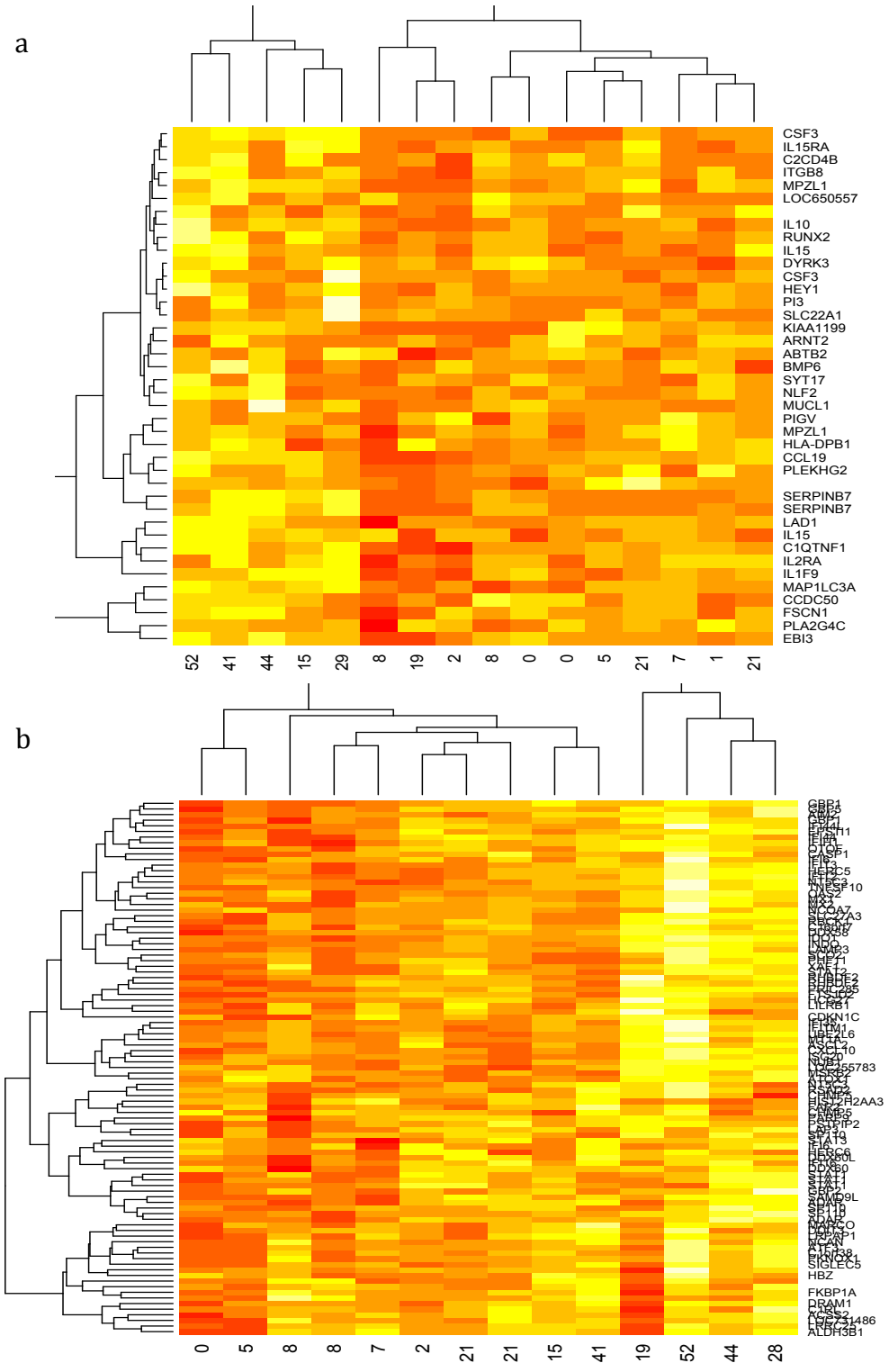


Figure 6.4.2 Gene expression (GEX) of *STAT1* is indicative of ELISpot results in unstimulated and Ag85A stimulated PBMC but not in BCG stimulated PBMC. Lines drawn at x=15 spots/million PBMC and y=9.5 (STAT1 log2 expression intensity). Blue: MVA85A group, red: candin group, circles: day 28, squares: day -7.

6.4.2 Response to vaccination: understanding the response to Ag85A

This study provided a large set of data through which to explore the factors affecting response to vaccination with MVA85A in South African infants. In order to reduce the complexity of the data, I used hierarchical clustering to group together genes showing similar expression levels and calculated an average expression for each set. I then used the generalized linear model package glmnet in R to downselect groups of genes with some predictive power for different outcome measures. In order to make this more accurate, I then reduced the original set of genes to clusters of interest and divided the genes into smaller clusters. This method allowed me to compare data from different timepoints and stimulations as well as including flow cytometry data.

This analysis was most successful when investigating the IFN-γ ELISpot response to Ag85A 28 days post vaccination in vaccinated volunteers. One gene cluster from day -7 unstimulated cells and three gene clusters from day 1 whole blood were identified as predictive of the response. Unsupervised clustering of samples using these genes for the two timepoints are shown in Figure 6.4.3.



ELISpot (85A 66p day 28)

Figure 6.4.3 Expression of select clusters of genes separates samples into ELISpot responders and non-responders. A. One gene cluster from unstimulated day -7 samples. B. Three clusters of genes from day 1 heelstick samples. Both heatmaps have ELISpot responses to Ag85A 66 peptide pools (spots/million PBMC) measured on day 28 on the x-axis.

Selecting just a few genes from each cluster allows clustering of the samples into two groups of infants: one group which develops a response to Ag85A post-vaccination that is measurable by IFN-γ ELISpot (responses of over 15 spots/million PBMC) and another which does not (responses of 15 spots/million PBMC or less). Unsupervised clustering of samples using these genes clusters samples into ELISpot responders or non-responders (Figure 6.4.4). A linear discriminant analysis incorporating two genes on day -7 (*IL15* and *IL2RA*) and 3 genes on day 1 (*IDO1*, *CASP1* and *STAT3*) classified the two groups with a cross-validation accuracy of 100% (Figure 6.4.5). Correlations for selected individual genes are also shown (Figure 6.4.6).

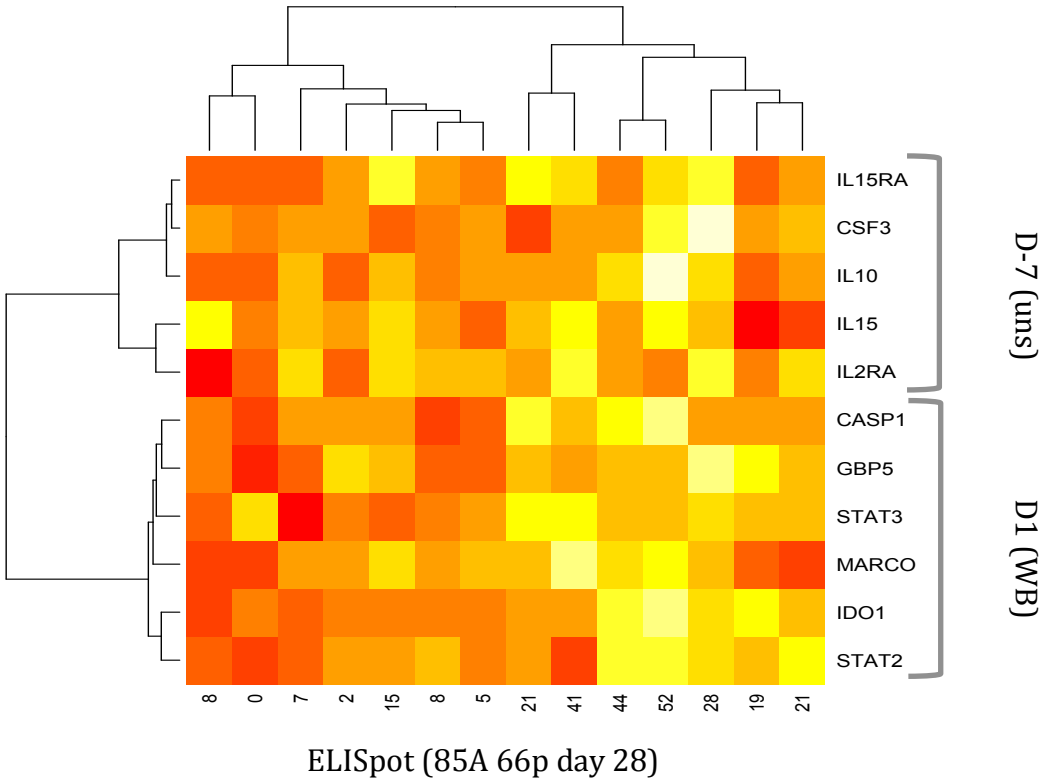


Figure 6.4.4 Samples cluster into responders and non-responders based on the expression of just a few genes from day -7 unstimulated PBMC and day 1 heelstick samples.

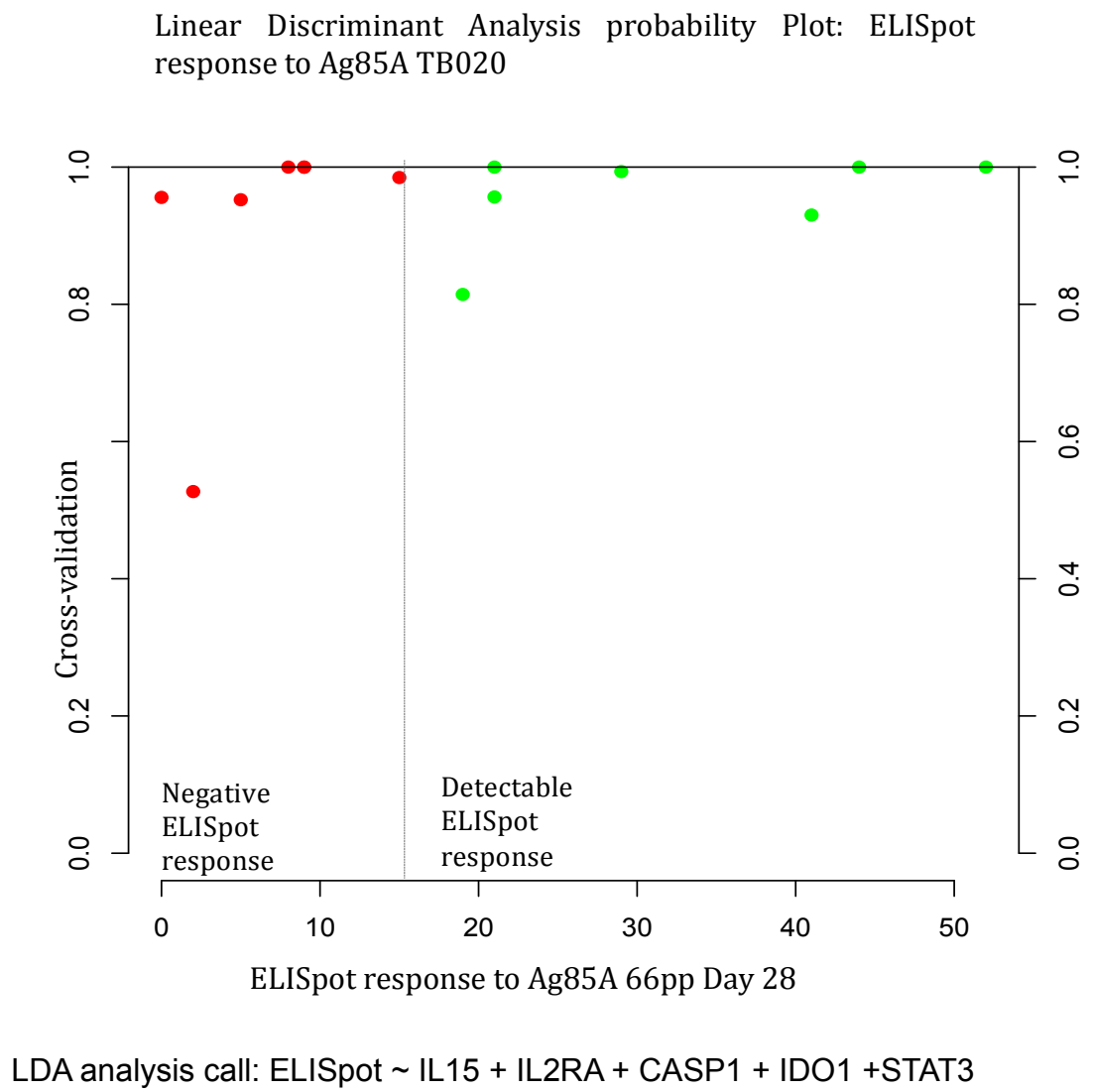


Figure 6.4.5 Probability plot of linear discriminant analysis. Linear discriminant analysis using 5 non-collinear genes from the heatmap shown in the previous figure. Positive ELISpot defined as greater than 15 spots/million PBMC. Cross-validation error rate was 0.

Correlations between gene expression in unstimulated samples and ELISpot response to Ag85A (day 28): *IDO1*, *STAT3* (day 1), *IL10*, *IL15* (day -7)

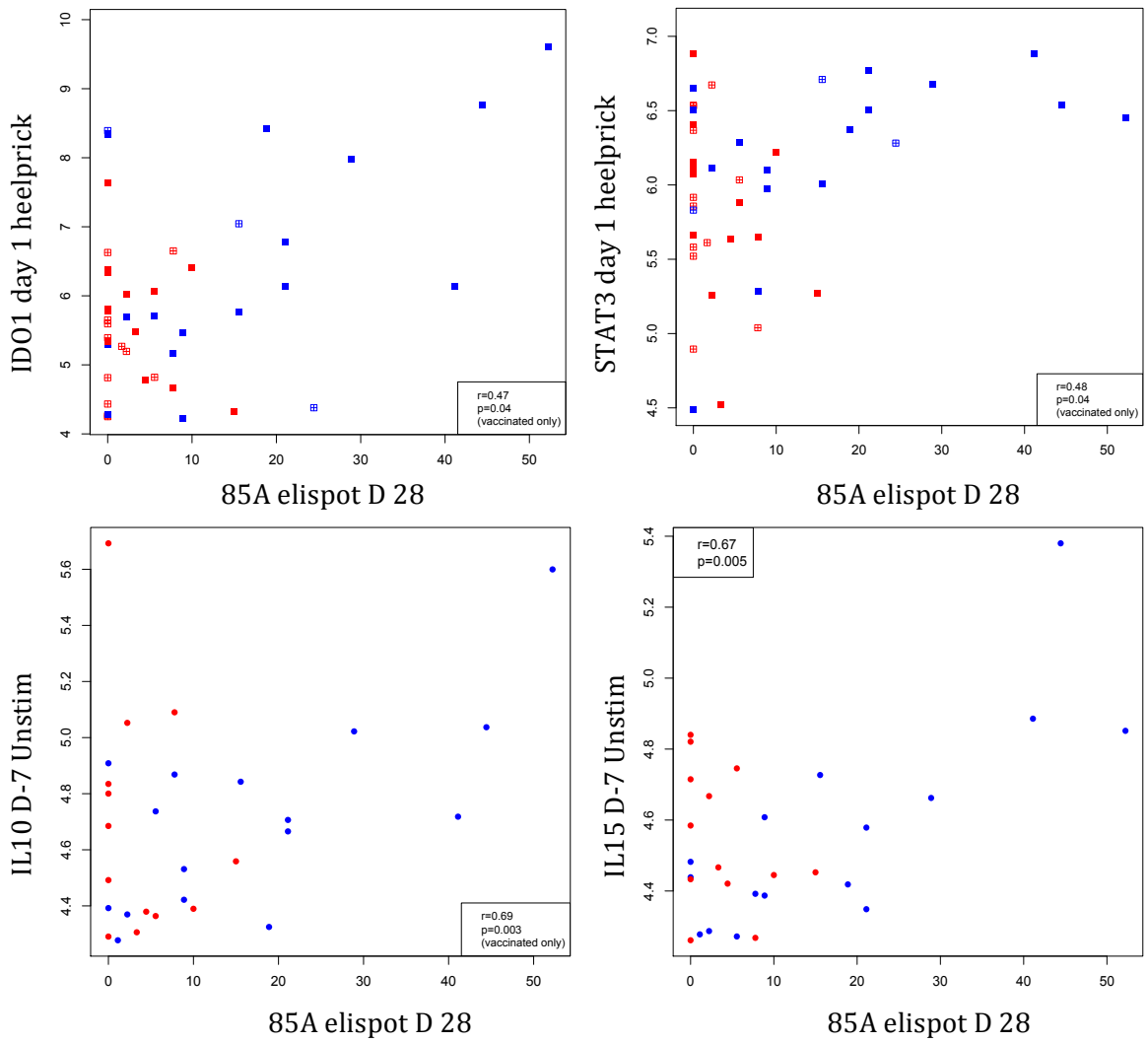


Figure 6.4.6 Expression of several genes pre- and 1 day post-vaccination correlates with ELISpot responses to Ag85A peptide pools measured 4 weeks post-vaccination (Spearman's rank correlation). Selected genes from the LDA analysis: IDO1 and STAT3 one day post-vaccination, IL10 and IL15 7 days pre-vaccination. Red: candin group, blue: MVA85A group, filled: both time points available, crossed centre only day 1 heelprick sample processed.

6.5 BCG stimulation of PBMC

Gene expression analysis was also carried out on BCG stimulated samples pre- and post-vaccination for 30 infants. In contrast to the samples stimulated with Ag85A peptides, I saw no changes in gene expression between samples from BCG only and

BCG-MVA85A infants. This was consistent with the ELISpot data in which we also observed no difference in the response to BCG or PPD between groups or timepoints.

Stimulation of these infant PBMCs with BCG led to differential expression of several thousand genes. Table 6.5.1 shows a subset of these genes showing the greatest fold changes and Table 6.4.4.2 shows the GO terms associated with the list of DEGs.

Table 6.5.1 Table of highly DEGs: BCG stimulated – unstimulated PBMC.

SYMBOL	logFC	AveExpr	adj.P.Val	fold change
CSF2	5.72	10.10	1.17E-53	52.56
TGFBI	-5.12	7.43	1.25E-39	-34.76
CSF1R	-4.80	9.00	1.50E-50	-27.81
LYZ	-4.56	7.78	7.91E-51	-23.66
CD14	-4.55	10.08	8.40E-48	-23.41
CCL7	-4.46	7.75	8.69E-39	-22.00
FPR3	-4.33	6.61	1.53E-39	-20.13
CCL20	3.77	10.58	6.56E-34	13.63
IFNG	3.76	6.48	8.49E-35	13.55
IL1F9	3.69	9.02	8.17E-32	12.90
TNF	3.67	10.46	3.83E-58	12.70
FCN1	-3.55	6.96	9.62E-29	-11.74
CXCL10	3.50	7.02	1.00E-21	11.33
AIF1	-3.30	6.82	5.39E-33	-9.84
MMP1	3.22	7.31	1.95E-22	9.33
F3	3.22	6.50	1.04E-38	9.29
CCL24	-3.04	7.00	8.65E-28	-8.25
GBP5	3.03	8.40	4.37E-31	8.16
VMO1	-2.92	6.24	4.42E-27	-7.57
CCL3L1	2.83	11.48	5.71E-25	7.13
CXCL2	2.80	11.32	1.61E-38	6.96
CSF3	2.78	5.90	1.24E-38	6.88
IL6	2.72	11.97	1.09E-23	6.57
IL1A	2.28	10.74	2.18E-23	4.84
IL24	1.82	7.79	4.78E-17	3.53
IL10	1.43	5.53	6.42E-21	2.69

Table 6.5.2 Table of enriched GO terms: BCG stimulated – unstimated PBMC.

Gene ontology term	Count	Benjamini
defense response	95	2.0E-20
lysosome	38	1.2E-16
cytokine-cytokine receptor interaction	58	5.4E-14
chemotaxis	38	9.4E-13
signal peptide	218	1.7E-10
regulation of cytokine production	34	3.0E-08
response to bacterium	35	3.5E-08
cell activation	43	7.4E-08
positive regulation of protein kinase cascade	31	2.0E-07
cell death	73	1.3E-06
leukocyte activation	36	1.9E-06
endocytosis	32	1.4E-05
regulation of apoptosis	75	1.4E-05
lymphocyte activation	29	4.5E-05
positive regulation of immune system process	32	5.9E-05
leukocyte chemotaxis	12	7.6E-05
Immunoglobulin domain	43	9.5E-05
carbohydrate binding	40	2.1E-04
regulation of interleukin-12 production	8	5.2E-04
positive regulation of cellular protein metabolic process	29	6.8E-04
NOD-like receptor signaling pathway	15	1.1E-03
positive regulation of protein metabolic process	29	1.3E-03
vesicle	57	2.3E-03
glycosidase	13	2.3E-03
membrane-bounded vesicle	49	5.0E-03
positive regulation of lymphocyte activation	15	9.1E-03
cytoplasmic vesicle	51	2.1E-02
cytoplasmic membrane-bounded vesicle	45	2.3E-02
caspase Recruitment	8	3.6E-02

In a previous study, Fletcher et al identified a gene signature of prospective risk of tuberculosis disease in 10-week old infants vaccinated with BCG at birth. They found infants clustered into two groups based on their response to *in vitro* stimulation with BCG. When infants were separated into two groups based on their response to BCG stimulation (weaker or stronger responders), it was possible to identify cases and controls using a 10-gene signature (Fletcher et al, submitted). None of the 60 infants

investigated here developed tuberculosis during the course of the study however we were interested to see whether the samples would separate into stronger and weaker responders using the “classifier” gene list identified by Fletcher et al (appendix section Table 8.2.1 Fletcher et al. classifier gene list) . Figure 6.5.1 shows clustering of samples according to the difference in expression between BCG-stimulated and unstimulated condition for each sample. As described by Fletcher et al, these infants also cluster into two groups on the basis of their response to BCG stimulation.

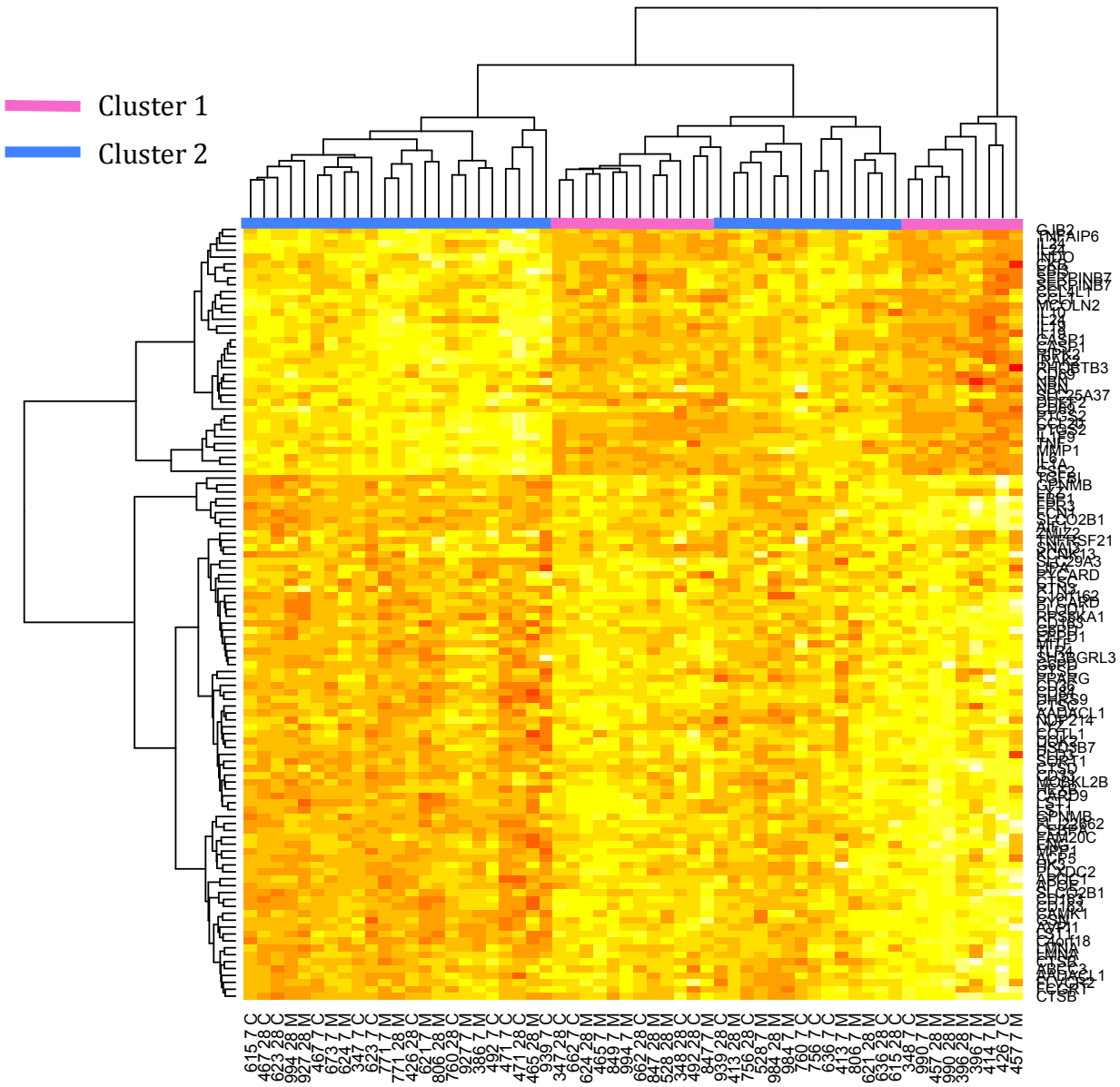


Figure 6.5.1 Infants cluster into two groups of "weaker" and "stronger" BCG responders. Heatmap shows unsupervised clustering of all PBMC samples (values for stimulated-unstimulated) using the list of "classifier" genes identified by Fletcher et al.

Looking at several of these genes individually, there is a significant association between the strength of the response to BCG and the ELISpot response to BCG at day -7 (ie those infants with greater upregulation of *TNF* and downregulation of *TGFBI* had higher ELISpot responses). Of the six genes shown in Figure 6.5.2, *IFNG* and *IL17F* are differentially expressed following BCG stimulation but are not part of the classifier list.

The associations at day 28 were weaker and remained significant only for IFN- γ and IL17F.

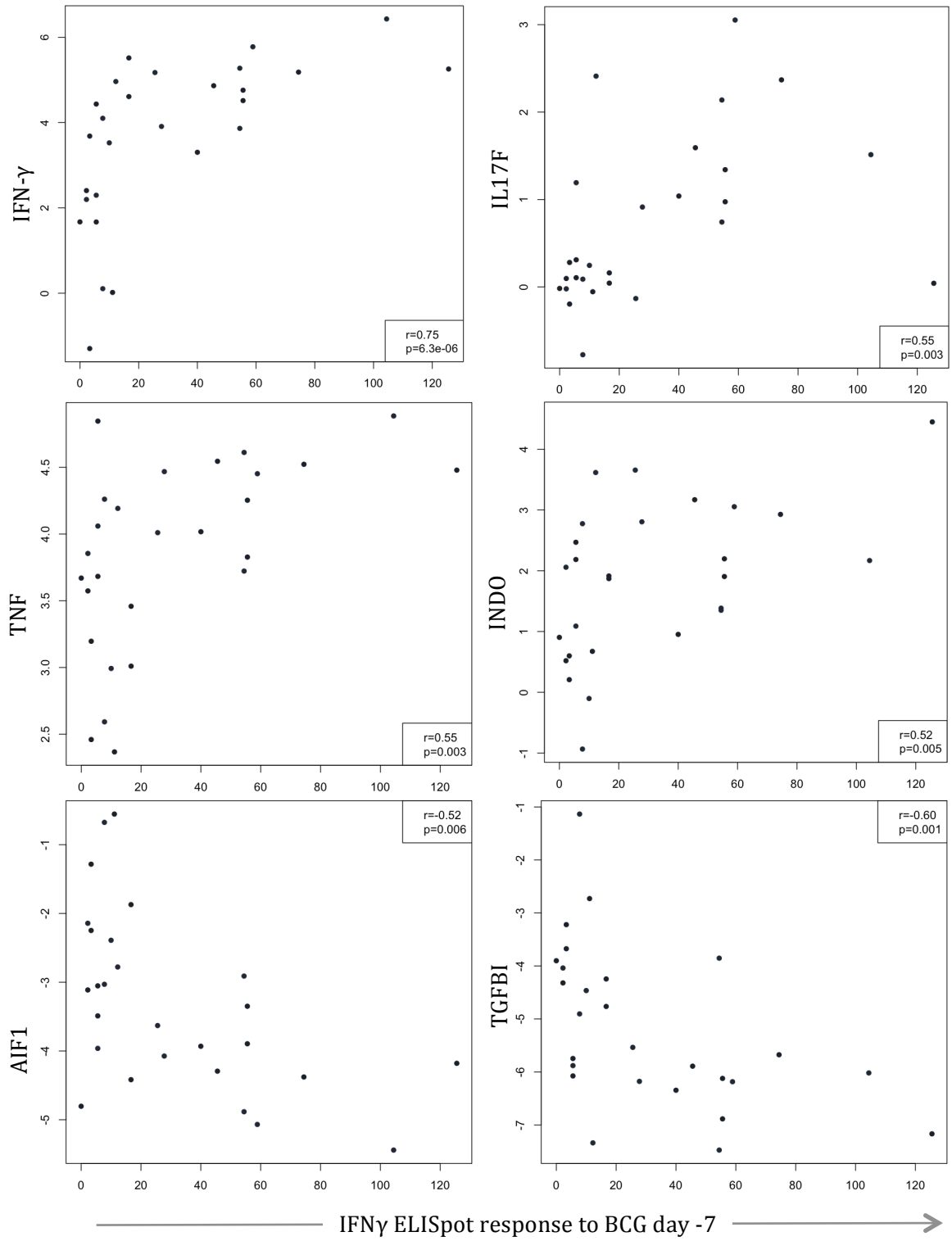


Figure 6.5.2 Correlations between fold change in response to BCG stimulation (day -7) and ELISpot response to BCG (day -7): *IFNG*, *IL17F, TNF, INDO, AIF1, TGFBI*.

6.6 Comparison to responses in UK adults

6.6.1 Innate immune response to vaccination: comparison with TB022

In both the UK adults and South African infants I saw associations between gene expression pre and 24-48hours post-vaccination with MVA85A and the IFN-γ ELISpot response to Ag85A peptide pools. This gave me an opportunity to compare the findings in the two groups, in particular with regards to genes involved in the regulation of the immune response, which seemed to be involved in both cases.

In TB022 (UK adults), higher expression of *STAT5B* on day 0 correlated positively with frequency of regulatory T cells one week post vaccination and both correlated inversely with IFN-γ ELISpot response to Ag85A (AUC). Low responses were also associated with higher levels of *CTLA4* and *IDO1* two days post-vaccination and an increase in circulating Tregs in the week following vaccination, as discussed in chapter 2.

By contrast, in the infants, the ELISpot response correlated positively with both IL10 pre-vaccination and *IDO1* post-vaccination. The relationship between the regulatory gene *IDO1* and other genes also differed in the two populations: in the UK adults *IDO1* expression at the innate timepoint (day two) was low in most volunteers and correlated with *CTLA4* expression but not *CXCL10*. In the infants however, one day post vaccination, the opposite was true (Figure 6.6.1). These data may be indicative of different regulatory mechanisms in these two populations and the different impact these have on an individual's response to vaccination.

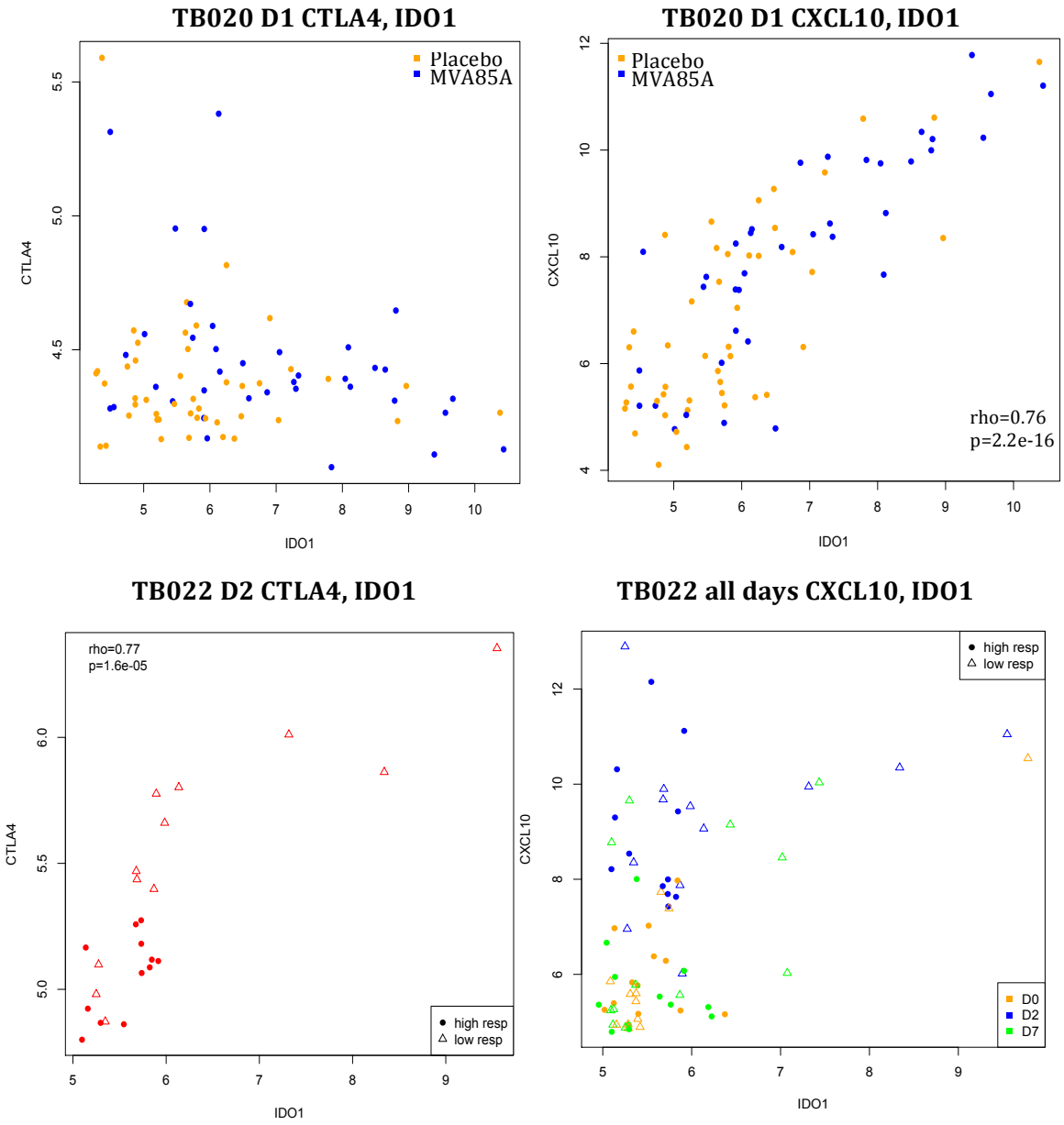


Figure 6.6.1 Differences in the relationship between the regulatory CTLA4 and IDO1 and the IFN-induced gene CXCL10 in South African infants (TB020) and UK adults (TB022). Spearman's rank correlation.

6.6.2 Response to in-vitro BCG stimulation: comparison with TB023

The list of DEGs between BCG stimulated and unstimulated PBMC were very similar for TB020 (infant efficacy trial) and TB023, the BCG challenge trial discussed in

chapter 3. The same genes feature in both lists, with huge fold changes in some cases although these are in general smaller in the infants than in either of the adult groups. Notably, IFN- γ expression is upregulated 45-200 fold in the adult samples but only 13 fold in the infant samples.

I also used the classifier list to look for differences in the response to BCG between adults. Although the samples also fall into two groups this is much less clear than in the South African infants, suggesting UK adults are more homogeneous in their response to BCG, with fewer at the extremes of the "stronger" and "weaker" responder phenotypes (Figure 6.6.2).

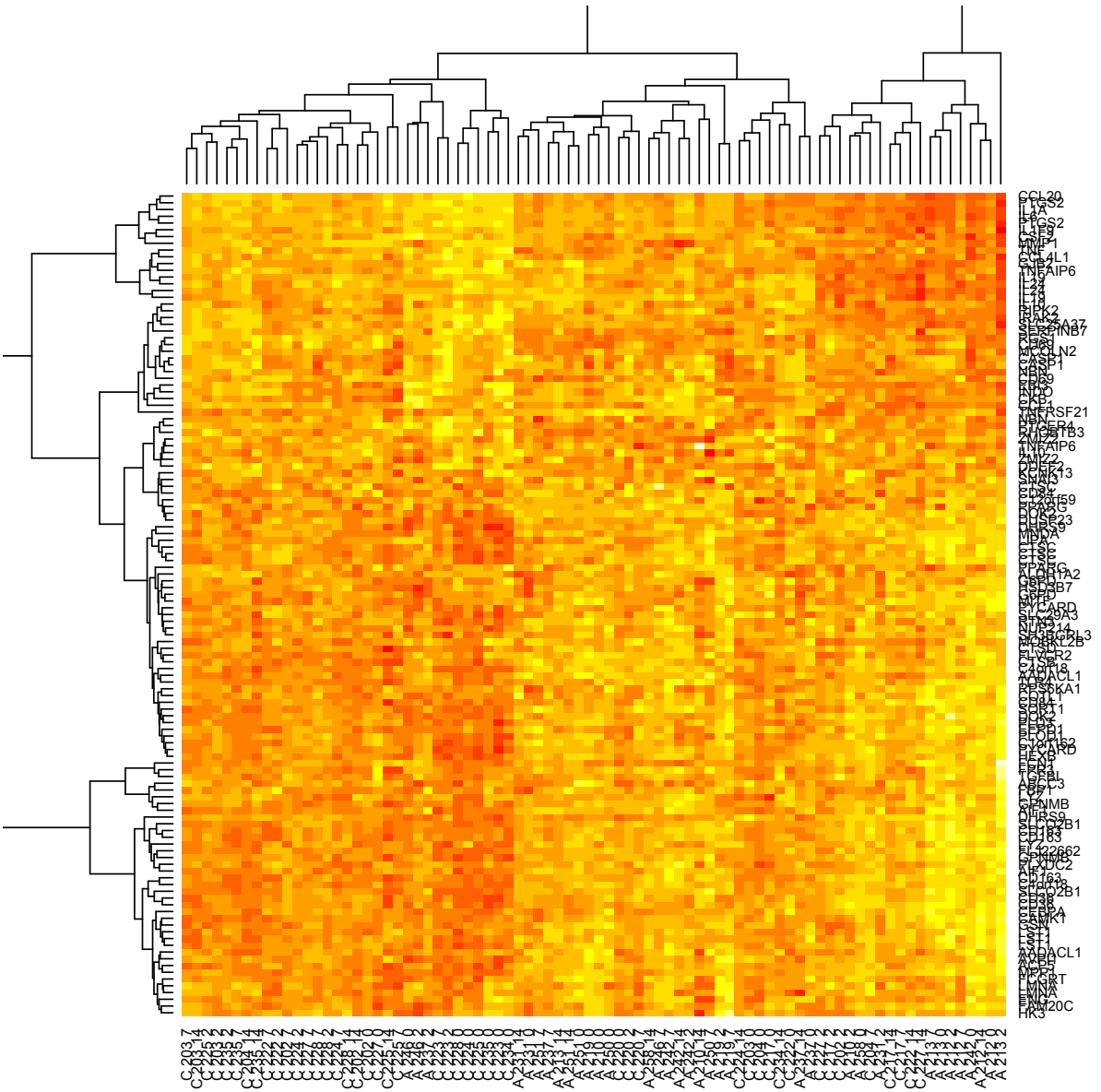


Figure 6.6.2 Hierarchical clustering of adult samples according to the classifier genes of Fletcher et al. Expression values for BCG stimulated – unstimulated in TB023 samples. These samples show less evidence of clustering into strong and weak responder phenotypes as compared to the RSA infants.

6.7 Discussion

In the experiments presented above, performing multiple assays in parallel on cells thawed at the same time gave me the opportunity to compare results of different assays and also profile the immune response to different stimuli in more depth. The results for antigen 85A stimulation were very different to what I observed after BCG

stimulation. In some cases the response is high, although still weaker than what was seen in the UK adults, but very one-dimensional. The results of three of the assays – ELISpot, Ki67 proliferation and gene expression microarray- correlate strongly in the Ag85A stimulated group. Additionally, the DEGs in the gene expression correlate strongly with each other, suggesting upregulation of a single pathway. A possible interpretation of this is that MVA induces very strong responses but that these are narrow, focused on the induction of IFN-γ and co-regulated genes. Use of the ELISpot, which measures specifically this response, to select vaccines may lead to a bias in vaccine selection towards candidates that promote strong induction of a single pathway rather than moderate induction of several. In the scenario where this response can induce protection this would be beneficial but problems may arise if multiple mechanisms are required for protection or if the response fails to be induced in a subset of vaccinees. This observation argues for the use of multiple, non-redundant assays early in the vaccine selection process.

The range in expression of inflammatory genes one day post-vaccination in the infant cohort, in both the vaccinated and placebo groups, was also surprising. By contrast, in UK adults a principal component analysis clearly separated the pre- and post-vaccination timepoints. All the UK adult volunteers made an innate inflammatory response to MVA85A vaccination which was not predictive of the adaptive response to Ag85A measured later in the trial. In the infants however, not all vaccinees had increased inflammation one day post-vaccination and low inflammation was associated with weak or absent antigen-specific responses four weeks later. The range of expression of inflammatory genes in the non-MVA85A vaccinated group was much greater in this cohort than in the previously studied UK adults and it is possible that

this may influence the ability of these infants to respond to vaccination and their susceptibility to disease. The infants in this trial are at an age of rapid changes in their ability to mount immune responses. Young infants and neonates, in whom overly vigorous immune responses may be life threatening, have been shown to have weaker Th1 responses and produce less IFN-γ than adults following vaccination with hepatitis B and the oral polio vaccine [193–195], although BCG appears to have a distinctive ability to induce strong Th1 responses in this age group [196]. Around 6 months of age, a sharp increase in the ability of infants to produce IFN-γ and associated cytokines has been reported [197]. The observed range in inflammatory responses may be a refection of the different stages of rapidly maturing immune systems of individuals within the cohort. Alternatively, or additionally, co-infection with or exposure to other pathogens is likely to affect the balance of immune responses.

In these infants, *IL2RA*, *IL15* and *IL10* pre-vaccination correlated with post-vaccination ELISpot responses to Ag85A, as did expression of *IDO1*, *CASP1* and *STAT3* post-vaccination. The association between IFN-γ responses to Ag85A induced by vaccination and the expression of several genes associated with regulation of the immune response pre- and post-vaccination was unexpected. Ascribing cause and effect is not possible and one explanation may be that inflammation and regulatory responses are closely coupled in this population and that this is the reason why the strongest responses to Ag85A are seen in infants that also have high expression of regulatory genes.

It is impossible to tell whether the differences observed in this study compared to adults in the UK are more attributable to age or to genetic or environmental

differences between the populations. Previous studies have found equivalent responses to BCG in adults and children in Africa [195,198] and that differences in responses to BCG were already present in young infants from the UK and Malawi [50]. In this study, infants from Malawi were found to make weaker Th1 responses to BCG stimulation than their UK counterparts. These studies suggest the differences are attributable to geographical differences rather than age. On the other hand it is also known that infant immune systems are markedly different from those of adults and undergo many changes in the first five years of life. Tregs are abundant in fetal tissue and cord blood and infants have been shown to make more IL-10 in response to TLR stimulation than adults [145,199]. Additionally, studies have shown that homing receptors on the surface of Tregs differ in infants and adults with the gut-homing receptor $\alpha_4\beta_7$ dominating in the first 18 months of life, before being largely replaced by CCR4 [144]. This suggests age-related differences are also likely to be important in the response to MVA85A, particularly as regulatory mechanisms appear to have important but opposite effects in UK adults and South African infants. The different associations of *IDO1* expression, with *IFNG* and *CXCL10* in the infants but not the adults, and vice versa compared to *CTLA4*, point to different mechanisms of immune regulation in the two cohorts. Conducting similar studies in African adults or infants from the UK is needed to help answer these questions.

In a previous study, Fletcher et al found that BCG-vaccinated South African infants separated into two groups based on their response to BCG, namely "stronger" and "weaker" responders, and that susceptibility to tuberculosis disease was associated with phenotypes at the two extremes of this scale. We also saw evidence of stronger and weaker BCG responders in this study however this cohort contained only infants

that did not become infected with tuberculosis during the follow-up period. Interestingly, this grouping was not seen in UK adults, suggesting most volunteers in this group had a more balanced response to BCG, which may be connected to the greater efficacy of BCG vaccination in this population. Again, it is not clear whether age or geography was the primary factor in the difference in grouping of the response and in the magnitude of the fold changes of DEGs in response to BCG stimulation in the two populations. In both populations however, higher IFN-γ production following BCG stimulation, as measured both by ELISpot and microarray, was also associated with increased production of *IL17F*. This is line with a number of studies that have shown BCG induces Th17 as well as Th1 responses and that both have a role in protection.

There is much that remains to be understood in the intricacies of BCG vaccination and tuberculosis around the world. In this chapter, I have tried to characterise the response to vaccination in South African infants, both to BCG and MVA85A, and compare it to previous data from UK adults. The differences between the two populations are many. The UK trials were conducted in adults with fully developed immune systems who, for the most part, received BCG in adolescence or adulthood, many years before their MVA85A boost. Aside from their genetic differences, this population lives in an area of higher prosperity, with little or no exposure to environmental mycobacteria or intestinal worms. The incidence of tuberculosis is now extremely low and BCG is highly efficacious in this population [180]. As a group, the volunteers from TB020 present an almost opposite picture: immature immune systems that develop in the context of a BCG vaccine given at birth, a high chance of exposure to *M.tb* in a country where prevalence is among the highest in the world and exposure to a multitude of pathogens that have become rare in the developed world.

Comparing the two populations has provided interesting and sometimes unexpected results. Future work on cases and controls from the efficacy trial will hopefully provide some answers in the search for a better understanding of what is required to protect against tuberculosis disease. Additionally, future studies in adults from South Africa or UK infants are needed to unravel the mechanisms behind the differences in immunogenicity seen between UK adults and South African infants. Ultimately, an understanding of the differences in this complex story and what underlies them is likely to be necessary before protection can be achieved in the diverse groups and areas of the world still plagued by tuberculosis.

CONCLUSIONS AND FUTURE WORK

7 CONCLUSIONS AND FUTURE WORK

This project began with the broad aim of characterising early changes in gene transcription following vaccination to investigate the reasons behind the variability in individuals' immune responses to tuberculosis vaccines. As the project developed, several more specific aims emerged:

f) To characterize the immune response to MVA85A vaccination by looking for changes in the transcriptome shortly after vaccination in UK adults.

g) To investigate these changes to further our understanding of the molecular mechanisms underlying the immunogenicity of MVA85A.

h) To characterise the changes in the transcriptome following vaccination with BCG and relate changes in gene expression with ability to control mycobacterial growth in a human *in-vivo* BCG challenge model

i) To characterise the response to vaccination with BCG and MVA85A in South African infants and link these findings with results from other immunological assays

j) To compare the factors affecting the immune response to vaccination in South African infants and adults in the UK to better understand the differences between these populations.

The growing availability of high-throughput technologies and their application to the field of vaccine research has led to an increase in what has been termed "systems vaccinology"; the study of innate and adaptive responses in vaccinated human volunteers using large-scale methodologies. This informs vaccine research as well as providing a relevant and informative model in which to study the events underpinning

the immune responses that develop in humans following vaccination and, by extension, following infection with viruses or bacteria [200]. As well as reducing the unknowns in vaccine design and enabling a more rational approach, these studies often lead to testable biological hypotheses that may in turn yield new insights into the complex molecular mechanisms of the human immune system.

It was interesting to see the large changes that occur in the transcriptome of UK adults vaccinated with MVA85A. Samples clustered clearly by time point and all volunteers made a strong innate response to the vaccine, as evidenced by the large changes in expression of genes such as CXCL10 in unstimulated PBMC two days post-vaccination. Surprisingly, the strength of this response was not related to the magnitude of the adaptive response as measured by IFN- γ ELISpot. This finding -that a strong innate response to vaccination did not always lead to a strong adaptive immune response- suggested more complex factors were involved.

Further analysis revealed associations between expression of genes on day of vaccination and two days later with the IFN- γ ELISpot response. This led to the hypothesis that expression of TLR1, TICAM2 and CD14 on day of vaccination and CTLA4 and IL2RA two days later were major determinants of the magnitude of the adaptive immune response to Ag85A peptide pools.

TLR2 had previously been implicated in responses to vaccinia virus and MVA and, as a viral ligand has never been identified, it was postulated that a host-derived ligand such as HMGB1 might be behind these observations [109,174]. Based on the genes identified in the microarray analysis and previously published studies in the literature, I hypothesized that release of HMGB1 from the nucleus during cell death occurred

following infection with MVA85A and that this molecule signaled through TLR2 to initiate an innate response. Experiments using TLR blocking antibodies in an *in-vitro* set-up and in mice supported this hypothesis whilst making it clear that other factors were also involved [201]. Given the wealth of immunomodulatory molecules encoded by MVA, this is perhaps unsurprising. Together these data suggest that, whilst multiple components are involved in the innate response to MVA, HMGB1 signalling through TLR2 is needed to mount a strong adaptive response. This may imply a need for a link between cell damage or stress and the development of a vigorous adaptive response. A similar proposal was made on the basis of data from a more in-depth study on the immune response to the vaccine YF-17D in which the viral-induced integrated stress response in dendritic cells stimulates antigen-specific CD8+ T cells [101,200]. These observations suggest a need to elucidate in more depth the effects of combinatorial signaling on the actions of DCs. Furthermore, if host-derived DAMPs do have different effects on the immune response to external PAMPs, how are these signals differentiated? Are TLR2 activation by HMGB1 and LPS equivalent? As vaccine strategies move towards using synthetic ligands to activate PRRs, this may become an important question.

The association of IL2RA and CTLA4 gene expression with IFN- γ ELISpot responses suggested involvement of CD4+ Treg cells in determining the magnitude of the adaptive response. Further investigation of related genes and cell populations by flow cytometry revealed an inverse correlation between Tregs and Treg-associated genes and the IFN- γ ELISpot response. Dampening of immune responses from Tregs following treatment or vaccination has been an issue in the cancer field [176] and research into how to mitigate these effects safely may also benefit the development of

vaccines against infectious diseases. The dynamics of immune regulation remain incompletely understood though it is becoming clear that the environment plays a large role in determining the extent of immune regulation occurring within an individual. Genetics and age also influence the regulatory environment and understanding these factors may prove crucial to unraveling the differences in responses to vaccination in different populations.

Having identified some factors affecting immunogenicity in samples from a trial conducted in UK adults, it was exciting to be able to compare these results in samples from South African infants. The indications are that immune mechanisms following vaccination in this population are very different and it will be fascinating to further explore these differences and their mechanistic basis in the future. In the infant samples, not all volunteers made an inflammatory response and there were not clear clusters of vaccinated and unvaccinated samples. Furthermore, the strength of the innate immune response to vaccination correlated with the magnitude of the IFN- γ ELISpot response to Ag85A peptides. Additionally, expression of regulatory genes such as IL10 pre-vaccination showed a positive correlation with magnitude of the ELISpot response. One day post vaccination, expression of CXCL10, IDO1 and IFN- γ were highly correlated in both vaccinated and unvaccinated infants, suggesting a coupling of inflammatory and regulatory responses that was absent in the UK adults. These observations suggest important differences in the factors that limit and/or promote the development of adaptive responses in these two populations.

Further work is needed to elucidate the reasons behind the differences observed in these two populations' responses to MVA85A. The relative contributions of

environment, age and genetics are presently unclear. To begin with, studying the immune response to MVA85A in a cohort of UK infants or adults or adolescents in Africa would be insightful. Many of the differences in the immune systems of adults and infants remain uncharacterized and much of the work done on infant immunity has been done in the context of atopy and allergic diseases in developed countries. Several studies are currently being conducted into the effect of infant and maternal parasitic infections on the immune responses to infection and vaccination and these large-scale studies will be a valuable source of information in answering these questions. Ultimately, this may lead to a better understanding of the cell types and receptors involved, what controls their relative abundance and how this contributes to the generation of an immune response. With more information, testable biological hypotheses can be generated so that the molecular reasons for these differences can be identified.

During this project I also looked at the responses of these two populations to BCG. The ubiquity of this vaccine and its enduring mysteriousness made this a fascinating challenge. Despite its widespread use for almost a century, basic questions on BCG – such as the extent of its effectiveness and how it is mediated- remain largely unanswered. Our shared evolutionary history with mycobacteria, and in particular *M.tb*, partly explains this complexity. In the data collected in UK adults, I studied the responses to BCG over a two week period in both mycobacterially-naïve and BCG-vaccinated volunteers. In contrast to MVA85A, the changes in the transcriptome of unstimulated cells were much smaller indicating a very different type of immune response. Surprisingly, the biggest changes were still two days post vaccination. I found that prior vaccination with BCG changed the magnitude but not the kinetics of

the immune response. Mycobacterial growth inhibition, as measured by the amount of BCG recovered from the a punch biopsy at the site of vaccination two weeks post-challenge, correlated with the amount of IFN- γ, IL-17F and IL-22 produced in BCG stimulated cells. Encouragingly, these findings are consistent with other recent studies indicating an important role for the IL-17 – IL-22 axis in addition to IFN- γ in immunity to mycobacteria [183–185]. The severity of the local reaction to BCG also correlated with ability to control BCG, perhaps highlighting the evolution of a compromise between immunity and immunopathology in mycobacterial infections.

The development of a BCG-challenge model to assess anti-mycobacterial immunity has huge potential. Future work should focus on extending this model to different settings to see whether it accurately reflects the differences in effectiveness of BCG vaccination and ability to control mycobacterial growth in diverse populations. If so, it could prove a powerful tool in down-selecting vaccines to take into clinical trials but also in teasing out the mechanisms involved in anti-mycobacterial immunity and why these are so variable.

UK adults and South African infants also differed in their response to stimulation with BCG. The South African infants were not part of a BCG "challenge" trial however I was able to study the changes in gene expression following *in vitro* stimulation of PBMC with BCG in infants 4-6 months after their BCG vaccination. As had previously been described, the infants clustered into two quite distinct groups based on whether they made "stronger" or "weaker" responses to BCG. By contrast, the adults from the UK trial all had a more balanced response. In the study that identified this dichotomy in responses in South African infants, individuals at either extreme of the spectrum were

more likely to develop tuberculosis, suggesting a balanced response to mycobacteria may be key to protection. The relative abundance of myeloid and lymphoid cell populations was key to determining this balance (Fletcher et al., submitted). Once again, the complex interplay of age, genetics and environmental factors makes it difficult to formulate precise hypotheses until these observations have been further elucidated. Further studies are needed to characterise responses to BCG in different age groups, different populations and different environments more precisely. All three are likely to play a role, as is the prevalence of HIV whose effect on the tuberculosis pandemic may be multifaceted and affect vaccine strategies and effectiveness in ways that are not yet understood.

Despite huge advances, many aspects of immune responses and immunity to mycobacteria remain mysterious and tuberculosis continues to have devastating effects in large parts of the world. Similarly, new studies are just beginning to uncover the complexities of human responses to vaccination. Future work should continue to focus on reducing the unknowns, as a lack of knowledge may mask protective responses in some cohorts, reducing our ability to select and improve vaccine candidates. In previous decades, vaccines were empirically selected on their ability to protect in vaccine trials and correlates of protection elucidated later from effective vaccines. The complexity of tuberculosis is such that this approach is not enough and it is imperative that we continue to extract as much immunological information from vaccine trials as possible. This can give new insights into the complex world of the human immune system and will also reduce noise from unknown factors when assessing vaccine efficacy in heterogeneous populations. The tuberculosis vaccine field suffered a disappointment in the efficacy results of MVA85A; the study is a testament

to the huge progress achieved in recent years yet also a reminder of the challenges facing the field and the large gaps in knowledge that remain. Understanding those differences, both in susceptibility to disease and response to vaccination, is likely to prove key to the development of a successful vaccine and vaccination strategy against tuberculosis. Characterising the differences will take time however the solution to developing a successful vaccine is likely to depend on an understanding of the variation and details in the diverse populations for whom the consequences of tuberculosis remain a daily reality.

APPENDIX

8 APPENDIX

8.1 Clinical trials data

Much of the work in this thesis made use of samples and data from clinical trials conducted at or in collaboration with The Jenner Institute in Oxford. The data from the trials have either been published or submitted and references are shown for each figure. Data used extensively as part of the research undertaken in this project is shown for ease of reference.

8.1.1 IFN-γ ELISpot data

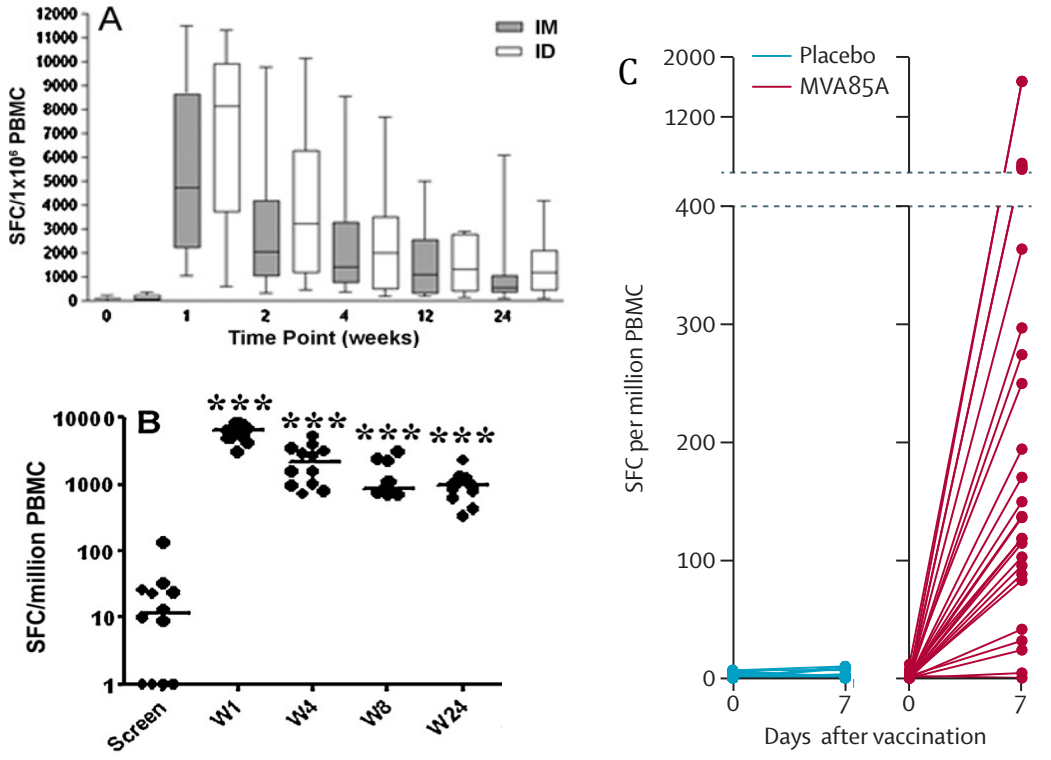


Figure 8.1.1 IFN- γ ELISpot responses to antigen 85A peptide pools measured during three clinical trials. A. TB022 MVA85A route comparison trial: ID vs IM [135]. B. TB009 dose escalation trial (high dose) [62]. C. TB020 Phase IIb efficacy trial in South African infants [43].

8.1.2 TB023 Challenge data

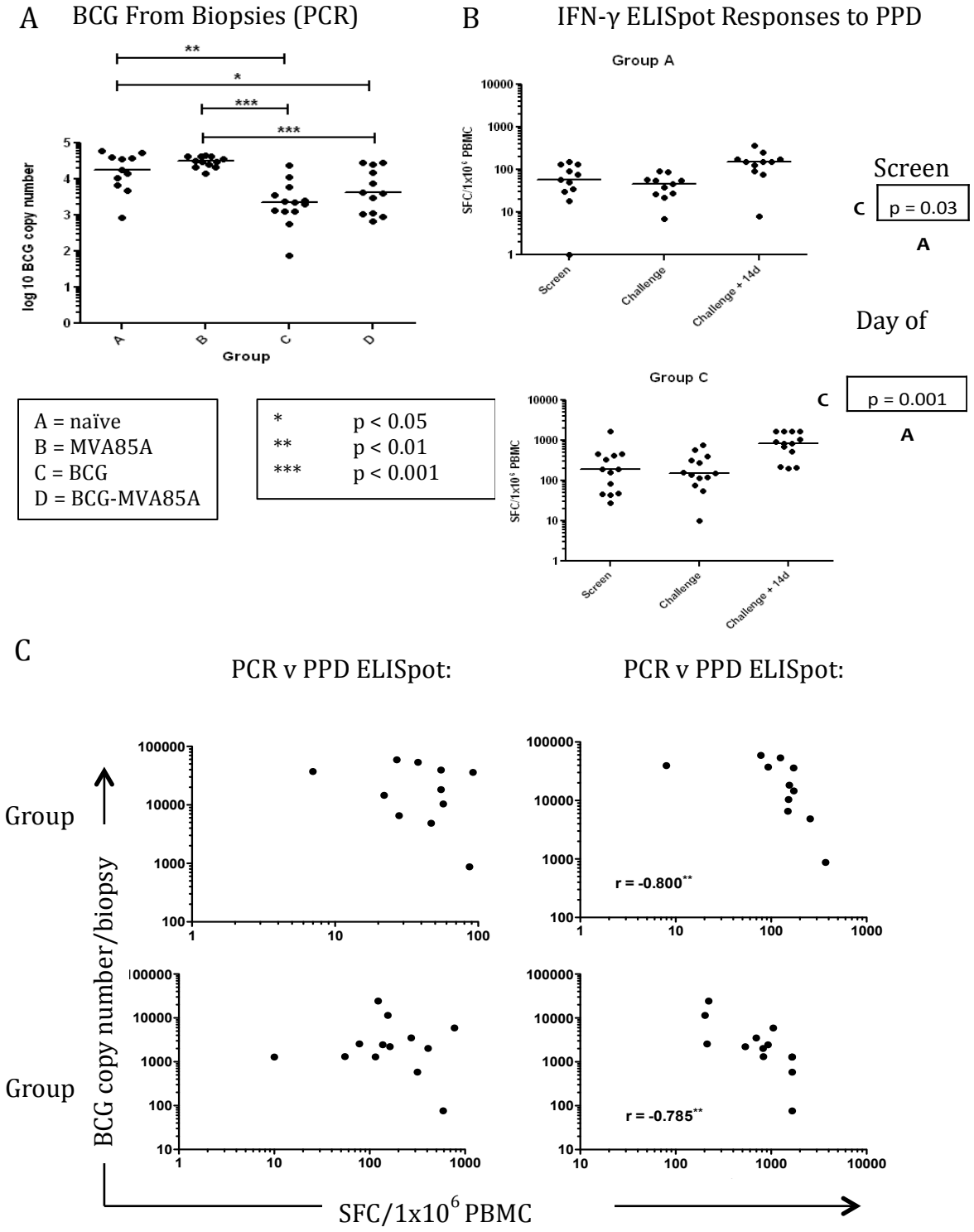


Figure 8.1.2 Data from the TB023 clinical trial to establish a BCG challenge model of mycobacterial growth. A. BCG copy number recovered from punch biopsies in each of the four groups. B. IFN- γ ELISpot responses to PPD-T in groups A and C. C. Correlations between BCG copy number (PCR) and ELISpot response to PPD-T for groups A (top) and C (bottom) (Harris et al., submitted).

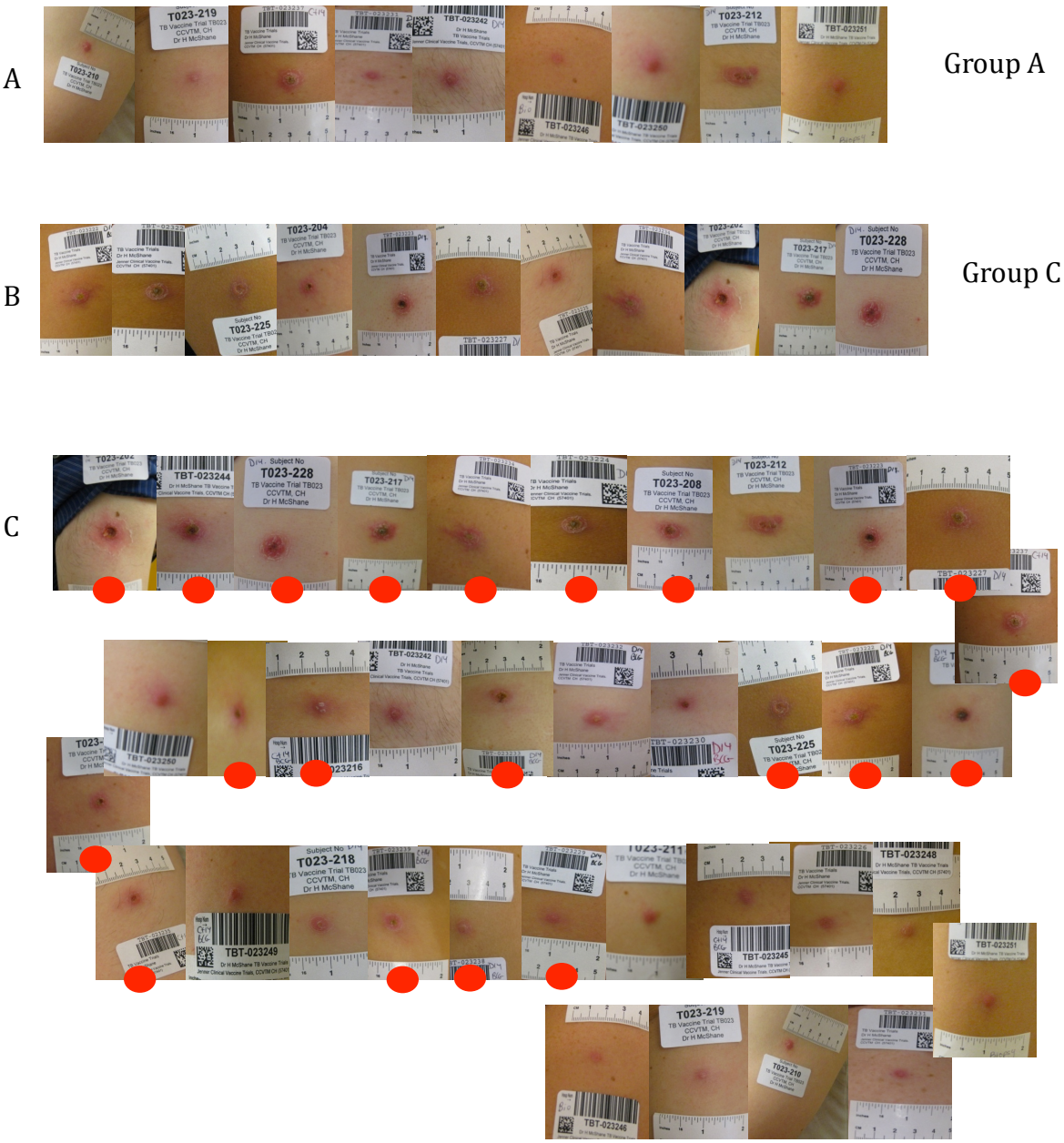


Figure 8.1.3 BCG “scar severity rank” performed by the trial clinician at the time of the trial. Subjects for whom photos were available were ranked based on the severity of local adverse reactions. A. Volunteers in group A. B. Volunteers in group C. C. Volunteers from all four groups, ranked by “severity” (most severe, top left, least severe, bottom left). Red circle indicates previous BCG vaccination (groups C and D) (Meyer, unpublished).

8.2 Classifier gene list for BCG response

The genes below form the classifier gene list identified by Fletcher et al. and used in Chapter 6.

Table 8.2.1 Fletcher et al. classifier gene list

FPR3	GJB2	ALDH1A2	CTSB
AVPI1	HSD3B7	APOE	CKB
IL1F9	PTGER4	FBP1	DUSP23
TGFBI	TNF	G6PD	IL24
AADACL1	TLR4	RGS1	TNFAIP6
PTGS2	FLVCR2	MMP1	HEXB
CSF2	LST1	AIF1	SNAI3
MNDA	CAMK1	FAM20C	RTN3
CD36	EBI3	GSN	MOBKL2B
CEBPA	GPNMB	RPS6KA1	CTSC
FLJ22662	CD84	CARD9	C4orf18
SLCO2B1	PLOD1	APOC1	CCL20
SERPINB7	RIPK2	EEPD1	CTSD
SORT1	IL19	CCL4L1	MITF
LST1	PYCARD	LYZ	KCNK13
DOK2	ENG	ACOT11	ZMIZ2
COTL1	ACP5	LMNA	NUP214
INDO	MCOLN2	IL6	TNFRSF21
DHRS9	CASP1	NBN	CD69
CCL1	ABCC3	SLC37A2	C12orf59
FCN1	DDEF2	SLC29A3	RHOBTB3
PPARG	CD163	HK3	IL12B
SLC25A37	PLD3	SH3BGRL3	IL10
PLXDC2	IL1A	C1orf162	CD33
LIPA	MPP1	IRAK2	FCGRT

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