Variation in T cell Immunity in Health

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Declaration

I, Emily Sarah Shaw, confirm that the work presented in this thesis is my own.

Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed: Emily Shaw
Abstract

Introduction

Bacillus Calmette–Guérin (BCG)-vaccination affords variable protection against tuberculosis (TB), which is unexplained. The project hypothesises that there is inter-individual variation in both the naïve T cell (NTC) response to a standard stimulation and in the ‘trained’ immune response of BCG-vaccine-primed T cells on secondary exposure to antigen and that relationships exist between the two. The aim of the study is to identify factors by which T cell vaccine design may be improved.

Methods

Blood was collected from 107 adults immediately prior to BCG-vaccination. NTCs were enriched from pre-BCG frozen/thawed peripheral blood mononuclear cells (PBMC), subjected to anti-CD3/CD28 stimulation and then characterised by fluorescence-activated cell-sorting (FACS) on markers of activation, differentiation and proliferation. Participants were then re-bled eight weeks later and their frozen/thawed PBMC incubated with purified protein derivative (PPD) in three separate assays before FACS to measure activation induced markers, Th1 cytokines and proliferation. A DNA sequencing pipeline and computational analysis were
employed to examine 67 participants’ peripheral blood TCR repertoires in response to BCG.

**Results**

There was inter-individual variation amongst all pre-vaccination NTC stimulation assay parameters, some of which were strongly positively correlated. In the post-BCG-vaccination assays, all parameters were significantly upregulated in response to incubation with PPD and demonstrated wide ranges of inter-individual variation. In both sets of assays, results were found to be reproducible and inter-individual variation surpassed technical noise. Post-BCG expanded TCRs generated clusters of homologous sequences, were shared between multiple participants and shared homology with annotated TB-specific TCRs.

**Discussion**

There is inter-individual variation in multiple parameters to standard NTC stimulation, possibly reflecting intrinsic variance in the T cell intracellular signalling capacity. BCG has variable T cell immunogenicity, which may bear relation to pre-vaccination parameters. BCG-vaccination induces a polyclonal population of T cells with the ability to recognise mycobacterial antigens. Improved understanding
of the T cell component to variable BCG vaccine efficacy may aid next generation TB vaccine development.
Impact Statement

The work presented in this thesis could be of beneficial use within the fields of immunology, vaccinology and mycobacteriology.

Academic insight is provided into variation between the immune systems of humans in health, in particular inter-individual variation between naïve T cells (NTCs) response to non-specific antigenic challenge and primed T cell response on re-exposure to mycobacterial antigens.

Methodology is provided for other researchers to achieve reproducible, standard, non-antigenic stimulation of NTCs via the T cell receptor (TCR) using beads coated with anti-CD3 and anti-CD28 monoclonal antibodies at maximal and sub-optimal levels. The quantification of natural variation between the responsiveness of NTCs between individuals is informative of sources of biological variation to researchers undertaking studies on human T cells.

With additional analyses, the data presented in the current study, may aid the identification of whether there are pre-vaccination NTC parameters predicative of Bacillus Calmette–Guérin (BCG) T cell immunogenicity. Such analyses may help to unravel the T cell contribution to inter-individual variation in BCG-induced protection afforded against tuberculosis (TB) and, with further investigation into
responsible genetic and epigenetic factors and intracellular pathways, could translate to inform the next generation of vaccinations against TB.

The peripheral blood TCRs before and after BCG-vaccination were also sequenced and analysed and provide a proof of concept that that peripheral blood TCRs may be used to identify BCG-induced signatures with the potential for the development of clinically-useful mycobacterial-vaccination response testing.
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# Abbreviations

- aAPC – artificial antigen presenting cell
- ACT – adoptive cell transfer
- ADAP – adhesion and degranulation promoting adaptor protein
- AIDS – acquired immune deficiency syndrome
- AIM – activation induced marker
- Anti-CD3 – monoclonal antibodies to CD3
- Anti-CD3/CD28 – monoclonal antibodies to CD3 and to CD28
- Anti-CD28 – monoclonal antibodies to CD28
- APC – antigen presenting cell
- AP-1– activator protein 1
- ARV – anti-retroviral
- BCG - Bacillus Calmette–Guérin
- Bcl-2 - B cell lymphoma 2
- BFA - brefeldin A
- BSA - bovine serum albumin
- BSB - brilliant stain buffer
- C - cytosine
- C region – constant region
- CCR7 - chemokine (C-C motif) receptor 7
- cDNA - complementary DNA
- CDR3 - complementarity determining region 3
- CFSE - carboxyfluorescein succinimidyl ester
- CMV - cytomegalovirus
- ConA - concanavalin A
- Conc. - concentration
- CTLA-4 - cytotoxic T lymphocyte antigen 4
- CTV - celltrace violet
- CYBB - cytochrome B-245 beta chain
- CV - coefficient of variation
- DAG - diacylglycerol
- DCs - dendritic cells
- DI – division index
- DMSO - dimethyl sulfoxide
- DNA – deoxyribonucleic acid
- DNase - deoxyribonuclease
- dNTPs - deoxynucleoside triphosphates
- DPBS+Ca/+/Mg - Dulbecco’s phosphate-buffered saline with calcium and magnesium
- DPBS-Ca/-Mg - Dulbecco’s phosphate-buffered saline without calcium and magnesium
- DTH - delayed type hypersensitivity
- EDTA - ethylenediaminetetraacetic acid
• ELISpot - enzyme-linked immune absorbent spot
• EPI - expanded programme on immunisation
• eQTL - expression quantitative trait loci
• ER - endoplasmic reticulum
• EtOH - ethanol
• FCS - fetal calf serum
• FACS - fluorescence-activated cell-sorting
• Fig. - figure
• FMO – frequency minus one
• G - guanine
• GAP - GTPase-activating protein
• GEF - guanine nucleotide exchange factor
• GLIPH - grouping of lymphocyte interactions by paratope hotspots
• GMT - geometric mean antibody titres
• Grb2 - growth factor receptor-bound protein 2
• GTP - guanosine triphosphate
• GWAS - genome-wide association studies
• G0 – cellular state outside of the replicative cell cycle
• G1 – intermediate phase between end of cell division in mitosis and beginning of DNA replication
• G2 – period of rapid cell growth and protein synthesis where cell prepares itself for mitosis
• HCC – hexamine cobalt chloride
• Hib - haemophilus influenzae type b
• HLA - human leukocyte antigen
• HLA-DR - human leukocyte antigen – DR isotype
• HIV – human immunodeficiency virus
• HIV-1 - human immunodeficiency virus-1
• H2O - water
• IBD - inflammatory bowel disease
• ICAM-1 - intercellular adhesion molecule 1
• ICH – UCL Institute of Child Health
• ICS – intracellular cytokine staining
• IGRA - interferon gamma release assay
• IFNGR1 - interferon gamma receptor 1
• IFNGR2 - interferon gamma receptor 2
• IFN-c - Interferon-c
• IFN-γ - interferon-γ
• IIT – UCL Institute of Immunity and Transplantation
• IKK - IκB kinase
• IL-2 – interleukin-2
• IL-2RA - α-chain of the IL-2 receptor
• IL-4 – interleukin-4
• IL-5 – interleukin-5
• IL-12B - interleukin 12B
• IL12RB1 - interleukin 12 receptor B1
• IL-13 – interleukin-13
• IL-17 – interleukin-17
• ImmVar – The Immune Variation Project
• IP3 - inositol 1,4,5-trisphosphate
• IQR – interquartile range
• IRF8 - interferon regulatory factor 8
• ISG15 - interferon-stimulated gene 15
• ITAMS - immunoreceptor tyrosine-based activation motifs
• ITSM - immunoreceptor tyrosine-based inhibitory motif
• J region – joining region
• L.monocytogenes - Listeria monocytogenes
• L/D – live/dead
• L/D Near IR - live/dead fixable near-infrared dead cell stain
• LAT - linker for Activation of T cells
• LCMV - lymphocytic choriomeningitis virus
• LD – laboratory donor
• LFA-1 - lymphocyte function-associated antigen 1
• LN2 - liquid nitrogen
• LTBI – latent tuberculosis infection
• M.avium – Mycobacterium avium
• M.bovis - Mycobacterium bovis
• M.fortuitum - Mycobacterium fortuitum
• M.kansasii - Mycobacterium kansasii
• M.tuberculosis - Mycobacterium tuberculosis
• MFI – mean fluorescence intensity
• MGIA - mycobacterial growth inhibition assay
• MIP1b - macrophage inflammatory proteins 1b
• MMR - measles, mumps and rubella vaccine
• MRC – Medical Research Council
• mRNA - messenger ribonucleic acid
• MSMD - Mendelian susceptibility to mycobacterial diseases
• Mtb - Mycobacterium tuberculosis
• mTOR - mammalian target of rapamycin pathway
• NEMO - nuclear factor-kappa B essential modulator
• NFAT - nuclear factor of activated T cells
• NFκB - nuclear factor κB
• NF-90 - nuclear factor-90
• NGS – next generation sequencing
• NK – natural killer cell
• NTC – naïve T cell
• OH – occupational health
- PBMC - peripheral blood mononuclear cells
- PCR – polymerase chain reaction
- PCV7 - 7-valent conjugated pneumococcal vaccine
- PCV13 - 13-valent conjugated pneumococcal vaccine
- PDK1 - phosphoinositide-dependent protein kinase-1
- PD-1 - programmed cell death 1
- PEG - polyethylene glycol
- PHA - phytohemagglutinin
- PHE – Public Health England
- PI – proliferation index
- PIL - participant information leaflet
- PIP2 - phosphatidylinositol 4,5-bisphosphate
- PIP3 - phosphatidylinositol 3,4,5-triphosphate
- PI3K - phosphoinositide 3-kinase
- PKC-θ - protein kinase C-θ
- PLC-γ - phospholipase C-γ
- PLWH- persons living with HIV
- PPD - purified protein derivative
- PTB – pulmonary tuberculosis
- QFT-Plus - Quantiferon Gold Plus
- QR - quality and/or quantity of a response to vaccination
- Q1 – first quartile
- Q3 – third quartile
- r – Pearson’s correlation coefficient
- RA - rheumatoid arthritis
- RasGRP - ras guanyl nucleotide releasing proteins
- REC – UCL research ethics committee
- RI – replication index
- RMS – root mean squared
- RNA - ribonucleic acid
- RPMI - Gibco Roswell Park Memorial Institute 1640 medium +L-glutamine
- RT – room temperature
- R10 - Gibco Roswell Park Memorial Institute 1640 medium +L-glutamine with the addition of 10% filtered FCS
- S – DNA synthesis phase of cell cycle
- SAE - severe adverse events
- SCID - severe combined immunodeficiency
- SCR - seroconversion rates
- SLP-76 - SH2-domain-containing leucocyte protein of 76 kDa
- SNPs - single-nucleotide polymorphisms
- Spearman’s rho (ρ) - Spearman’s rank correlation coefficient
- SSI - Statens Serum Institut
- STAT1 - signal transducer and activator of transcription 1
- STAT5 - signal transducer and activator of transcription-5
• STIM1 - stromal interaction molecule 1
• TAK1 - transforming growth factor β-activated kinase 1
• TB - tuberculosis
• TCR – T cell receptor
• TEMRA - CD45RA+CCR7- effector memory T cells
• Th – T helper cell
• Th1 – T helper type 1 cell
• Th2 – T helper type 2 cell
• TIV - trivalent inactivated influenza vaccine
• TNFα - tumor necrosis factor α
• TRAF-6 - tumour necrosis factor receptor-associated factor 6
• Treg - regulatory T cell
• TSC - tuberous sclerosis complex
• TST – tuberculin skin testing
• TCM – central memory T cell
• TEM – effector memory T cell
• UCL – University College London
• UK – United Kingdom
• UKHSA – United Kingdom Health Security Agency
• UIN - unique identification number
• UN - United Nations
• UMI – unique molecular identifier
• V region – variable region
• WASp - Wiskott-Aldrich syndrome protein
• WHO – World Health organisation
• WIP - WASp-interacting protein
• YF17D - yellow fever vaccine 17D
• ZAP-70 - zeta-chain-associated protein kinase-70
• α3+28 - beads coated with anti-CD3 and anti-CD28 monoclonal antibodies
• °C - degrees Celsius
• Δ - delta
• μ - mean
• ρ – Spearman’s rank-order correlation
• σ - standard deviation
Chapter 1 Introduction

1.1 Pre-Introduction

The project explores heterogenicity in immune systems in humans, focusing in particular on inter-individual variation in T cell functionality. The project seeks to explore possible relationships between an individual’s naïve T cell (NTC) phenotypic responses to non-specific stimulation and their subsequent ‘trained’ response to cognate antigenic stimulation. It is hypothesised that there may be intrinsic biological differences between healthy individuals’ NTCs, which determines their responsiveness to primary challenge and resultant differentiation, and therefore aspects of ‘trained’ immune response to secondary exposure to antigen.

The ensuing introductory chapter details pertinent background information and context underpinning the concepts, objectives and methodology within the current study, with the key topics covered summarised in Fig. 1.1. First, an overview is provided of recent studies exploring inter-individual variation between the immune subset composition of ‘healthy’ individuals. Next, examples are given of studies measuring the cellular phenotype and functionality responses of individuals to infective or vaccine challenges, where variations were observed between individuals.
The current project specifically seeks to explore heterogeneity in T cell responsiveness and therefore the subsequent paragraphs provide detail on T cell biology including the T cell receptor (TCR) and relevant T cell signalling pathways which are mobilised on TCR-engagement and T cell activation. Next, an account is given of conditions by which NTCs may be non-specifically stimulated *in vitro* in the absence of cognate antigen, through the addition of monoclonal antibodies against CD3 and CD28 (anti-CD3 and anti-CD28) which provide a platform by which to quantify an individual’s NTC responsiveness. A rationale is then provided on the phenotypic markers of activation, differentiation and proliferation selected to measure T cell response to non-specific stimulation.

In the second part of the project, T cells are primed *in vivo* and then the ‘trained’ immune response is quantified through *in vitro* phenotypic responses to secondary exposure to antigen. Therefore, next in this opening chapter, vaccines and systems vaccinology are introduced. Subsequently, the rationale for adopting the Bacillus Calmette-Guérin (BCG)-vaccination for the priming event is provided. There is a review of the literature on the variability of immunogenicity and efficacy in BCG and a summary is provided on the evidence that mycobacteria, including the attenuated *Mycobacterium Bovis* BCG, provoke a predominantly T cell response. It is currently unknown what component inter - individual T cell variation contributes towards BCG efficacy and immunogenicity. There is no gold standard laboratory
test for recipient BCG response and so an account of the multivariant surrogates of protection adopted in this study is provided.

In a third arm to the current study, peripheral blood TCRs before and after BCG-vaccination are analysed and a background to this methodology is provided.

The ultimate aim of the current study is to identify factors by which T cell vaccine design may be improved.

The objectives of the current study are:

(1) To quantify inter-individual variation in baseline NTC responsiveness to non-specific stimulation via the TCR.

(2) To quantify, in the same individuals, the inter-individual variation in response of memory T-cells to cognate antigen to which they have been pre-primed.

(3) To establish if there are associations between aspects of the measured cellular-response of NTCs to generic stimulation and the adaptive T cell response to antigen.

(4) To utilise sequencing of the peripheral blood TCRs before and after vaccination to identify any BCG-vaccine-induced signature.
Figure 1.1 Flowchart of concepts and background information introduced in Chapter 1.
1.2 ‘Immunotypes’ and Inter-individual Variation in Human Immune Systems in Health

The current project is concerned with variation between the immune systems of humans in health, investigating the contribution natural variation between host T cells may make to outcomes following antigenic challenge. It is known that the immune systems of ‘healthy’ individuals appear to be both highly variable between people and remarkably stable for each individual⁴. For example, multiple studies have performed replicate peripheral lymphocyte subset sampling of volunteer subjects for up to eight years and have demonstrated greater inter-subject than intra-subject variability in the total frequency and relative proportions of the different immune cells composing each individual’s immune system (an example of which is presented in Fig. 1.2), with each subject appearing to have a longitudinally stable characteristic lymphocyte subsets profile¹-⁶. Carr et al immune profiled 638 Belgium-residents in good health aged 2 to 86 years for up to three years and reported a high level of inter-subject variation in circulating immune cellular subsets, (the frequency of undifferentiated (naïve) or precursor cell types providing the largest component of the inter-subject variation) and stable population baselines in a longitudinal sub-cohort of 177 individuals. The majority (84%) of the variation seen in the study was explicable by a model that included both inter-individual and intra-individual variation, with just 1.4% attributable to the intra-individual variation between visits⁶.
Figure 1.2 An example reproduced from Brodin and Davis of inter-individual variation in distributions of six immune cell subset populations, from a Stanford cohort (n=398) of healthy adults, numbers indicative of minimum and maximal values observed\(^1\).
Further, individual immune systems appear to demonstrate a transient and highly elastic response to immunological and microbiological disturbances. An antigenic challenge may produce rapid expansion of cell populations and increase in serum cytokine concentrations of many orders of magnitude. However, whilst there may be cell division and functional changes within the responder clones (which undergo expansion followed by differentiation to produce a small population of memory cells), the long-term overall cell subset structure of the immune system remains stable, with individuals returning to steady state subsequent to the challenge (a hypothetical simplified example is illustrated in Fig. 1.3). Carr et al proceeded to describe a sub-cohort of 22 Belgium-residents who experienced traveler’s diarrhoea and an independent cohort of 32 English subjects who received the influenza vaccination. Rather than such challenges acting as a ‘reset’ on the immunological landscape with individuals stabilising at an alternative equilibrium point afterwards, the ‘immunological distance’ between longitudinal samples from both groups were no greater than that of individuals who were continuously healthy\(^6\). There do appear to be exceptions to intra-individual longitudinal stability. Seasonal changes in gene expression patterns for specific immune cell subpopulations over the course of the year have been described\(^7\). Further, extremes of age deliver a potent impact on both the innate and adaptive immune response including an age-associated decline in naïve T-cells expressing the CD4 co-receptor (driven primarily by failure of their peripheral replication) and reduced vaccine-responsiveness\(^8\)\textsuperscript{,12}. 
Figure 1.3 A illustration of intra-individual temporal stability found in most immune parameters over the course of weeks, including in response to an antigenic challenge where subsequent measurements tend to return to a stable baseline level\(^1\).
By measuring immune cell population frequencies and their responses to varied challenges it has been demonstrated that each healthy human has their own characteristic immune system, their ‘immunotype’. Immunotypes appear to occur on spectra, as opposed to clusters of like-performing individuals. On these continuums there will be healthy individuals with extreme values of specific cell types, but they are not outliers in the context of their collective immune cell composition. Kaczorowski et al performed a meta-analysis of immune cell composition data from 1575 healthy individuals across multiple cohorts and found no evidence of discrete groups of individuals based on their immune cell composition. Instead the study demonstrated a continuous distribution of immune cell subset populations, for example, in the ratios of T-cells expressing the CD4 or CD8 co-receptors among healthy individuals varying broadly from 0.34 to 16 (where the ‘normal’ ratio is considered approximately 2)\textsuperscript{13}.

\textit{A complex system, such as the immune system, probably uses adaptive strategies, compensatory pathways and functional redundancy to maintain its vital functions even in ‘outlier’ individuals [Petter Brodin & Mark Davis, 2017]}\textsuperscript{1}

The current study transcends simply measuring variation in baseline immune subset composition, and concerns itself with the variations between individuals in cellular phenotype and functionality in responses to antigenic challenge, which is commonly engendered in studies but infrequently commented upon. For example,
Martin et al, adopted surrogate activation markers examining genetically diverse mice to identify high inter-individual variability in both the magnitude of pathogen-specific CD8+ T-cell responses and the T-cell-mediated protection against re-infection in lymphocytic choriomeningitis virus (LCMV) and *Listeria monocytogenes* infection\textsuperscript{14,15}. Additional examples include several human studies which have identified inter-subject variation in immune signatures in response to the live-attenuated yellow fever (YF) vaccine 17D (YF17D). Gaucher et al demonstrated high variability between individuals in both CD4+ and CD8+ T cell proliferation and the expression of cytokines by CD4+ T cell subsets following in vitro stimulation with YF17D-derived peptide pools two-months subsequent to YF17D-vaccination\textsuperscript{16}. Querec et al identified that from two weeks post-vaccination, the CD8+ T cells of healthy human YF17D-recipients demonstrated activation (measured by expression of CD38 and Human Leukocyte Antigen – DR isotype, HLA-DR) varying more than tenfold between individuals\textsuperscript{17}. Akondy et al performed longitudinal analysis of YF17D-induced, antigen-specific CD8+ T cells, tracking their differentiation, and identifying inter-individual variation in the magnitude, phenotype and functionality of CD8+ T cell responses\textsuperscript{18}. A sample of plots from these three YF17D studies are reproduced in Fig. 1.4.
Figure 1.4 Examples of inter-individual variation in magnitude of phenotypic T cell response to antigenic challenge. (A) is reproduced from Gaucher et al and summarises T helper cell (Th1 and Th2) cytokine expression and carboxyfluorescein succinimidyl ester (CFSE)-labeled proliferative response by CD4+ and CD8+ T cells following 24hr incubation with 22 YF17D-derived peptide pools of six volunteers 60 days after YF17D-vaccination16. (B) is reproduced from Querec et al and depicts percentage cell antigenic-specific HLA-DR+CD38+ expression by peripheral CD3+CD8+ T cells in response to YF17D peptides on days 15 and 60 in 15 YF17D-vaccinees17. (C) is reproduced from Akondy et al and depicts expression of markers of activation, differentiation and proliferation by peripheral A2-NS4B+CD8+ cells in up to 15 YF17D-vaccinees on days 11, 14, 30 and 90 (individuals depicted with open circles, group mean given by horizontal line)19.
The observation of variation in structure and functionality of immune systems between individuals, might lead one to postulate that whilst patients born with monogenic deficiencies may present more overtly in early life with increased risk of severe infections, more subtle immune variation may contribute towards otherwise seemingly healthy individuals producing noteworthy immune responses such as autoimmune conditions, systemic inflammatory response syndrome (SIRS) in response to microbiological challenge or, most pertinent to this project, sub-optimal response to vaccination\textsuperscript{20-22}. Nuances within the immune states of a population are increasingly being appreciated, as we begin to shift towards precision and bespoke medicine\textsuperscript{23}.

To experimentally investigate molecular pathways and epigenetic factors underlying inter-individual variation in phenotypic T cell responses to stimulation is beyond the scope of the current project. The substantial variation demonstrated in the ‘immunotypes’ of humans and their immune system responses to challenges is considered to be the result of a vast array of heritable and non-heritable host factors: genetic diversity, varied environmental exposures and, effectively, the biographies of those individuals\textsuperscript{1}. With regard to the contribution of heritable factors towards inter-individual immune variation, genome-wide association studies (GWAS) have associated genetic loci with individual immune system measurements such as the frequencies of specific immune cell or the concentration of a specific cytokine, as well as identification of genetic risk variants for
autoimmune conditions\textsuperscript{24,25}. Gene expression profiling of immune cells suggests that approximately 25% of overall variation in immune function between individuals may be attributed to heritable factors, though heritability is more of a factor in some immune features than others\textsuperscript{26}. For example, Brodin et al recruited 105 healthy twin pairs and found that the phosphorylation (reversible covalent attachment of a phosphate group) of the transcription factor signal transducer and activator of transcription-5 (STAT5) following stimulation of CD4+ and CD8+ T cells with the interleukin-2 (IL-2) and IL-7 was almost entirely explicable by heritable factors. However, the group identified that 58% of all measurements of blood cell frequencies and functions and soluble factors had <20% of their total variance explained by heritable influences\textsuperscript{5}.

Host non-heritable factors thought to influence the human immune system response to antigenic challenges are listed in Table 1.1. Carr et al identified the largest influence on immunological variation as co-habitation, with 50% less immunological variation between the 70 pairs of individuals who shared a local environment as co-parents than between people in the wider population\textsuperscript{6}. Cytomegalovirus (CMV) produces a life-long chronic infection in humans and the virus is considered an important modulator of the host immune system, with each reactivation resulting in approximately 10% of the T cell repertoire becoming CMV-reactive\textsuperscript{27}. In monozygotic twins discordant for CMV, 119 out of 204 immune cell frequencies and serum proteins had a reduced twin-twin correlation
compared with CMV-negative monozygotic twins\textsuperscript{5}. With regard to vaccine efficacy, host factors which may contribute towards response include age (many vaccines have lower immunogenicity in neonates and elderly); sex (females have an overall tendency to generate higher antibody responses); ethnicity (different ethnic groups sharing geographical location exhibit variable responses to vaccination) and genetics (twin studies estimate the degree of heritability 36-90\% for humoral responses, and 39-90\% for cellular responses)\textsuperscript{28}. In addition to host genetic and non-genetic factors, factors directly related to the antigenic challenge itself will influence the host response to challenge for example pathogen factors, vaccine factors (vaccine product, vaccine strain, adjuvants and vaccine dose) and vaccine administration factors (administration schedule, site of administration and route of administration).
| Intrinsic factors | Age  
|                  | Sex  
|                  | Co-morbidities |
| Perinatal factors | Gestational age  
|                  | Birth weight |
|                  | Feeding method (breast or formula feeding) |
|                  | Maternal antibodies |
|                  | Maternal infections |
|                  | Other maternal factors |
| Extrinsic factors | Concomitant acute bacterial or viral infections  
|                  | Helminthic infections |
|                  | Pre-existing immunity |
|                  | Gut microbiota |
|                  | Chronic viral infections incl. latent CMV and EBV |
|                  | Antibiotics |
| Environmental factors | Geographic location  
|                  | Season |
|                  | Co-habitation |
|                  | Family size |
|                  | Toxins |
| Behavioural factors | Smoking  
|                  | Alcohol consumption |
|                  | Exercise |
|                  | Sleep |
|                  | Psychological stress |
| Nutritional factors | Body mass index (BMI)  
|                  | Nutritional status |
|                  | Micronutrients (vitamin A, D, E and Zinc) |
|                  | Enteropathy (pathology/inflammation of the intestine) |

Table 1.1 Examples of host non-heritable factors which may influence an individual’s immune response to an antigenic challenge$^{5,28-31}$. 

1.3 T Cells, the T Cell Receptor and T Cell Signalling

Lymphocytes (T and B cells) generate adaptive immune responses in vertebrates, both through their ability to respond to a vast array of antigens and by conferring immunological memory. These properties are facilitated in T cells by TCRs. Individual T cells are clonal, typically expressing only one type of receptor and each T cell displays approximately 30,000 identical TCRs unique to that T cell at the cell surface. Clonal amplification via division is an integral feature of immune responses. Each TCR is composed of a TCRα and a TCRβ chain, the TCRα:β heterodimer linked by a disulphide bond, which spans the T cell membrane and provides one antigen-binding site. TCRs have an amino-terminal variable (V) region and a constant (C) region and the combination of the α and β variable regions provide exquisite specificity for a particular, non-self-antigen. The variable transmembrane TCRα:β heterodimer of a T cell is combined with further cell surface molecules, the CD3γ, CD3δ and CD3ε protein chains, each with extracellular immunoglobulin-like domains (together forming the CD3 complex) and ζ chains to form the TCR-CD3 complex. The TCR-CD3 complex is responsible for transducing the TCR signal into the T cell, leading to gene activation, differentiation and proliferation. The receptor α chain interacts with one CD3δ:CD3ε dimer and one ζ dimer, whilst the receptor β chain interacts with one CD3γ:CD3ε. The TCR-CD3 complex structure contains eight subunits,
consisting of four dimeric modules, TCRαβ, CD3γε, CD3δε' and CD3ζζ' with 1:1:1:1 stoichiometry. The transmembrane segment contains an eight-helix-bundle, at the centre of which are the two transmembrane helices of TCRαβ. A simplified illustration of the TCR-CD3 complex is presented in Fig. 1.5. A minority of T cells display alternative receptors structurally similar to TCRαβ, designated γδ.
Figure 1.5 The basic structure of a typical T cell receptor.\textsuperscript{32}
The remarkably diverse TCRs (estimated to arise from a potential range of >$10^{15}$) are generated in the early development of T cells from common bone marrow derived lymphoid progenitor cells. Diversity between the TCRs of different T cells is achieved predominantly through a series of stochastic genomic rearrangements of the germline TCR locus, with non-template addition and removal of deoxyribonucleic acid (DNA) base pairs. Imprecise TCRα locus rearrangements in variability (Vα) and joining (Jα) gene segments in alpha chains and TCRβ locus rearrangements in Vβ, diversity (Dβ) and Jβ segments in beta chains (see Fig. 1.6) achieve an extensive array of disparate sequences in the complementarity determining region 3 (CDR3), the most variable portion of the molecule and crucial in peptide recognition. Within the thymus, developing T cells bearing novel TCRs are tested both by positive selection (only cells bearing TCRs able to recognise antigen combined with self-major histocompatibility complex (MHC) molecules are allowed to mature) and negative selection (cells intolerant of self-antigens are removed). Only approximately 1-2% of the TCR sequences between two healthy and unrelated individuals are thought to be shared. Shared TCR molecules between individuals may be considered ‘public’ whilst ‘private’ T cell responses involves little TCR sharing.
Figure 1.6 Hypothetical examples of human T cell receptor (TCR) gene rearrangement to generate the functional genes encoding for the T cell surface αβ heterodimer. The α-chain deoxyribonucleic acid (DNA) (in which the TCR-δ locus is also embedded), undergoes variable (V) - joining (J) recombination and the TCR-α transcript is produced where V, J and constant (C) segments connect directly after the intron sequences are spliced out. The β-chain DNA undergoes two-step recombination, first diversity (D) β to Jβ, and then Vβ to Dβ-Jβ rearrangement. The intervening sequences are then cut off, generating the TCR-β chain transcript with V, D, J and C region adjacent.\(^\text{39}\).
The TCR recognises the foreign antigen when presented by an antigen presenting cell (APC) bound to a MHC, illustrated in Fig. 1.7. T cells may be categorised as belonging to one of two major classes, determined by their expression of either CD4 or CD8 cell-surface proteins. T cells which express CD4 are referred to as T helper cells, as their function is to activate other cells, whilst CD8 is expressed by cytotoxic T cells. CD4 recognises MHC class II molecules and CD8 recognises MHC class I molecules. MHC molecules are transmembrane glycoproteins expressed on cell surfaces which present short peptide fragments of protein antigens. In the course of antigen recognition, CD4 or CD8 associate with their own cell’s TCR and also to the cell which is presenting the antigen, by binding to the invariant site on the MHC portion of the composite peptide: MHC ligand, away from the peptide-binding site. In this way, CD4 and CD8 are co-receptors, and this binding contributes to the overall effectiveness of the T cell response.
Figure 1.7 TCRs recognise foreign antigen when presented by an antigen presenting cell (APC) bound to a major histocompatibility complex (MHC). CD4 co-receptors recognise MHC class II molecules whereas CD8 co-receptors recognise MHC class I molecules.\textsuperscript{32}
Prior to activation, T cells are relatively functionally dormant and are NTCs. When the TCRs of NTCs encounter their cognate antigen, those rare antigen-specific clones within the NTC repertoire may activate, proliferate and differentiation into several different functional types of effector and memory T cells. NTCs enable a host to combat new, previously unencountered pathogens, whilst memory T cells are enriched to counter recall antigens. Whilst activated CD8+ cells differentiate into cytotoxic effector cells to kill their target cells (providing defense against intracellular pathogens, especially viruses), there are several subsets of effector T cells into which CD4+ T helper cells may differentiate, which act to orchestrate different immune functions. The main CD4+ effector subsets include T helper 1 (Th1) cells (broadly, release interferon-γ (IFN-γ) and tumor necrosis factor α (TNFα) to target viruses and intracellular microbes), Th2 cells (produce interleukin-4 (IL-4), IL-5 and IL-13 to counter parasites), Th17 cells (release IL-17 family cytokines at mucosal interfaces to contribute antifungal and antibacterial defenses), follicular Th cells (aid B-cells in the production of high-affinity immunoglobulins) and regulatory T (Treg) cells (suppress T cell and innate immune cell activity to suppress auto-immunity during immune responses). Memory T cells are long-lived, persisting after an antigen has been cleared, and provide long-term immunity by remaining capable of rapid amplification of effector functions and proliferation on re-encounter of the antigen to which they have been pre-primed.
T cells migrate between blood, lymph and secondary lymphoid tissue, surveying the APCs for their specific antigen. T cells are able to respond to their specific antigen even when sparsely present: the number of peptide:MHC complexes displayed by APCs specific for a particular TCR is likely to be very low. The engagement of the extracellular region of the transmembrane portion of TCR of a dormant naïve T cell by its specific antigen is transduced into intracellular signalling, mediated by invariant accessory proteins, transmitting the information via multiple downstream pathways, summarised for CD4+ T cells in Fig 1.8 and described in detail in the paragraphs subsequent to this. The cascades of intracellular signalling results in the cells restructuring their actin cytoskeleton, activating transcription factors, increased appropriate protein synthesis followed by rapid cell division and differentiation of the lymphocytes into an expanded population of metabolically active effector T cells\textsuperscript{32}.\textsuperscript{32}
Figure 1.8 Overview of CD4+ T cell receptor signalling cascades, reproduced from Hwang et al\textsuperscript{40}. 
The initial intracellular signalling event in the signalling cascade following ligand binding to the TCR is mediated by tyrosine phosphorylation within cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs) of the CD3ε, γ, δ and ζ chains. Each TCR has a total of 10 ITAMs (the CD3γ, CD3δ and two CD3ε each containing one ITAM and each of the homodimer ζ chains have three each). The Src-family kinase Lck is constitutively associated with the cytoplasmic domains of the co-receptors CD4 and CD8 and when the TCR in naïve and effector T-cells binds its antigen, Lck phosphorylates the two tyrosine residues of each ITAM. A further role of the co-receptors in TCR signalling may be to stabilise interactions between the receptor and the peptide:MHC complex by increasing its duration.32

The next step in the TCR signalling pathway is the recruitment and activation of zeta-chain-associated protein kinase-70 (ZAP-70, ζ-chain-associated protein). Lck-phosphorylated ITAMs bind ZAP-70, which is then itself phosphorylated by Lck. Activated ZAP-70 phosphorylates Linker for Activation of T cells (LAT) and SH2-domain-containing leucocyte protein of 76 kDa (SLP-76), recruiting them to the activated TCR complex. LAT and SLP-76 can be linked by the adaptor protein Grb2-related adaptor downstream of Shc (Gads) to form the LAT:Gads:SLP-76 complex. A second essential event that occurs rapidly following ZAP-70 activation is the recruitment and phosphorylation of the membrane lipid Phosphatidylinositol 4,5-bisphosphate (PIP2) in the plasma membrane by phosphoinositide 3-kinase (PI3K) to generate Phosphatidylinositol 3,4,5-triphosphate (PIP3)32.
On formation of the LAT:Gads:SLP-76 complex and activation of PIP_3, the TCR-signalling pathway branches into four downstream modules (all of which mediate cellular changes to enable the T-cell to activate):

(1) Phosphorylation of phospholipase C-γ (PLC-γ) (by the Tec-family kinase Itk) which catalyses the breakdown of PIP_2 to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) which triggers T-cell transcription factor activation via (at least three) pathways:

(i) IP_3 binds endoplasmic reticulum (ER) membrane Ca^{2+} channel receptors, triggering depletion of ER Ca^{2+} stores, which triggers oligomer clustering of stromal interaction molecule 1 (STIM1) in the ER membrane. The STIM1 oligomers bind the ORAI1 calcium channels of the plasma membrane, allowing calcium into the cell, inducing a conformational change in calmodulin. Calmodulin then activates calcineurin which dephosphorylates Nuclear Factor of Activated T cells (NFAT). NFAT then enters the nucleus where it is a transcription factor for genes for T-cell activation such as the IL-2 gene.

(ii) DAG binds to the C1 domain of Ras guanyl nucleotide releasing proteins (RasGRP) which activates the small guanosine triphosphate (GTP)ase Ras. Phosphorylated LAT and SLP-76 also recruits the adaptor protein Growth factor receptor-bound protein 2 (Grb2) which recruits the guanine-exchange factor Sos, which also activate Ras. Activated Ras triggers a three-kinase Raf/MEK1/Erk
cascade pathway which helps generate the T cell activation transcription factor Activator protein 1 (AP-1).

(iii) DAG also recruits Protein kinase C-θ (PKC-θ) which phosphorylates CARMA1, which then oligomerises to form a multi-subunit complex which activates tumour necrosis factor receptor-associated factor 6 (TRAF-6), which activates transforming growth factor β-activated kinase 1 (TAK1), which activates IκB kinase (IKK), which phosphorylates IκB, causing its ubiquination and degradation, which results in active transcription factor Nuclear Factor κB (NFκB) to the nucleus.

The transcription factors NFAT, AP-1 and NFκB, which, for example, bind to their control sites in the IL-2 promoter for the gene for the cytokine IL-2 (essential for promoting T-cell proliferation and differentiation into effector cells).

(2) Phosphorylation of a serine/threonine kinase Akt by phosphoinositide-dependent protein kinase-1 (PDK1), which enhances T-cell activation by:

(i) Triggering a cascade which may inhibit cell death by the phosphorylation of the pro-apoptotic protein Bad (blocking its inhibition of the anti-apoptotic protein B cell lymphoma 2 (Bcl-2)).

(ii) Regulating the expression of homing and adhesion receptors so that the activated T cell may migrate.

(iii) Stimulating the cell’s metabolism by increasing the activity of glycolytic enzymes and by inducing the upregulation of T cell membrane nutrient transporters.
(iv) Inactivating the tuberous sclerosis complex (TSC) (a GTPase-activating protein (GAP) for the small GTPase Rheb), leading to Rheb activation, which activates the mammalian target of rapamycin (mTOR) pathway, enhancing macromolecular biosynthesis and cellular metabolism.

(v) Phosphorylating nuclear factor-90 (NF-90), which translocates from the nucleus to the cytoplasm, stabilising IL-2 messenger ribonucleic acid (mRNA), enhancing IL-2 synthesis.

(3) Recruitment of the adhesion and degranulation promoting adaptor protein (ADAP) to the LAT:Gads:SLP-76 scaffold complex. ADAP then recruits SKA55 and RIAM. The ADAP:SKAP55:RIAM complex binds to the small GTPase Rap1, activating Rap1 at the site of TCR signalling. GTP-bound Rap1 then promotes lymphocyte function-associated antigen 1 (LFA-1) aggregation and the conformational change that converts LFA-1 into a high-affinity binding partner for intercellular adhesion molecule 1 (ICAM-1). This promote the stability of the T cell-APC interaction and localising active signalling complexes into this ‘immune synapse’ region.

(4) A guanine nucleotide exchange factor (GEF) Vav is recruited to PIP3 and the LAT:Gads:SLP-76 scaffold complex and activated, which in turn activates Rho-family GTPases, including Cdc42, which induces a conformational change in the Wiskott-Aldrich syndrome protein (WASp), which is also recruited to the LAT:Gads:SLP-76 scaffold complex by binding to the small adaptor Nck. The active form of WASp binds to WASp-interacting protein (WIP), and together these
proteins recruit Arp2/3 leading to actin polymerisation and cytoskeleton reorganisation aiding formation of a stable immune synapse, for example, to ensure effective interactions between CD4+ T cells and B cells.\textsuperscript{32}

Deficiencies in several signalling components including Lck, Zap-70, Itk, CD45, CARMA1 and ORAI1 have been found to be mutated in cases of severe combined immunodeficiency (SCID).\textsuperscript{32}

Signalling via the TCR alone is insufficient to activate a NTC and simultaneous additional signals are required to interact with naïve T cell CD28 co-stimulatory receptors. This enhances TCR induction of transcription factors as well as PI3K activation to achieve T-cell cell proliferation, cytokine production and cell survival. If the co-stimulatory ligands B7.1 (CD80) and B7.2 (CD86) present on the surface of APCs engage CD28 simultaneously to TCR engagement, co-stimulatory signals ensure maximal activation of three of the four TCR signalling modules (PIP3, Itk and Lck). Thus, the full activation of PLC-γ leading to transcription factor activation requires signals emanating from both the TCR and CD28. Further, CD28 stimulation induces phosphorylation of NF-90 resulting in increased IL-2 mRNA stability.\textsuperscript{32}

Immune checkpoint receptors or inhibitory receptors (for example, cytotoxic T lymphocyte antigen 4 (CTLA-4 or CD152) and programmed cell death 1 (PD-1)
are expressed by conventional T cells to downregulate activation signalling and can stimulate apoptosis, thereby preventing potentially destructive inflammation or self-reactive T cells (autoimmunity). CTLA-4 directly antagonises the costimulatory receptor CD28, possibly through competition, given CTLA-4 binds the CD28 ligands CD80 and CD86 with greater affinity than CD28. Mice genetically-deficient in CTLA-4 die young from an immune dysregulation and autoimmunity, the later likely to be the result of excessive CD28 stimulation by CD80 and CD86. PD-1 is extracellularly expressed by all T cells on activation. Ligation of the PD-1 receptor by PD-1 causes phosphorylation of immunoreceptor tyrosine-based inhibitory motif (ITSM) and recruitment of SHP2, resulting in the dephosphorylation of PI3K and AKT resulting in reduced NFκB activity and therefore decreased T cell activation and preventing autoimmunity.

1.4 Inter-individual Variance in Naïve T-cell Responsiveness

Inherent biological variations between the baseline, pre-perturbation immune systems of individuals and the effects of this on that individual’s immune responses appear to transcend just differences in cell subpopulation frequencies (as already described in the opening paragraphs of this Introduction). A phenomenon about which little is currently known, and the subject of the first arm of this thesis, is inter-individual variation in NTC response in type and magnitude to a standard, non-antigen-specific stimulation. One could speculate, any such measurable
variation may reflect intrinsic biological variance between individuals in their T cell intracellular signalling capacity, which may have genetic and nongenetic components. Akin to the diversification of MHC alleles, human T cell responsiveness in overall efficacy and functional bias may have diversified through selection from exposure of different ancestries to different types of pathogens. One geographical location may bias towards a Th phenotype being advantageous, whilst in another it may result in sub-optimal responses to the dominant pathogens, or carry a penalty in terms of self/non-self-discrimination and autoimmune diseases. Characterisation of baseline inter-individual variation between healthy-individuals pre-pathogen or vaccine challenge may identify individuals with outlier immunotypes, unresponsive to currently available vaccinations; help to construct systems models; identify pertinent biomarkers; and help characterise pathological states; but also, crucially may reveal correlates for post-perturbation responses and unlock critical molecular and cellular pathways for targeted future therapeutic development.

T cell response to TCR-engagement by antigen may include activation, clonal expansion, differentiation into effector cells, apoptosis or anergy. T cells may be artificially and polyclonally activated by a standardised mimic of cognate antigen recognition and the variability in response can be characterised by profiling the activated T cells. Generic T cell stimulation via the TCR-CD3 complex and CD28 co-receptor is achieved in the first arm of this project by culturing NTCs in the
presence of the recognised expansion platform of anti-CD3 and anti-CD28 monoclonal antibodies bound to a solid surface such as a bead. Anti-CD3/anti-CD28 beads (anti-CD3/CD28) may be referred to as artificial antigen-presenting cells (aAPCs) (see Fig. 1.9). The technique has been utilised as an immunotherapy strategy in clinical settings, to expand antigen-specific cells ex vivo, whilst maintaining their functional capacity before adoptive cell transfer (ACT) into a patient with the goal of treating a virus or tumour. Anti-CD3/CD28 stimulation mimics recognition of cognate antigen in a standard manner, is independent of T cell specificity and triggers the cascade of intracellular signals resulting in increased cellular metabolism, cell division and opposition to cell death. Further, anti-CD3/CD28-induced activation may also be performed independent of constitutional APCs (which may vary in functional capacity between individuals, a potential source of confounding if seeking to measure an individual’s T cell responsiveness) and also by-passes the potential influence of human leukocyte antigen (HLA) polymorphism. Highly purified human NTCs will not proliferate in response to ligation of CD3 alone and co-stimulation with anti-CD28 antibody in addition to anti-CD3 is required.
Figure 1.9 Generic T cell stimulation via the TCR-CD3 complex and CD28 coreceptor achieved by culturing naïve T cells (NTCs) in the presence of anti-CD3 and anti-CD28 monoclonal antibodies bound to a solid surface such as a bead (also known as an artificial antigen-presenting cell, aAPC).
Inter-subject variation in NTC response in type and magnitude to a standard, non-antigen-specific stimulation, the subject of the first part of the present study, has hitherto only had a cursory examination in the literature. Patel et al in 2017, published a small study on the response of frozen/thawed whole peripheral blood mononuclear cells (PBMCs) CD8+ T cell to stimulation with commercial anti-CD3/CD28 Dynabeads in six laboratory donors. The concentrations and ratios of anti-CD3 and anti-CD28 on such commercial Dynabeads is not made publicly available (private correspondence with ThermoFisher), but preliminary assays described in Chapter 3 are suggestive of commercial Dynabeads holding sufficient anti-CD3 and anti-CD28 that maximal T cell stimulation is achieved. In the Patel study, CD8+ T cell response to incubation with Dynabeads was ascertained by flow cytometry for the markers IFN-γ, macrophage inflammatory proteins 1b (MIP1b), perforin and granzyme B and corroborated by alternative antibody-based assay technologies to detect soluble cytokines. A range of between 1.2- to 100-fold increases in expression of functional markers was described depending on donor and stimulation time point, suggestive of considerable inter-individual variation in response to stimulation, though ‘data were analysed for general patterns of change’ and no statistical tests were run. The Immune Variation (ImmVar) Project recruited a cohort of healthy, multi-ethnic residents of metropolitan Boston, with the aim of investigating variability in immunological functional responses between individuals. In a seminal 2014 study, Ye et al isolated the peripheral CD4+ T cells of 348 ImmVar subjects and stimulated them with anti-CD3/CD28 beads,
observing a high degree of reproducible interindividual variation following patterns more complex than simple Th1/2/17 partitions (see Fig. 1.10). In addition, a quantitative and qualitative ‘response index’ was calculated using gene expression profiling to quantify transcriptional responses to the stimulation. NanoString direct molecule counting was employed to measure 236 transcripts (those thought to best capture the responses and their variance across donors and activation states, together with transcripts of known importance, including 16 key-defining cytokines). Of the 1750 induced and 456 repressed genes (fold change >1.68), proliferation and effector genes were represented; cytokines displayed the most variability (but with minimal or no evidence of cis genetic control); and cytokine receptors were less variable (but for which several expression quantitative trait loci (eQTLs) were detected). The authors found variation between individuals in their general immune ‘responsiveness’, with a widespread correlation between all induced cytokines. Data were related to dense single-nucleotide polymorphisms (SNPs) from the participants, to identify the genetic contributions to the variation and eQTLs controlling these responses were fine-mapped: 39 loci were identified associated in cis with gene activation in T cells, explaining on average 25% of the repeatable variation (the remaining 75% possibly due to environmental influences and immunologic history). The authors proposed that genetic fluctuations are tolerated at cytokine receptor loci, but cytokines themselves might vary with environmental cues or the individual’s immunological history.\textsuperscript{54}
Figure 1.10 Reproduced from Ye et al, a heatmap of 16 cytokines clustered by expression across 348 individuals following 48 hours incubation with beads coated with anti-CD3 and anti-CD28 monoclonal antibodies (here referred to as \( \alpha 3+28 \)) and demonstrative of inter-individual variation in T cell response\(^{54}\).
The first arm of the present study presented in Chapter 4, seeks to further expand on the aforementioned published studies by quantifying inter-individual variation in integral naïve CD4+ and CD8+ T cell responsiveness to non-specific stimulation. The response parameters measured include markers of activation, differentiation and proliferation. The heritable, intrinsic and/or environmental factors driving any inter-individual variation is beyond the scope of the present study. There is however an exploration for any relationship between the measured variation in baseline responsiveness of an individual’s NTCs and subsequent functionality of that individual’s T-cells. A multiparametric matrix of intrinsic T cell ‘responsiveness’ in approximately one hundred study participants, is constructed from NTC anti-CD3/CD28 stimulation assays and subsequent multichromatic fluorescence-activated cell sorting (FACS) flow cytometry for markers of activation, proliferation and differentiation.

Activation markers are surface proteins upregulated soon after peptide-MHC ligand engagement of the TCR and the subsequent signaling cascades which determine T cell response. The present study utilises surface expression of CD25 and PD-1 to quantify NTC activation. Prior to activation, NTCs express an IL-2 receptor composed only of β- and γ-chains, which engender only modest binding of IL-2. Following antigen recognition by the TCR, NTCs synthesize both IL-2 and also the α-chain of the IL-2 receptor (IL-2RA, also known as CD25). The addition of CD25 to the β and γ heterodimer to form a trimeric IL-2 receptor
provides it with a much higher affinity for IL-2, so that the activated T-cell may respond to very low IL-2 concentrations\textsuperscript{32}. The nature and function of PD-1 has already been described in an earlier paragraph.

The present study utilises both Ki67 and cell trace labelling (CellTrace Violet, CTV) to measure NTC proliferation. In the event of an NTC TCR binding its cognate antigen, the T cell transitions from a small, quiescent state with condensed chromatin (G\textsubscript{0}) to re-enter the G\textsubscript{1} phase of the cell cycle and rapidly divide and amplify in numbers to build the required antigen-specific effector T cell numbers. Ki-67 is a cellular protein which spatially organises heterochromatin, thereby controlling gene expression and is strictly associated with proliferation, present during all active phases of the cell cycle (G\textsubscript{1}, S, G\textsubscript{2}, and mitosis), but not detectable in G\textsubscript{0} resting cells\textsuperscript{55}. Using cell trace labelling of cell membranes with a fluorescent dye, subsequent dilution of the fluorescence concentration by half each time the cell divides, measurable using flow cytometry, enables cell tracking over multiple generations.

Broadly speaking, cellular differentiation of T cells from naïve, to memory and effector cells may be indicated according to their expression of CD45RA (the naïve cell marker) and chemokine (C-C motif) receptor 7 (CCR7, which recruits cells to the lymph nodes), although the use of these markers is by no means exhaustive to categorise all T cell subsets\textsuperscript{56,57}. Activated T cells switch the isoform of CD45
expressed from CD45RA to CD45RO and effector memory cells (T_{EM}) also down regulate CCR7. Therefore, cells may be subdivided into naïve (CD45RA+CCR7+), central memory T cells (T_{CM}, CD45RA-CCR7+), and effector memory T cells (T_{EM}, CD45RA-CCR7-)\textsuperscript{58-62}. There is also a subset of CD45RA+CCR7- effector memory T cells, TEMRA, thought to re-express CD45RA following antigenic stimulation, of unknown function not included in the analyses of the present project\textsuperscript{63}.

Collectively, these response parameters measured in the present study by multichromatic flow cytometry following NTC stimulation by anti-CD3/CD28 will provide a multi-dimensional picture of each individual’s intrinsic ‘responsiveness’. Cytokine response to NTC stimulation was not measured on account of NTCs being known to primarily produce IL-2 alone (which has been demonstrated to be crucial for proliferation) and secreting only low quantities of IFN. This is contrast to differentiated effector T cells, which produce diverse cytokines (the character of which are dependent on the mature effector phenotype)\textsuperscript{32}.

When seeking to establish divergence in the immune systems of a population, it is crucial to delineate the relative proportion of inter-, intra- (temporal) and technical variations. Total observed variation in any measured parameters may be attributed to (i) subject-to-subject variation, (ii) variation within a person over time and (iii)
technical or measurement noise. We may seek to estimate the contribution from the third category with technical repeats.

1.5 Vaccines and Systems Vaccinology

The second arm of the present study seeks to examine for inter-individual variation in vivo NTC response to a standard T cell priming vaccination via subsequent in vitro mature T cell phenotypic response to recall antigen (in the same individuals in whom NTC response to anti-CD3/CD28 was being measured). A vaccine is an innocuous preparation derived directly or indirectly from a pathogen, and then inoculated into a subject to stimulate a host immune response. From a public health perspective, the goal of vaccination is the generation of long-lasting and protective immunity. The quality and/or quantity of a response to vaccination (QR) is commonly measured using surrogate correlates of protection, such as geometric mean antibody titres (GMTs), seroconversion rates (SCRs), sero-protection rates (SPRs), functional antibodies (by flow cytometric opsonophagocytosis assays), antibody avidity, B and T cell activation, lymphoproliferation and cytokine responses. Such surrogate markers are preferentially used in the stead of protection against the pathogen itself (although some studies have employed infection challenges where no or only weak correlates of protection are known, such as in malaria). Healthy individuals display
substantial variation in their immune response to vaccinations with consequences for both efficacy and duration of protection. For example, B cell antibody responses induced by vaccination may vary between individuals by over 10-fold for yellow fever vaccination; 40-fold for 7- and 13-valent conjugated pneumococcal (PCV7 and PCV13) and haemophilus influenzae type b (Hib) vaccinations; and over 100-fold for trivalent inactivated influenza vaccine (TIV) and hepatitis B vaccination and with some ‘healthy’ candidate vaccinees being ‘non-responders’.\textsuperscript{12,17,28,68}

Vaccination presents the opportunity to provide a precisely synchronised perturbation of the immune system with easy access to subsequent blood samples to decipher the resultant immune response from the immediate moments to decades after vaccination. Wrammert et al found that 50-80\% of circulating plasmablasts were specific for antigens in the vaccine seven days after an influenza virus vaccination\textsuperscript{69}. Virtually all individuals have protective antibody titres against diphtheria for 25 years and tetanus for 50 years following vaccination\textsuperscript{70}. A compelling option to establish if there are aspects of the measured cellular-response of NTCs to non-specific stimulation which are predictive of the adaptive T cell response to antigen would be to employ systems vaccinology and modelling in analysis of the data. Systems biology is the practice of collecting dense measurements (‘big data’) of system states from which models are constructed, with an aim to accurately predict responses to perturbation\textsuperscript{71}. In place of adopting
group averages to model immune system behaviour, systems approaches can explore inter-individual variation by generating distinguishable clusters of phenotypes within and across individuals to discover underlying immune states\textsuperscript{72}. Within a population, vaccination may be deployed as a model perturbation and then the variation in quality or quantity of the response comprehensively analysed with the aim of defining new mechanisms and correlates of protective immunity\textsuperscript{73}. Multiplexed, high-throughput technologies, for example ultra-high-dimensional flow cytometry, massively parallel sequencing of the T-cell receptor repertoire, transcriptomics, proteomics, metabolomics, cytokine profiling and mass cytometry can make ‘omic’ measurements of biomarkers from the same individual and subsequent computational analysis can facilitate integration of hierarchical levels of information and provide a deconvolution of complex biological systems\textsuperscript{30}. This technique of ‘systems vaccinology’ may utilise mathematical or machine-learning models to combine information from multiple parameters to generate potential pre-vaccination correlates or predictors of quality immune responses also accounting for inter-subject variability beyond that attributable to observable intrinsic factors\textsuperscript{17,64,68}. Tsang et al utilised multimodal data sets and natural population variation in 63 individuals before and following vaccination with TIV and developed models able to predict the humoral responses using baseline PBMC subpopulation frequencies alone (independent of age, gender, initial serology and the specificity of the cell populations for vaccine antigens)\textsuperscript{4}. 


Systems vaccinology is increasingly appreciated as pertinent science as it facilitates
the study of the human immune system in vivo. Humans are genetically diverse,
are afflicted by a myriad of diseases, lend themselves to medically-translational
research and afford the potential of discovering novel human biology.

*We don’t have to look for a model organism anymore. Because we are the model
organisms.* [Sydney Brenner, 2008]

Understanding which parameters of an immune response vary between individuals,
and how this variation influences the immune system is expected to improve our
understanding of the mechanisms of immunological protection and pathogenesis.
This knowledge, may support new approaches for risk stratification, therapies
targeting the immune system and rational design of vaccination, rather than
development by ‘trial and error’.

1.6 Inter-individual Variance in Bacillus Calmette-Guérin Immunogenicity

For the present study, a suitable vaccine was sought which offers protection
primarily via T cell cytotoxicity or T cell activation of macrophages. This was in
order that each participants’ post-vaccination trained T cell phenotypic responses
to cognate antigenic stimulation could then be measured.
Excluding pure polysaccharide vaccines, all vaccines elicit T cell responses. In the majority of vaccines known as ‘humoral vaccines’ (for example, the measles, mumps and rubella (MMR) vaccine), the T cell responses are essential for generating antibody isotype switching, affinity maturation and high titres of neutralising antibodies against extracellular organisms, viruses or toxins\textsuperscript{76}.

In contrast, ‘T cell vaccines’ are considered to be any which offer protection via direct T cell activity and largely independent of antibody production. Development of T cell vaccines is challenging as the targeted pathogens tend to be highly evolved to combat immune detection and clearance; natural infection with these pathogens can be chronic and/or recurrent and does not tend to induce protective immunity; and strong and durable CD4+/CD8+ cell-mediated immunity is not efficiently generated by the current vaccine technology. The BCG vaccine remains the only licensed T cell vaccine in the world\textsuperscript{77}. Other cell-mediated vaccines currently remain more experimental with only modicums of success (including MRKA\textsuperscript{d}5 HIV-1 gag/pol/nef vaccine (against human immunodeficiency virus, HIV) and a universal influenza vaccine)\textsuperscript{78-81}.

*Mycobacterium Tuberculosis* (Mt\textsubscript{b}), a bacterial pathogen spread by respiratory droplets and able to afflict any part of the human body, remains one of the biggest infectious causes of mortality worldwide. The World Health Organisation (WHO) estimate two billion people worldwide harbour latent tuberculosis infection (LTBI)
and tuberculosis (TB) was estimated as the cause of 1.4 million deaths and 10 million new cases of disease in 2019[^82]. The live attenuated BCG vaccine, derived from *M. bovis*, is currently the only licensed vaccine available for the prevention of disease related to mycobacterial infection and is one of the most widely distributed of all vaccines, with >3 billion BCG doses administered to date worldwide and to >80% of neonates in TB-endemic countries (as part of the WHO Expanded Programme on Immunisation (EPI)). However, despite its widespread use, on account of BCG’s incomplete and inconsistent efficacy, it is estimated that millions of BCG-vaccinated individuals remain unprotected from TB[^83,84]. Whilst in immunologically naïve animal models BCG consistently confers effective protection against TB and in human children there is evidence that BCG affords protection against disseminated forms of TB, considerable variation is found in BCG-induced protection against human adult pulmonary TB between trials in different settings[^85-87]. A Mangtani et al 2014 systematic review, from which Fig. 1.12 is reproduced, meta-analyses and meta-regression examining the association of trial, setting and design with BCG-efficacy against TB found protection against pulmonary TB (PTB) ranged from 0% in the Chingleput trial in South India to 80% in the UK Medical Research Council (MRC) trial. The protective effect of BCG was, on average, greater in trials conducted at latitudes farthest from the equator, and, in general, absent or low in trials closer to the equator (latitudes <20° and 20°–40°[^88]).
Figure 1.12 Reproduced from Mangtani et al, meta-analyses derived rate ratios and 95% confidence intervals for pulmonary tuberculosis (PTB) following Bacillus Calmette-Guérin (BCG)-vaccination, stratified by latitude of study location and ordered by year of study start.
Why BCG-induced immunity against TB is so variable between hosts has been much debated over the last 70 years, but hitherto remains unexplained, and is pertinent to the present study, which seeks to explore the contribution of individuals’ T cell vaccine responses to the vaccine’s immunogenicity. The prevailing hypotheses regarding the likely source of variable BCG efficacy between trials, first proposed in the 1960s, identify the close relatedness of the species within the Mycobacterium genus, to which both *M. bovis* and *M. tuberculosis* belong, but also includes *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium fortuitum*, and *Mycobacterium kansasii* and many other non- or mildly-pathogenic soil and water saprophytes (so called environmental mycobacteria). Shared features within the Mycobacterium genus include lipid-rich outer cell walls, genomes with a high guanine-cytosine (GC) content, multiple similar gene families and cross-reactivity with high conservation of some of the major immunodominant antigens. Sensitisation to environmental mycobacteria has been reported to occur more in tropical or temperate latitudes and less at higher latitudes. Repeated exposure to these environmental mycobacteria induces immune reactivity, which may ‘mask’ or ‘block’ the effects of BCG. This may explain why the level of protection afforded by BCG was found to offer no additional benefit when used at low latitudes unless there is vaccination of neonates (before environmental sensitisation has been able to occur) or stringent testing for and exclusion of previously sensitised subjects.
The ‘masking hypothesis’ proposes that environmental mycobacterial sensitisation confers substantive protection against TB, thus inducing comparable protection in both unvaccinated and BCG-vaccinated subjects, resulting in lower measured BCG-induced protection. Palmer and Long exposed Guinea pigs to *M.fortuitum, M.avium* or *M.kansasii*, resulting in 1.5, 50 or 85% as much protection as did BCG alone, respectively, and the combination of environmental mycobacteria exposure followed by BCG did not result in more protection than BCG alone\textsuperscript{101}. A 2006 sub-analysis of the hitherto labelled zero-protection 1968 Chingleput trial, found a subset of over 40,000 subjects who were non-reactors to PPD and in whom BCG did actually confer a low level of protection (29 – 34\%\textsuperscript{104}). The ‘blocking hypothesis’ postulates that environmental mycobacterial sensitisation prior to BCG-vaccination actually prevents immune response to the BCG, for example, by stopping BCG multiplication or by causing an accelerated BCG-clearance by pre-primed immunity\textsuperscript{105}. Prior acquisition of immune reactivity to mycobacteria is demonstrable by tuberculin skin testing (TST), where tuberculin purified protein derivative (PPD) consisting of Mtb extracts is injected under the skin and previous sensitisation to mycobacteria elicits a delayed type hypersensitivity (DTH) response (swelling and reddening at the site of injection 24-48 hours later)\textsuperscript{106}.

However, more recent discoveries are problematic to the environmental sensitisation hypotheses. For example, it is no longer considered accurate to state environmental mycobacteria decrease with latitude, as it is now appreciated that
there are hundreds of different mycobacteria with different geographical distributions\textsuperscript{105}. With that said, the magnitude of human exposure to environmental mycobacteria may still vary between countries at low and higher latitudes.

Alternative hypotheses to environmental sensitisation, proposed to explain variable BCG-efficacy across the globe include genotypic differences between infecting mycobacteria; variable exposure to ultraviolet light (which has a mycobacterial killing effect); the effect of vitamin D levels, helminthic infections and poor nutrition on the immune system in low-income settings; variable CD4+ T cell activation in BCG-recipients caused by, for example, CMV infection; and different strains of BCG administered in the different trials\textsuperscript{88,107-110}.

With regard to different strains of BCG, BCG is derived from a virulent strain of \textit{M. bovis}, which was attenuated by 231 serial passages on a potato medium from 1908 – 1921 at the Pasteur Institute in Lille, France. During the first half of the 20\textsuperscript{th} Century, the substantial global demand for BCG was met by disseminating the initial strain from the original European sources to multiple local laboratories. This occurred prior to the introduction of standardised seed lots, with further passages on a variety of synthetic media, resulting in subsequently identified differences in genetic, transcriptomic, proteomic, phenotypic and antigenic properties between local BCG strains. It was not until the 1950s and 1960s that lyophilisation was
employed to stabilise BCG products\textsuperscript{111,112,113}. It is postulated that the many different strains of BCG used in the different trials have widely differing potency, although this was not sustained by Mangtani et al (assessed in terms of Brosch’s attenuation lineage and with respect to year the trial was started) and to-date, none of the SNPs, duplications and deletions found between BCG strains have been directly proven to have affected vaccine efficacy\textsuperscript{88,114-117}.

None of the above cited hypotheses regarding the variable efficacy of BCG between trials can satisfactorily explain the considerable inter-individual variability in BCG immunogenicity and efficacy found within same-setting cohorts, including within case-matched randomised controlled trials (RCTs). Finan et al reported variation of up to 10 log-fold in whole blood IFN-\(\gamma\), IL-5 and IL-13 responses to mycobacterial antigens in 236 healthy Gambian infants which had been BCG-vaccinated at birth\textsuperscript{118}. Genetic variation between BCG study participants is likely to play a role. Multiple studies are suggestive that the immunogenicity of both BCG-vaccination and mycobacterial infection are heritable and it is suspected that host genetic variation contributes towards BCG-afforded protection against TB\textsuperscript{119-123}. Smith et al used the ‘Collaborative Cross’, a panel of recombinant inbred murine lines, and reported that in mice BCG-efficacy and TB-susceptibility are genetically dissociable, that BCG-efficacy correlated with intrinsic immune biases in the strains and that BCG-vaccination only afforded protection, reducing the Mtb loads by 10- to 100-fold, in a subset of genotypes (B6 mice, recombinants CC001 and
CC002 and the wild-derived WSB line). BCG was not protective against pulmonary TB in any of the other genotypes. SNP cytokine response genes have been reported to be associated with human response to mycobacteria-antigens and BCG-vaccination. Significant heritability in Interferon-c (IFN-c) and IL-13 responses to mycobacterial antigens was identified by a study comparing BCG-vaccination responses between monozygotic and dizygotic twins.

What over 100 years of BCG has taught us is that we do not understand how the BCG vaccine works. We do not know whether the variability and inadequate protection that have plagued the use of the BCG vaccine...will also effect the efficacy of new recombinant BCG vaccines [Hazel Dockrell, 2022].

The present study seeks to examine the T cell contribution towards inter-individual variation seen in BCG immunogenicity between individuals. An improved understanding of why BCG vaccine efficacy varies to such a great extent, including any T-cell component to this, would be critical to informing the development of the next generation of vaccines against tuberculosis, especially given many candidate vaccines have been designed to boost the protection provided by BCG.
1.7 Evidence for the Role of T cells in the Immune Response to Mycobacteria

The present study seeks to identify the T cell component to inter-individual variation in BCG immunogenicity. It is therefore important to qualify that BCG is indeed a vaccine which mediates some or all of its activity against subsequent mycobacterial challenge through the priming of T cells.

It is well established that T cell responses are critical in the human immune response to mycobacteria (both in BCG vaccination and Mtb infection)\textsuperscript{132,133}. Mtb is a facultative intracellular pathogen able to replicate within human monocytes and macrophages\textsuperscript{134}. Immune response to Mtb infection includes macrophage anti-mycobacterial action, mediated by the cytokines secreted by T-helper cells, and T-cell-mediated hypersensitivity, which manifests as caseous tissue destruction\textsuperscript{135}. The most compelling evidence for the contribution of Th cell response to mycobacteria is listed in Table 1.3.
Table 1.3. Evidence that T helper (Th) cell immunity is integral in host response to mycobacteria

<table>
<thead>
<tr>
<th>Evidence T helper (Th) cell immunity is integral in host response to mycobacteria</th>
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<tbody>
<tr>
<td>Increased rates of mycobacterial infections in Mendelian susceptibility to mycobacterial disease (MSMD)</td>
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<tr>
<td>Increased rates of mycobacterial infections in persons living with human immunodeficiency virus (HIV, PLWH)</td>
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<tr>
<td>Increased rates of mycobacterial infections in recipients of anti-Tumour Necrosis Factor α (TNFα) biological agents</td>
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<tr>
<td>In vivo delayed-type hypersensitivity (DTH) response to tuberculin skin test (TST)</td>
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<tr>
<td>In vitro demonstration of CD4+ T cell mediated cytolysis of mycobacterial-infected macrophages</td>
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Table 1.3. Evidence that T helper (Th) cell immunity is integral in host response to mycobacteria
Evidence that intact CD4+ T cell function is requisite for host response to mycobacteria is provided by specific patient groups listed below in whom it is compromised.

Firstly, Mendelian susceptibility to mycobacterial diseases (MSMD) is a collection of rare, inherited disorders with an underlying loss-of-function mutation in genes all involved in production or response to IFN-γ (for example in interferon gamma receptor 1 (IFNGR1), IFNGR2, signal transducer and activator of transcription 1 (STAT1), interleukin 12B (IL-12B), interleukin 12 receptor B1 (IL12RB1), interferon-stimulated gene 15 (ISG15), interferon regulatory factor 8 (IRF8) and nuclear factor-kappa B essential modulator (NEMO)). IFN-γ is secreted by activated CD4+ T cells, as well as CD8 expressing and natural killer (NK) cells, and serves multiple functions including the promotion of macrophage activation, to enhance antigen presentation and in regulation of Th1/Th2 balance. MSMD is characterised by host predisposition to various infectious diseases including from weakly virulent mycobacteria including the BCG vaccination itself.

Secondly, infection with human immunodeficiency virus-1 (HIV-1), which primarily targets and depletes CD4+ T cells, presents the single biggest risk factor for developing TB disease, increasing the relative risk approximately 100-fold. In advanced acquired immunodeficiency syndrome (AIDS, as a result of infection with HIV-1), Mtb infection typically presents as disseminated disease. However,
even after CD4+ T cell counts recover on antiretroviral therapy (ART), the incidence rate of PTB remains higher in persons living with HIV (PLWH) than in the general population.\textsuperscript{138,139}

Thirdly, recipients of anti-TNFα therapy (either soluble TNFα receptors or anti-TNFα monoclonal antibodies to intercept the physiological action of TNF-α, a key Th1 cytokine against intracellular infection), used in the treatment of autoimmune conditions including rheumatoid arthritis (RA) and inflammatory bowel diseases (IBD), have an increased relative risk of TB infection from 1.5 to 17.\textsuperscript{140}

Further evidence that CD4+ T cell immunity is central to host response to mycobacteria is drawn from experimental clinical and laboratory data. Hoft et al documented \textit{in vivo} DTH to TST in human volunteer recipients of intradermal BCG-vaccination, (as well as \textit{in vitro} T cell proliferation and IFN-γ release to mycobacterial antigens)\textsuperscript{106}. Pithie et al identified that PPD-stimulated CD4+ T cells, both from healthy TST-positive, BCG-vaccinated participants and from patients with recently diagnosed active TB demonstrated high levels of \textit{in vitro} cytolysis of PPD-pulsed autologous macrophage, which was not found in the T cells of unvaccinated individuals.\textsuperscript{141} Chan et al developed murine models in which IFN-γ stimulated-macrophages restricted growth of Mtb, although this has not been replicated in human cells.\textsuperscript{142}
Beyond CD4+ T cells playing a pivotal role in host response to mycobacteria, CD8+ T cells are also activated following mycobacterial stimulation, and may have a cytolytic response to mycobacterial antigens. Both adoptive transfer and selective depletion in murine models are suggestive of CD8+ T cell-mediated cytolysis against Mtb infected cells\textsuperscript{142-144}. Tan et al identified in human blood and alveolar lymphocytes which had been pulsed by PPD and expanded with IL-2, CD8+ T cells with mycobacterium-specific cytotoxic activity\textsuperscript{145}. Turner and Dockrell cultured human PBMC with \textit{M.bovis} BCG for a week and subsequently isolated the CD8+ T cells, which were demonstrated to be cytotoxic against cells infected with live \textit{M.bovis} BCG, dead \textit{M.bovis} BCG, and to a lesser degree, PPD\textsuperscript{146}.

Finally, it is increasingly apparent that lymphocytic response to mycobacterial antigens is complex and extends beyond expansion of just the classical antigen specific CD4+ and CD8+ T cells, with non-classical gamma delta T cells, CD1 and HLA-E restricted T-cells also playing a role\textsuperscript{147-149}. Further, there is increasing evidence that the humoral response to BCG may be of functional importance in protection against TB, with BCG-induced antibodies opsonizing mycobacteria and enhancing macrophage phagocytosis \textit{in vitro}\textsuperscript{150}. However, examining the post-BCG-vaccination response of CD4–CD8+ T cells or B cells is beyond the scope of the present study.
1.8 Laboratory Assays for Bacillus Calmette-Guérin Immunogenicity

The second arm of the present study seeks to identify inter-individual variation in T cell priming by BCG-vaccine in human recipients. This is complicated given BCG produces many potential antigens. There is currently no gold standard measure of BCG immunogenicity. Antibody levels, typically measured as surrogates of vaccine efficacy, are less relevant to BCG, which is a predominantly T cell-mediated vaccine. An experimental technique for estimating BCG-immunogenicity, Mycobacterial Growth Inhibition Assay (MGIA), measure the ability of vaccinee PBMC to control mycobacterial growth in vitro, although this is yet to demonstrate correlation with in vivo protection against TB disease. T-cell vaccines are most commonly assessed by cytokine-based approaches, including enzyme-linked immune absorbent spot (ELISpot) and much evidence suggests IFN-γ-based assays provide a good indicator of BCG-immunogenicity. However, assays detecting IFN-γ-releasing cells alone have their limitations: they likely only identify a small, biased, proportion of the vaccine-specific T cell response. Further, IFN-γ has not been shown in vitro to stimulate human macrophages to restrict or kill Mtb. Given increasing evidence that T cell responses to Mtb are highly heterogenous with different functional capacity and includes IFN-γ-independent responses, limiting measurements to one or more cytokines may potentially underestimate the BCG-immunogenicity in that host. Therefore, the present study utilises several indirect in vitro mycobacterial antigen T
cell-stimulation assays to capture a comprehensive set of CD4+ and CD8+ T cell antigen-induced phenotypic attributes (namely activation, Th1-cytokine production and proliferation) in BCG-vaccinated individuals, to construct a multi-dimensional profile of vaccine-immunogenicity in each subject\textsuperscript{156,157}. Given that outside of the context of an acute infection, typically less than 1% of peripheral memory T-cell cells are antigen-specific, any assays employed to measure immunogenicity need to be both highly sensitive and specific, as well as allow for the processing of large cell samples to facilitate detection of adequate numbers of rare antigen-specific events within the many non-target cells.

One of the assays employed in the present study to ascertain T cell priming by BCG is the measurement of upregulation of activation-induced markers (AIM) following incubation of post-vaccination PBMC with PPD. Over the last two decades, multiple groups have employed AIM to delineate antigen-specific T cells of interest. AIM do not require prior knowledge of the epitope or require restricting HLA-type. AIM are uniformly upregulated, TCR-stimulated surface markers, measured independently of cytokine-production. AIM may capture both cytokine-producing CD4+ T cells missed by current intracellular staining protocols for less-frequency recognised effector T cell subsets, thus demonstrating greater sensitivity, a broader insight into T-cell response to vaccination and a higher signal-to-noise-ratio\textsuperscript{156}. 
CD4+ T cell AIM include the co-expression of OX40 (also known as CD134) and CD25 by CD4+ T cells. OX40 is a member of the TNF-Receptor superfamily, which regulates effector CD4 T cells by sustaining clonal expansion and prolonging cell survival on mediating signaling from its ligand OX40L (also known as CD252) on APCs. The nature and function of CD25 has already been described in an earlier paragraph. Another CD4+ T cell AIM is CD40 Ligand (CD40L, also known as CD154), a member of the TNF superfamily, upregulated by CD4+ T cells following TCR-engagement to function as an immune regulator by signaling to CD40 on B-cells, dendritic cells (DC), monocytes and macrophages. The CD4+ T cell co-expression of OX40 and CD25 and the expression of CD40L have previously been identified in response to Mtb antigens, including in LTBI\textsuperscript{158-160}. CD40L is only fleetingly expressed on the cell surface of CD4+ T cells before immediate downregulation on its interaction with CD40 but may be detected intracellularly by adding the Golgi inhibitor brefeldin A (BFA) to the culture, which completely blocks CD40L surface expression\textsuperscript{161,162}.

AIM have also been utilised to study antigenic-specific responses in CD8+ T-cells, including their co-expression of CD69 and 4-1BB (also known as CD137)\textsuperscript{163,164}. 4-1BB belongs to the TNF-Receptor superfamily and is a co-stimulatory molecule, and on binding its ligant 4-1BBL on professional APCs, acts to amplify initial TCR-activating signals with anti-apoptotic functions, whilst promoting T-cell proliferation and T-cell survival\textsuperscript{165,166}. Whilst undetectable on unstimulated CD8+
T cells, 4-1BB becomes uniformly upregulated on activation regardless of cell subset or functionality, with peak expression at 24 hours\textsuperscript{167}.

Interestingly, it may be unnecessary to measure more than one set of AIM in each T cell subset. Bowyer et al compared CD4+ T cell expression of various AIM in a phase 1a clinical trial for candidate viral-vectored Ebola vaccines and found the frequencies comparable and highly correlated. Of further interest, the group did not find AIM to correlate with the frequency of intracellular cytokine-production, suggestive that AIM provides additional insight into antigen-specific responses, not detected by more traditional methods\textsuperscript{168}.

1.9 Probing the TCR Repertoire for a BCG-Induced Response

The third arm of the present study seeks to interrogate the host TCR repertoire before and after BCG-inoculation. It examines whether there is an expanded TCR signature subsequent to BCG vaccination detectable in peripheral blood and common to and shared between vaccinees and whether the post-BCG expanded TCRs are homologous to previously recognised Mtb-specific TCRs. TCRs have previously been examined to characterise the T-cell adaptive response to pathological events, such as cancer, but to date, there has only been a small handful of studies published on TCR sequencing in the context of vaccination.
(including in five recipients of the hepatitis B vaccine and seven recipients of the yellow fever vaccine)\textsuperscript{169,170}.

The typical human’s T cell armoury consists of an estimated 100 billion T cells with approximately one billion different TCRs (out of a potential $>10^{15}$ possible TCRs)\textsuperscript{34}. Each individual TCR consists of an $\alpha$- and a $\beta$-chain and is fashioned via the recombination of VDJ genes in the thymus (see Fig. 1.6). The TCR determines a T cell’s specificity (which epitope the T cell will recognise). Using high throughput next generation sequencing (NGS), it is possible to amplify, sequence and analyse a sample containing millions of different TCRs before and after a biological event, to explore the dynamic impact of that perturbation on the host’s adaptive immune response via the character and abundance of an individual’s TCRs\textsuperscript{171,172}. The CDR3 region of the TCR$\alpha$:\(\beta\) chain is the most variable parts of the sequence (since it contains the imprecise join between variable region genes) and is the TCR region which makes closest contact with the epitope. Further, TCRs which recognise the same antigen have been demonstrated to share similar CDR3 sequences\textsuperscript{35,159,173}. The CDR3 region is therefore the primary focus of most TCR repertoire analyses. Many different TCRs may recognise the same epitope but two unrelated, healthy individuals typically share only about 1-2\% of their repertoire\textsuperscript{35-37}.
Algorithms may be employed to cluster TCRs with a high probability of sharing specificity due to conserved motifs and comparable CDR3 sequences\textsuperscript{174}. For example, Glanville et al used the Grouping of Lymphocyte Interactions by Paratope Hotspots (GLIPH) algorithm to identify approximately 5700 TCR sequences enriched for Mtb specificities from the reactive CD4+ T cells of 22 individuals with LTBI\textsuperscript{159}. The antigen specificity of sequenced TCRs may be subsequently identified through an analysis of TCR sequences using a panel of peptide and MHC-tetramer-sorted cells and structural data\textsuperscript{175}. Expanded TCR sequences can also be checked to see if they cluster with TCRs previously identified as specific for a pathogen’s epitopes\textsuperscript{176}. Should employment of these analyses identify peripheral-blood, BCG-induced TCR signatures, they may inform our understanding of mechanisms of action of the BCG and may be developed as a laboratory test for BCG response. Further it

1.10 Research Questions and Aims of the project

The current project asks the questions, are some individuals’ NTCs inherently more responsive to activation than those of others and does an individual’s NTC responsiveness bear a relationship to their subsequent memory response to cognate antigen? Baseline inter-individual variation in NTC responsiveness to generic stimulation via the TCR will be quantified. Then, in the same individuals, the inter-individual variation in response of memory T cells to cognate antigen to
which they have been pre-primed will be ascertained. It will be examined if there is a relationship between any aspects of the measured cellular-response to generic stimulation and adaptive T cell response to antigen. If associations between the two states are identified, the current study could provide the foundations for a systems vaccinology study employing modelling to identify if there are NTC pre-vaccination predictors of vaccine immunogenicity. The non-specific TCR stimulation will be achieved using anti-CD3/CD28 beads, the priming event by BCG-vaccination and the cognate antigen by PPD. Any variation elicited may potentially reflect variation in TCR intracellular signalling machinery. Sequencing of the peripheral blood TCRs before and after vaccination will also be examined for any vaccine-induced signature. The heritable, intrinsic and/or environmental factors driving any inter-individual variation is beyond the scope of the present study, which instead seeks to look downstream of the causative factors, at the subsequent functionality of the T-cells. The ultimate aim of this project is to identify factors by which T-cell vaccine design may be improved.

Variations can lead to drastic differences in the responsiveness of the immune systems, such as wide differences in the immunogenicity and efficacy of vaccines. Therefore, a major challenge for human immunology is to embrace this diversity by probing the immune system in diverse human populations, with a view to obtaining mechanistic insights about the factors that lead to this variation. [Pulendren & Davis, 2020]
1.11 Project Hypothesis

There is a relationship between an individual’s baseline NTC responsiveness to generic stimulation via the TCR and the response of their memory T-cells to cognate antigen.
Chapter 2 Materials and Methods

2.1 Ethics

University College London (UCL) Research Ethics Committee (REC) approval was granted in July 2018, UCL-REC Project ID Number 13545/001. The Data Protection Registration Number is: Z6364106/2018/05/102. The study was conducted in accordance with the Declaration of Helsinki Ethical Principles for Medical Research involving human subjects.

2.2 Study subjects

2.2.1 Subjects for Preliminary Assays

Blood was taken from a total of 24 healthy, adult, laboratory donors, who provided written consent, in order to perform preliminary assays to optimise the protocols for the tissue culture assays. With regard to the preliminary assays in which PPD was a stimulatory condition, blood was used from the laboratory donors who reported that they had been BCG-vaccinated.

2.2.2 Study Subject Recruitment
It was not possible to calculate the required number-of-participants sample size using a power analysis, given the effect size is unknown. Recruitment was targeted at 100 subjects, as this is in the same vicinity to other analogous systems vaccinology studies and was feasible given available resources and time\textsuperscript{4,12,68}.

Recruitment to the study occurred between September 2018 to June 2019 and was primarily via UCL Occupational Health (OH), London, United Kingdom (UK). During routine OH consultations, BCG-naïve healthcare students were advised of the study and given a recruitment leaflet by OH providers. Recruitment was also facilitated by poster displayed in UCL Medical School and by word-of-mouth between healthcare students. On expression of interest, potential participants were given or emailed a participant information leaflet (PIL), any questions were answered via email or on the telephone and participants were given at least 24 hours to consider the information. If they agreed to proceed, participants could withdraw at any time without giving a reason and without penalty. Provision was not made for non-English speakers as good English is a condition of entry for UK Medical Schools.

Although BCG vaccination is predominately administered to children, recruitment was of young adults aged 18 – 25yo with occupational risk for TB-exposure (in accordance with Public Health England (PHE, now UK Health Security Agency,
UKHSA) guidelines) for logistic constraints (for example required blood volumes)\textsuperscript{177}.

This study sought only BCG-naïve participants. In the UK in 2005, the BCG stopped being offered universally to all children in secondary schools and was replaced with a targeted programme to infants deemed at higher risk. Therefore, given that the potential study participants were born between 1993 and 2000, BCG-vaccination was neither routinely offered to them as infants nor as teenagers, although policy was variable between regions and some of our participants were born or lived abroad as children\textsuperscript{178}. Further, from 2015 until 2019, there was a national shortage of the UK-licensed, PHE-contracted Statens Serum Institut (SSI, Denmark) BCG-vaccine on account of SSI selling its vaccine production business, resulting in many University OH departments being unable to offer BCG-vaccination to unvaccinated healthcare students during this period\textsuperscript{179}. The combination of both the BCG 2005 change in policy and 2015 to 2019 supply difficulties resulted in the majority of UCL healthcare students being BCG-naïve during the period of study recruitment (September 2018 to June 2019).

Inclusion and exclusion criteria for recruitment to the study are listed in Table 2.1. Participants were asked if they were aware of having been previously BCG-vaccinated; they were asked to check with their families, their health records and
their GP surgery; their limbs were checked for a BCG-scar. All candidates had already been routinely screened for HIV-1 infection by OH.
<table>
<thead>
<tr>
<th>Inclusion criteria</th>
<th>Exclusion criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age 18 to 25 years</td>
<td>Age &lt;18 or &gt;25 years</td>
</tr>
<tr>
<td>Resident of United Kingdom (UK)</td>
<td>Human Immunodeficiency Virus-1 (HIV-1) infection</td>
</tr>
<tr>
<td>Healthcare student</td>
<td>Pregnant or breast-feeding</td>
</tr>
<tr>
<td>In good health</td>
<td>Diabetes Mellitus (DM)</td>
</tr>
<tr>
<td>No previous vaccination with Bacillus Calmette-Guérin (BCG)</td>
<td>History of an autoimmune disease</td>
</tr>
<tr>
<td></td>
<td>History of significant generalised skin condition</td>
</tr>
<tr>
<td></td>
<td>History of malignancy</td>
</tr>
<tr>
<td></td>
<td>Immunomodulatory drugs</td>
</tr>
<tr>
<td></td>
<td>Confirmed anaphylactic reaction to similar vaccine components</td>
</tr>
<tr>
<td></td>
<td>Past history of tuberculosis (TB) or latent tuberculosis infection (LTBI)</td>
</tr>
<tr>
<td></td>
<td>Keloid scarring</td>
</tr>
</tbody>
</table>

Table 2.1 Study inclusion and exclusion criteria.
Fig. 2.1 provides a pictorial summary of subject numbers. 142 potential candidates expressed an interest in participating in the study, of which 121 decided to proceed (21 opted not to proceed or believed they did not meet the inclusion/exclusion criteria). Subsequently on face-to-face assessment 12 potential candidates did not meet inclusion/exclusion criteria (including eight with evidence of previous BCG-vaccination).

Of the remaining 109 potential candidates, if it had not already been performed within the last six weeks by OH, an up-to-date peripheral blood Interferon Gamma Release Assay (IGRA) Quantiferon Gold Plus (QFT-Plus; QIAGEN) was performed for evaluation of IFN-γ T cell response to Mtb peptides CFP-10 and ESAT-6 to screen for LTBI\textsuperscript{180}. Subsequently, two further candidates could not be included in the study on the grounds of inadequate IGRA results. No individuals had a positive IGRA result.

107 candidates met all study conditions. Written consent for participation was obtained and they were enrolled in the study. A medical questionnaire was completed by a doctor or research nurse. Each participant was allocated a unique identification number (UIN) on enrolment. None of the 107 participants withdrew or were lost to follow-up. Participant costs were met by flat rate re-imbursement of £50, paid after the second blood-draw.
Figure 2.1 Summary diagram of subject numbers.
2.3 Bacillus Calmette-Guérin vaccination

The BCG used in the present study was InterVax Limited preparation, imported by PHE from the Canadian firm InterVax and manufactured by BB-NCIPD Ltd, Bulgaria. InterVax BCG is unlicensed in the UK, as InterVax have never applied for UK Marketing Authorisation. However, the InterVax preparation has been WHO prequalified for >25 years, is used by United Nations (UN) organisations for immunisation against TB, is used extensively in over 100 countries, has a good safety record, is thought to be as effective as the SSI BCG vaccine and the Medicines and Healthcare Products Regulatory Agency did not object to PHE importing InterVax vaccine.

Prior to the arrival of participants on their day of vaccination, ampoules of freeze-dried BCG were reconstituted with saline diluent and administered within 6 hours of reconstitution, with the ampoule stored away from the light between 2 and 8 degrees Celsius (°C) between doses. 0.1ml InterVax BCG was administered intradermally using a sterile 26-guage needle on the upper left arm by an experienced research nurse181.

Eight weeks after vaccination, the BCG site was examined. No participants experienced more than the common local reactions to the BCG-vaccination and there were no severe adverse events (SAE).
2.4 Sample Taking

The experimental pipeline is summarised pictorially in Fig. 2.2. Immediately prior to BCG vaccination, 55ml peripheral blood was collected in lithium heparinised vacutainers and 3ml in a Tempus Blood ribonucleic acid (RNA) Tube (ThermoFisher Scientific). Eight weeks after vaccination, a further 55ml peripheral blood was collected in lithium heparinised vacutainers and 3ml in a Tempus Blood RNA Tube.
Figure 2.2 Summary of experimental pipeline.
2.5 Peripheral Blood Mononuclear Cell Preparation and Cryopreservation

Within two hours of blood taking via venesection, PBMC preparations were performed and the cells then cryopreserved. The pre- and post-vaccination 55ml heparinised blood samples of each participant were diluted with half volume of Gibco Dulbecco’s phosphate-buffered saline with calcium and magnesium (DPBS +Ca/+Mg) (ThermoFisher Scientific). Using Falcon 50ml conical centrifuge tubes (ThermoFisher Scientific), the diluted blood was then layered onto Ficoll-Paque PLUS Media (Cytiva, formerly GE Healthcare Life Sciences), using 25ml of diluted blood per 15ml of Ficoll, followed by density gradient centrifugation. PBMCs were then obtained from the buffy coat and washed three times using DPBS +Ca/+Mg.

A microscopy viable cell count was performed with 0.4% Trypan Blue Solution (Elabscience). The cells were then resuspended in freshly made 90% filtered Fetal Calf Serum (FCS; Biosera) and 10% Dimethyl sulfoxide (DMSO; Sigma-Aldrich) solution at a concentration of 10 million cells/ml in 1ml cryovial aliquots.

Cryovials were placed immediately onto ice and then transferred as soon as possible (to minimise exposure of cells to potentially toxic DMSO) into a Nalgene Mr. Frosty Cryo 1°C Freezing Container (ThermoFisher Scientific, to reduce the cooling rate to -1°C/hr to prevent intracellular ice crystal formation) and into a -80°C freezer for cryopreservation. After 48 hours, cryovials were transferred into liquid nitrogen (LN₂) storage. The FCS used for all donors and all experiments was all sourced from the same batch.
2.6 Thawing and Washing of Frozen Peripheral Blood Mononuclear Cells

The numbers of samples thawed for individual experiments ranged from six donors (NTC assay) to a maximum of 16 donors (AIM, intracellular cytokine staining (ICS) and proliferation assays), with thawing staggered in waves (to minimise exposure of thawing cells to potentially toxic DMSO), with a maximum of four donors’ cryovials thawed at a time. Immediately prior to their use in experiments, 20M PBMCs (two cryovials) per donor per assay were removed from the LN$_2$ tanks and thawed in a 37°C water bath, with gentle agitation until only a small ice crystal remained (occurs in seconds). The thawed cells were immediately Pasteur pipetted to 40ml pre-warmed pre-prepared Gibco Roswell Park Memorial Institute (RPMI) 1640 Medium +L-glutamine (ThermoFisher Scientific) with the addition of 10% filtered FCS (hereafter referred to as R10) and washed a total of three times. Again, the preceding steps were undertaken as fast as possible due to the toxicity of DMSO and to minimise time outside of the incubator. After washing, the cells were rested in R10 in an incubator at 37°C 5% CO$_2$ for an hour (NTC, AIM and proliferation assays) or for three hours (ICS assay; longer to facilitate correct incubation time)$^{183}$. A microscopy viable cell count was performed with Trypan Blue.

2.7 Pre-vaccination Naïve T Cell Stimulation Assays
2.7.1 Naïve T Cell enrichment and Cell Tracker Staining

After resting, pre-vaccination PBMCs were washed twice and re-suspended in pre-prepared MACS buffer solution (Gibco Dulbecco’s phosphate-buffered saline without calcium and magnesium (DPBS -Ca/-Mg) (ThermoFisher Scientific), 0.5% FCS and 2mM Ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich)). A Naïve Pan T cell isolation kit was then used with LS+ positive selection columns and midiMACS separators (all Miltenyi Biotec) to enrich for NTCs by negative selection, as per manufacturer’s protocol184.

FACS performed on the effluent from the LS+ columns (stained and fixed on the day of enrichment) were routinely >95% CD3+CCR7+CD45RA+, see Fig. 2.3, confirming purity of the NTCs. Following enrichment, a viable NTC count was performed with Trypan blue. NTCs were then labelled with in vitro CellTrace Violet (CTV; ThermoFisher Scientific) as per manufacturer’s protocol before being resuspended in R10185.
Figure 2.3 FACS gating strategy to demonstrate purity of effluent following naïve T cell (NTCs) enrichment in one donor. NTCs were identified as CD45RA+CCR7+; T_{CM} as CD45RA-CCR7+; and T_{EM} as CD45RA-CCR7-. Cells were gated on single lymphocytes, then dead cells were excluded. In the example donor, NTC purity was 98% of cells. Staining and FACS details given later.
2.7.2 Preparation of anti-CD3 and anti-CD28 Monoclonal Antibody

Stimulation

Initial study plans were to stimulate the NTCs with anti-CD3 monoclonal antibody immobilised by adsorption onto the surface of culture plates, in addition to soluble anti-CD28 monoclonal antibody, as has been reported previously\textsuperscript{186}. 29 iterations of preliminary assays were trialled on 20 laboratory donors employing variable concentrations of immobilised mouse anti-human CD3 OKT3 (0.0156µg/ml to 10µg/ml) and immobilised or soluble mouse anti-human CD28 CD28.2 (1-2µg/ml) (both ThermoFisher Scientific) on Nunclon Delta surface-treated 96 well flat microwell plates (Merck). The plates were prepared through the addition of the appropriate concentration of antibody (achieved through serial dilution in 100µl PBS -Ca/-Mg in each well, making the plates airtight with sealing Nescofilm (Termofisher Scientific) and overnight incubation at 2 - 8°C overnight. The following day, plates were removed from refrigeration and the wells washed twice with PBS -Ca/-Mg before suspended cells were pipetted into the wells. Both fresh and frozen/thawed cells and both whole PBMC and enriched NTC were cultured in such conditions, 0.2M cells in 200µl R10 per well with 1-2µg/ml soluble anti-CD28 (if not already plated) for either two- or six-days at 37°C in 5% CO\textsubscript{2}.

However, as detailed in Chapter 3, plate-immobilised antibodies generated only a weak and irreproducible T cell proliferation. These assays were therefore abandoned in favour of using anti-CD3/CD28 beads.
Streptavidin M-280 Dynabeads, biotinylated mouse anti-human CD3 OKT3 and biotinylated mouse anti-human CD28 CD28.2 (all ThermoFisher Scientific) were used to prepare anti-CD3/CD28 beads in accordance with the manufacturer’s instructions\textsuperscript{187}. Four ‘strengths’ of beads were produced, with the ratio of antibodies at one-part anti-CD3 to five parts anti-CD28\textsuperscript{188}. To vary the concentrations of antibodies adhered to the streptavidin Dynabeads, serial dilutions of solutions of biotinylated anti-CD3 and anti-CD28 at 1:5 ratio were produced followed by the addition of a standard volume of beads. The maximal strength of bead, hitherto referred to as ‘fully-saturated anti-CD3/CD28 beads’, was produced by incubating the streptavidin Dynabeads with a two-fold greater volume of antibodies than the manufacturer cited as the capacity of that volume of Dynabeads. The next strength of bead, hitherto referred to as ‘half-saturated anti-CD3/CD28 beads’ were produced by the incubation of the streptavidin Dynabeads with half the volume of antibodies as that volume of Dynabeads are maximally able to bind. The third strength of bead, hitherto referred to as ‘quarter-saturated anti-CD3/CD28 beads’, were produced by the incubation of the streptavidin Dynabeads with one quarter the volume of antibodies as that volume of Dynabeads were able to maximally bind. The minimum strength of bead, hitherto referred to as ‘one-eighth-saturated anti-CD3/CD28 beads’ was produced by the incubation of the streptavidin Dynabeads with one eighth the volume of antibodies as that volume of Dynabeads are able to maximally bind. The binding
of the biotinylated antibodies to the streptavidin-coated beads was optimised by rotating the suspensions at room temperature for three hours. Finally, the beads were washed four times and resuspended in PBS with 0.01% bovine serum albumin (BSA) and stored at 2 - 8°C. Fresh batches of homemade beads were prepared every three months⁴⁹. Estimates of the concentrations of antibodies which the different strengths of beads deliver to cell suspensions when added in accordance with the study protocol are given in Table 2.2. Concentrations of each antibody present during NTC stimulation for each strength of bead was estimated by calculating the mass of antibody that was expected to have bound to the anti-CD3/CD28 beads during their synthesis and the number of beads used during each NTC stimulation.
Table 2.2 The concentrations of anti-CD3 and anti-CD28 the varying strengths of anti-CD3/CD28 beads were estimated to deliver when added to cell suspensions in accordance with the study protocol.

<table>
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<tr>
<th>‘Strength’ of anti-CD3/CD28 bead</th>
<th>Conc. of anti-CD3 provided to experimental well (ng/ml)</th>
<th>Conc. of anti-CD28 provided to experimental well (ng/ml)</th>
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</thead>
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<tr>
<td>Fully-saturated</td>
<td>42</td>
<td>208</td>
</tr>
<tr>
<td>Half-saturated</td>
<td>21</td>
<td>104</td>
</tr>
<tr>
<td>One quarter saturated</td>
<td>10</td>
<td>52</td>
</tr>
<tr>
<td>One eighth saturated</td>
<td>5</td>
<td>26</td>
</tr>
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</table>
In the next set of preliminary assays, performed using the homemade anti-CD3/CD28 beads, the CTV-labelled, enriched NTCs of nine laboratory donors were incubated in 96 well flat-bottomed plates with 0.1M cells in 200µl R10 per well. The cells were incubated in duplicate for each donor and each condition with either fully-, half- or quarter-saturated anti-CD3/CD28 beads, using a 3:1 bead to cell ratio. The 3:1 bead to cell ratio for the homemade beads was selected as the streptavidin Dynabeads are approximately one quarter the surface area of the commercial Dynabeads, which recommend a 1:1 bead to cell ratio. Following addition of beads to each well, cell suspensions were thoroughly mixed with a p200 to ensure even distribution. Control conditions also included for each donor were CTV-unlabelled and CTV-labelled negative controls (media only) and a positive control with commercial Human T-Expander anti-CD3/CD28 Dynabeads (ThermoFisher Scientific, using a 1:1 bead to cell ratio). With regard to the latter, the concentration of antibodies and ratio of anti-CD3 and anti-CD28 bound to the commercial Dynabeads is not publicly made available (private correspondence with ThermoFisher). Otherwise empty wells surrounding the experimental wells were filled with 200µl PBS -Ca/-Mg to prevent excess evaporation and ensure even heat distribution for all wells. The plates were then incubated at 37°C in 5% CO₂ for 67 hours. The experiment was repeated for each of the donors on three separate occasions (using PBMC obtained from a single bleeding point, but thawed on three separate occasions).
For the definitive experiments testing the 107 study participants, the above NTC assay was replicated, except the anti-CD3/CD28 bead wells were in triplicate rather than duplicate; the condition of half-saturated anti-CD3/CD28 beads was omitted; the commercial Dynabeads were substituted with the fully-saturated anti-CD3/CD28 beads to serve as positive controls; and the experiment was repeated for a sub-sample of 11 participants (representing ten per cent of the cohort) on a second occasion in replicate experiments (cells sourced from the same PBMC preparations of the same participants, but thawed and established for incubation on separate days and with anti-CD3/CD28 beads sourced from alternate batches). Experiments were also repeated up to three times for any participant where the assay failed (<50% viable cells on FACS and/or positive control failed). All of the NTC stimulation assays were undertaken in a total of 24 separate experiments.

2.7.3 Pre-vaccination Naïve T Cell Stimulation Assay Cell Staining

The antibody-fluorochrome staining panel and ratios for the pre-vaccination NTC stimulation assay is given in Fig. 2.4. Following 67hrs incubation, the NTC stimulation plates were removed from the incubator, the cells washed twice in PBS-Ca/-Mg, before each pellet was resuspended with Live/Dead Fixable Near-Infrared Dead Cell Stain (L/D Near-IR; ThermoFisher Scientific) in 50:50 PBS -Ca/-Mg /brilliant stain buffer (BSB; BD Biosciences) and incubated for 20mins at 2 - 8°C. The cells were then washed again, resuspended with CD3-BUV805 (BD
Biosciences), CD4-BUV395 (BD Biosciences), CD8-AlexaFluor700 (BD Biosciences) and CD45RA-FITC (BD Biosciences), CCR7-BV605 (Biolegend), PD-1-PE (Biolegend), CD25-PE/Dazzle (Biolegend) and CD69-BV786 (Biolegend) in 50:50 PBS -Ca/-Mg /BSB and incubated for 30mins at room temperature (RT). The cells were then washed, fixed for 45mins at RT, then permeabilised for intra-nuclear staining with the addition of Ki67-PE-Cy7 (ThermoFisher Scientific) for 30mins using the FoxP3/Transcription Factor Staining Buffer Set (ThermoFisher Scientific), before a final wash. For all assays, all antibodies were mouse anti-human and, as far as possible, sourced from the same batches.
<table>
<thead>
<tr>
<th>Laser Name</th>
<th>Wavelength</th>
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<td>PE</td>
<td>PD-1</td>
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</tr>
</tbody>
</table>

Figure 2.4 The antibody-fluorochrome staining panel for the pre-vaccination Naïve T Cell (NTC) stimulation assay.
2.8 Post-vaccination Purified Protein Derivative Stimulation Assay

2.8.1 Purified Protein Derivative-stimulation

Three separate assays and staining panels were utilised in the post-vaccination PPD stimulation experiments.

For the AIM assay, once the PBMC had been thawed, washed and rested as described in section 2.6, 1M cells of whole PBMC in 200μl pre-warmed R10 were plated per well in 96-well round bottomed plates, with each condition for each donor in duplicate or triplicate (dependent on cell numbers). 10μg/ml azide-free Tuberculin PPD for in vitro testing (AJ Vaccines) was added to the wells and thoroughly mixed with a p200 pipette. Further conditions included for each donor were a negative control well (media only) and positive control well (commercial Dynabeads). Each plate was incubated for 24hrs at 37°C in 5% CO₂. Given the heterogenous nature of PPD, PPD for all the experiments was sourced from a well-vortexed single vial from the same batch.

For the ICS assay, the same protocol was followed as per the extracellular AIM assay except with the addition of 1ng/ml BFA (PeproTech) (for cytokine and CD40L-trapping in the antigen-responsive cells) to each well with good p200 pipette mixing two hours after the addition of the stimulating conditions.
Incubation was then for a further 16hrs (18hrs total incubation: 2hrs without BFA and 16hrs with BFA).

For the proliferation & late activation assay, the same protocol was followed as per the extracellular AIM assay except following the thaw, wash and rest, the whole PBMC was CTV-stained (as before); cells were plated at 0.2M cells/well; negative controls included both CTV-unlabelled and CTV-labelled cells; and the plates were incubated for 117hrs (approximately five days) in total, with a media change performed on day two.

2.8.2 Post-vaccination Purified Protein Derivative-stimulation Assays Cell Staining

The antibody-fluorochrome staining panels and ratios for the post-vaccination PPD-stimulation assays are given in Fig. 2.5. For all post-vaccination assays, on removal from the incubator, the cells were washed twice in PBS and each pellet resuspended in L/D Near-IR in 50:50 PBS -Ca/-Mg/BSB and incubated for 20mins at 2 to 8°C. For the AIM assay, the cells were then washed again, resuspended in CD3-BUV805, CD4-BUV395, CD8-BV786 (BD Biosciences) and OX40-PE (BD Biosciences), CD25-APC (BD Biosciences), BV421-4-1BB (BD Biosciences), CD69-PE-Cy7 (BD Biosciences), CD45RA-FITC and CCR7-BV605
in 50:50 PBS-Ca/-Mg/BSB and incubated for 30mins at RT. The cells were then washed and fixed in 1% formaldehyde for 20mins at 4°C, before a final wash.

For the ICS assay, after the Live/Dead (L/D) stain and wash, a surface stain was performed by resuspension with CD3-BUV805, CD4-BUV395, CD8-BV786 and CD19-APC-Cy7 (BD Biosciences) (the latter in the dump channel with L/D) in 50:50 PBS -Ca/-Mg/BSB and incubated for 30mins at RT, before washing. The cells were then fixed for 20mins at 4°C and then permeabilised (to facilitate the access of the fluorochrome-conjugated antibodies to detect the trapped cytokines or CD40L) with the addition of TNFα-FITC (BD Biosciences), IFN-γ-V450 (BD Biosciences), IL-2-APC (BD Biosciences) and CD40L-PECy7 (Biolegend) using the Cytofix/Cytoperm, Fix/Perm Solution Kit (BD Biosciences) for 30mins at 4°C, before a final wash.

For the proliferation & late activation assay, after the L/D stain and wash, a surface stain was performed with resuspension with CD3-BUV805, CD4-BUV395, CD8a-AF700 (BD Biosciences) and PD-1-PE in 50:50 PBS -Ca/-Mg/BSB and incubated for 30mins at RT, before a further wash. Then cells were fixed at RT for 45mins, then permeabilised with Ki67-PE-Cy7 for 30 minutes using the FoxP3/Transcription Factor Staining Buffer Set before a final wash.
## Extracellular Activation Induced Marker (AIM) Assay on BD LSR II Multicolour Flow Cytometer

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### Intracellular Cytokine Staining (ICS) Assay on BD LSR II Multicolour Flow Cytometer

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Figure 2.5 The antibody-fluorochrome staining panels and ratios for the post-vaccination purified protein derivative-stimulation assays, (A) activation induced marker (AIM) assay panel, (B) intracellular cytokine staining (ICS) assay panel and (C) the proliferation & late activation assay panel.
2.9 Flow Cytometry

Cells were acquired on a BD LSRII multicolour flow cytometer using BD FACSDiva software (version 8.0). All flow cytometry was undertaken within 24hrs of staining. For both the pre-vaccination NTC assay and the post-vaccination proliferation assay, where >1% of T cell subsets were predicted to express the markers of interest, ≥0.1M events were collected for each sample. For the post-vaccination AIM and ICS assays, the frequency of antigen-specific T-cells for some donors was predicted to be as low as <0.1% (considered the general detection limit of flow cytometry), and so ≥1M events collection was targeted. As all flow cytometry was conducted over many months, a single flow cytometer was used and CS&T research beads (BD bioscience) from a single batch were used weekly during cytometer setup to ensure all data, including MFIIs, were comparable between experiments across time. Analysis was performed with FlowJo software (version 10.8.0) with fluorescence compensation optimised using compensation controls run for every experiment, using single-stained cells for L/D and CTV and single-stained CompBeads anti-mouse Ig,K compensation particles for all other fluorochromes (BD Biosciences). Fig 2.6 provides illustrative compensation plots for the different antibody-fluorochromes used in each of the assays.
Figure 2.6 Illustrative compensation control matrices from the four stimulation assays. Selective fluorochromes are provided for each assay including for the fluorochromes for the parameters which were detected on the same laser and where the parameters were found to correlate. Plots are depicted for cells which have undergone stimulation with the quarter-saturated beads (naïve T cell (NTC) assay) or commercial Dynabeads (the post-vaccination assays) and have been gated for lymphocytes, single cells, live cells, CD3+ and CD4+. The turquoise dots represent overlay of the cells without application of compensation (uncompensated), the black dots represent the cells with compensation applied.

(A) The pre-vaccination NTC stimulation assay, illustrative of the fluorochromes detected by the yellow-green laser (PE-Cy7 – Ki67, PE-Dazzle – CD25 and PE – PD-1). (B) The post-vaccination activation induced marker (AIM) assay, illustrative of the fluorochromes detected by the yellow-green laser (PE-Cy7 – CD69 and PE – OX40). (C) The post-vaccination intracellular cytokine staining (ICS) assay, illustrative of the fluorochromes detected by the blue laser (FITC - TNFα) and violet laser (V450 – IFN-γ). (D) The post-vaccination proliferation and later activation assay, illustrative of the fluorochromes detected by the yellow-green laser (PE-Cy7 – Ki67 and PE-PD-1).
The gating strategies for the assays are shown in Fig. 2.7. The gate boundaries were set with the aid of fluorescence minus one (FMO) plots, examples of which are shown in Fig 2.8. A generous lymphocyte gate was drawn (to capture dividing cells) and then doublet cell populations were excluded by plotting forward scatter area versus forward scatter height. Viable cells were identified as L/D Near-IR-low cells. CD3-CD4-CD8- cells were excluded on the grounds that CD3 may be down-regulated on activation, but cells expressing neither CD3 nor a T-cell subset co-receptor were unlikely to be relevant. T cell subsets were then gated as CD4+CD8- or CD8+CD4- and then the percentage of cells within each subset expressing the marker of interest was determined.
(B)

Lymphocytes → Single cells → Live → CD4 and CD8

Activation Induced Markers

CD4

- CD25 - APC
- OX40 - PE

CD8

- 4-1BB
- CD69

Unstimulated → Stimulated with purified protein derivative → Stimulated with commercial Dynabeads
(C)

Intracellular Cytokine Staining

- **IFN-γ**
  - IFN-γ - V450

- **TNFα**
  - TNFα - FITC

- **IL-2**
  - IL-2 - APC

Intracellular Activation Induced Marker

- **CD40L**
  - Unstimulated
  - Stimulated with purified protein derivative
  - Stimulated with commercial Dynabeads
Figure 2.7 Sample flow cytometry gating strategies for each of the assays, including single donor examples for the markers of interest under the examined conditions. (A) The pre-vaccination naïve T cell (NTC) stimulation assay; (B) the post-vaccination activation induced marker (AIM) assay; (C) the intracellular cytokine staining (ICS) assay; and (D) the proliferation & late activation assay.
Figure 2.8 Example fluorescence minus one (FMO) gating strategies for (A) the pre-vaccination naïve T cell (NTC) stimulation assay; and (B) the intracellular cytokine staining (ICS) assay.
**2.10 Proliferation Modelling**

The FlowJo ‘proliferation modelling’ function was used to determine CTV-measured proliferation indexes in the pre-vaccination NTC stimulation assay and the post-vaccination proliferation & late activation assay (see Fig 2.9). For each participant, the same initial gating was performed as previously (size, singlet, L/D, CD4+ and CD8+). Next, the ‘Generation 0’ peak was determined using the undivided cells in the CTV-stained negative control. The platform was then able to model and generate statistics for each of the donors’ stimulated samples as the cells divided. Manual adjustments were required to minimise the Root Mean Squared (RMS) value (which represents the difference between the proliferation model and raw data). Proliferation indexes were then generated which included:

- **Percentage Divided** - the percentage of that subset of cells that entered division (same as precursor frequency);

- **Division Index (DI)** - a measure of the average number of divisions undergone by the cells (including the undivided cells);

- **Proliferation Index (PI)** - a measure of the average number of divisions by dividing cells (excludes the undivided cells);

- **Replication Index (RI)** - a measure of the fold-expansion of only the responding cells (excludes the undivided cells)\(^{189}\).
Figure 2.9 Examples from two individual participants of CellTrace Violet (CTV) modelling function. (A) In the pre-vaccination anti-CD3/CD28 beads naïve T cell (NTC) stimulation assay and (B) in the post-vaccination purified protein derivative (PPD) proliferation & late activation assay. For each participant/condition, the same initial gating was performed as previously (size, singlet, live/dead, CD4+ and CD8+), then the FlowJo ‘proliferation modelling’ function was utilised. The ‘Generation 0’ peak was determined using the undivided cells in the CTV-stained negative control (far-right panels). For the stimulated samples, gates were created on the population representing each of the proliferation peaks (central panels). Manual adjustments were made to minimise the Root Mean Squared (RMS) value (which represents the difference between the proliferation model (indicated by red line) and raw data (indicated by black line). ‘Background’ cell autofluorescence can be accounted for in the model, guided by the peak of CTV-unstained cells (far left panels).
2.11 Statistical Analyses

GraphPad Prism (version 9.3.0, GraphPad Software Inc., La Jolla, CA, USA) was used to perform statistical analyses. For the NTC stimulation assay, subtraction of background levels from the unstimulated, negative control condition was not required gives the enriched NTCs generated negligible background noise (<1% of T cell subset expressing the marker). As the PPD-stimulated post-vaccination assays used whole PBMC (as opposed to enriched monocultures of NTCs), some background expression of the markers by unstimulated cells was measurable in most individuals, and so delta values of expression (percentage of cells expressing the marker in PPD-stimulated minus percentage of cells expressing the marker in unstimulated PBMC) are given as well as absolute values.

For the pre-vaccination NTC assay and post-vaccination PPD-stimulation assays, the expression of each marker for the given condition is given as the percentage of T-cell subset cells expressing that marker. For duplicate or triplicate technical repeats for the same donor in the same experiment, the mean average of those samples is given with the standard deviation. Across all datasets, the values reported are the minimum value, the maximum value, the median (the value separating the higher half from the lower half of data) and the interquartile range (IQR) (calculated as the third quartile (Q3, the value under which 75% of data points are found when arranged in increasing order) minus the first quartile (Q1,
the value under which 25% of data points are found when they are arranged in increasing order). The data were treated as non-parametric with Mann-Whitney analyses used for comparison between groups and the Wilcoxon signed-rank test was used for response comparison within groups. P values <0.05 were considered to be statistically significant, with the symbol * indicative of p<0.05, ** p <0.01, *** p <0.001, **** p <0.0001.

For all assays, estimates were made of the contribution of technical noise within and between experiments and the magnitude of inter-individual variability in T cell expression of Ki67, using the coefficient of variation (Cv), defined as the ratio of the standard deviation (σ) to the mean (μ):

\[
C_v = \frac{\sigma}{\mu} \times 100\%
\]

In the first instance, each individual participant’s variance within the same experimental plate duplicate or triplicate repeats was calculated. This is hitherto referred to as the intra-individual, intra-experimental Cv. Next, the mean average of each individual participant’s same experimental plate duplicate or triplicate repeats was taken and the variation was calculated between different-day replicate experiments. This is hitherto referred to as the intra-individual, inter-experimental Cv. For the preliminary assays this was done separately for the fully, half and quarter anti-CD3/CD28 beads and commercial anti-CD3/CD28 Dynabeads.
These two measures of variation help quantify the intra-experimental and inter-experimental technical noise respectively in the assays.

Next the inter-individual coefficient of variation was calculated between individuals both within experiments (the inter-individual, intra-experimental $C_V$) and between all plates (the inter-individual, all experiment $C_V$). For all measure of variance, the median and IQR were calculated. Comparison between the intra-individual, intra-experimental $C_V$, the intra-individual, inter-experimental $C_V$ and the inter-individual, intra-experimental $C_V$ were made using the Mann-Whitney two-tailed unpaired test. The inter-individual, all experiment $C_V$ was a single numerical value and so did not lend itself to comparisons of the other measures of variance using the Mann-Whitney two-tailed unpaired test. It was therefore tested to see if it was outside of the intra-individual, non-parametric confidence bounds at 95% and 99% (and therefore significant at that level) or not outside the bounds (in which case not significant at the level).

To establish if there were linear relationships between the measured parameters, including between the pre-vaccination parameters, between the post-vaccination parameters and between all pre- and post-vaccination parameters, Spearman’s rank correlation coefficients (Spearman’s rho, $\rho$), a nonparametric measure of statistical dependence between each set of measured parameters, generated for each pair of parameters and correlation matrixes were constructed.
A value of \( r=+1 \) is indicative of a perfect positive correlation, +0.7 suggests a strong positive correlation, +0.5 a moderate positive correlation, +0.3 a weak positive correlation, and 0 indicates no correlation, whilst negative correlations generate \( r \)-values of below 0 to -1.

### 2.12 Whole Blood pre- and post-vaccination T Cell Receptor - sequencing

The TCR response to BCG-vaccination was investigated using our previously published and well-established in-house integrated experimental and computational pipeline which entails polymerase chain reaction (PCR) amplification of peripheral blood TCR alpha and beta chains using a robotic semi-high-throughput approach and subsequent massively parallel sequencing and computational analysis\(^{171,172,190-192}\).

### 2.12.1 Ribonucleic Acid Extractions

3ml whole blood was collected in RNA stabilising-solution both immediately pre- and eight-weeks post BCG-vaccination using TEMPUS RNA tubes (ThermoFisher Scientific). These specimens were frozen at -80°C within 2hrs of collection. Paired pre- and post- samples were subsequently thawed for RNA extraction, performed using a RNeasy extraction kit and silica membrane columns (both Qiagen) in accordance with the manufacturer’s instructions\(^{193}\). Post-
extraction RNA was then quantified using the Qubit with RNA BR reagents (ThermoFisher Scientific) and integrity was assessed Tapestation (Agilent Genomics), before samples were stored at −80°C.

2.12.2 T Cell Receptor Library Preparation and Sequencing

Paired pre- and post- extracted RNA samples were next thawed for polymerase chain reaction (PCR) amplification of TCR α and β chains. First, deoxyribonuclease (DNase) treatment was performed to remove residual DNA: 500ng RNA (in 8µl suspension) was mixed with 1µl RQ1 DNase (Promega), and 1µl RQ1 10× Buffer and incubated for 30mins at 37°C. DNase was then inactivated by the addition of 1µl RQ1 DNase stop buffer for 10min at 65°C.

Next, reverse transcription of the RNA into complementary DNA (cDNA) was performed using primers close to the 5' end of the constant region. 11µl of DNase treated RNA was combined with 4µl RNase-free water, 1.5ul of 10mM stock solutions of deoxynucleoside triphosphates (dNTPs) (Promega) and 1.5ul of 10uM stock solutions of reverse transcription primers alpha RC2 and beta RC2. The primer mix was denatured by 5min heating to 65°C followed by rapid cooling on ice. Next were added 1.5µl Superscript (SSIV) reverse transcriptase (Invitrogen ThermoFisher), 1.5µl RNasin Ribonuclease Inhibitor (Promega), 1.5µl 0.1M
dithiothreitol, and 6 µl of 5×stock Superscript SSIV buffer, which were incubated for 20 mins at 55°C, and then for 10 mins at 80°C.

The cDNA was then purified: 30µL of Agencourt AMPure XP magnetic beads (Beckman Coulter) were added to each 30ul sample in a 96-well plate, mixed well and stood at room temperature (RT) for 5 min, before being stood on a magnetic stand for 2 min and the supernatant removed, leaving the beads behind. The beads were then washed twice using 180µL of 80% ethanol (EtOH) for 30 s on each wash. After the second wash, the plate was allowed to air dry for 5 min. The DNA was then eluted into 12µL of molecular grade H₂O and mixed thoroughly, ensuring all the beads are immersed in the water.

The next step was ligation. Here a single-stranded oligonucleotide with the Illumina SP2 sequencing primer, and a unique molecular identifier (UMI) consisting of two sets of six random nucleotides separated by spacers is ligated to the 3' end of the single-stranded cDNA using T4 RNA Ligase I. 12µl of purified cDNA was added to a ligation mixture of 2µl T4 RNA ligase 1 (NEB), 3µl T4 RNA ligase buffer, 3µl 1mg/ml bovine serum albumin (BSA)/10mM hexamine cobalt chloride (HCC) mixture, 1µl 10mM ATP, 1µl 10µM stock 6N_I8.1_6N_M13_2 ligation oligonucleotide, 10µl 50% stock solution polyethylene glycol (PEG) 8000, mixed manually and incubated for 20–24 hr at 16°C, followed by incubation at 65°C for 10 min (before being stored at 4°C until
This ligation step is critical as it facilitates amplification of all possible rearrangements using a single set of primers per locus, whilst introducing a UMI to label each starting cDNA molecule. The UMI may later be utilised to correct for sequence errors as well as mitigate variable PCR amplification efficiency, generating more accurate measures from the sample of the actual TCR frequency.

Next, a second purification step was performed to remove as much of the ligation oligonucleotide as possible. 30ul of ligation mix was diluted with 70ul RNase-free water and mixed well with 50ul AMPure XP beads and left to stand for 5min at RT before the beads were collected on a magnetic stand for 2min and the supernatant liquid aspirated and discarded. The beads were then washed twice using 300µl of 80% EtOH and air-dried for approximately 5min. The DNA was then eluted in 33 µl molecular grade water (H₂O), the plate placed back on the magnetic stand for 2mins and 31ul of sample transferred to a new plate.

Next the first four rounds of PCR were performed. To 31µl of the purified ligation sample was added 1µl 10mM stock of dNTPs, 2.5µl 10µM stock SP2_M13 primers, 2.5µl 10µM stock Alpha RC1 primers, 2.5µl 10µM stock Beta RC1 primers, 0.5µl Phusion High Fidelity proofreading DNA polymerase (Thermofisher), and 10µl, 5×stock Phusion HF buffer. The first of the three added primers, SP2_M13, extends the ligated fragment to include the Illumina primer SP2. Meanwhile, the other two primers, αRC1 and βRC1 (the latter a mixture of
βRC1.1 and βRC1.2), hybridize to the constant region of the α and β genes, respectively, providing nested primers for the reverse transcription step, thus crucially increasing the specificity of the amplification step. PCR cycling parameters were heated lid (typically 105°C), 98°C 3min denaturation step, followed by 4 cycles of 98°C, 15s denaturation, 69°C, 30s annealing, and 72°C, 40s extension; followed by 72°C, 5min final extension, then held at 10°C until purification.

A third purification step was then performed by adding 40ul AMPure XP beads to the 50ul PCR mixture and repeating the earlier bead purification protocol before eluting in 65ul water, and transferring 31μL of the DNA into two separate wells in a new plate, to process α and β chains separately from this point onwards.

Next, an extension PCR was performed for incorporation of the Illumina sequencing adaptors P5 and P7, the sequencing primer SP1, and two indexing sequences (to facilitate multiplexing of multiple samples on the same sequencing run). 31μL of purified PCR1 ligation mixture was combined with 10μL Phusion HF buffer, 1μL 10mM stock dNTPs, 2.5μL10μM primer SP1-P5, 2.5μL 1μM stock primer SP1-6HN-IX-aRC1 or SP1-6HN-IX-bRC1 (IX indicating an index number), 2.5μL10μM stock P7-L-X (X indicating an index number) and 0.5μL Phusion High Fidelity proofreading DNA polymerase (Thermofisher) (with all samples in a library allocated different combinations of SP1 and SP2 indices, to
facilitate the computational pipeline to identify each sample unambiguously). PCR cycling parameters were heated lid, 98°C 3min denaturation step, followed by 6 cycles of 98°C, 15s denaturation, 69°C, 30s annealing, and 72°C, 40s extension; held at 10°C.

In a fourth purification step, the 50ul PCR2 sample was mixed with 40μL AMPure XP beads, proceeding as before, followed by elution in 30μL water before transferring 27.2μL of the DNA in a QPCR (MicroAmp Fast Optical) plate (ThermoFisher Scientific).

For the third PCR, 27.2μL of PCR2 sample were mixed with 10ul 5xHF buffer, 5ul SYBR Green stock solution (twice diluted SYBR Green I Nucleic Acid Gel Stain; ThermoFisher), 1.25μL 10mM of each dNTP, 1μL ROX reference dye (Thermofisher), 2.5μL 10μM P5, 2.5μL 10μM P7 and 0.5μL Phusion Polymerase. The inclusion of SYBR Green with PCR3 enabled a qPCR to track the reaction progress so the cycler could be stopped on the signal reaching a predetermined threshold (thus reducing the risk of over-amplification).

In a final purification step, the 50ul PCR3 sample was added to 40μL XP AMPure beads as before, with subsequent elution in 30μL water and then transfer 28μL of the DNA into a new plate.
The final post-PCR concentration of each DNA sample was quantified by spectroscopy (dsDNA high-sensitivity Qubit kit, ThermoFisher Scientific) and analyzed by micro-electrophoresis using a high-sensitivity D1000 TapeStation screen tape (Agilent). The final purified product was mixed with samples with other indexes (up to 80 samples run in parallel), to give the final library for sequencing in accordance with the Illumina NextSeq protocol.171,172

2.12.3 Computational Preparation of Sequenced Files

Computational preparation of the Illumina files to ready them for analysis was conducted using our suite of Python (version 3.7) ‘Decombinator’ (version 4.0.3) software tools, publicly available at https://github.com/innate2adaptive/Decombinator. The ‘Demultiplexor’ script was able to identify sequences from multiple samples sequenced together and separate them into individual biological samples in a series of FASTQ files. Next, the ‘Decombinator’ script used a string-matching algorithm to identify in the FASTQ reads, rearranged TCR chains and assign V and J genes by identifying V and J gene specific tag-matches (allowing a maximum of a single base pair mismatch). The output was CSV files in which each line corresponds to a distinct TCR sequence. The next script, ‘Collapsinator’, combed the Decombinator CSV files to provide qualitative and quantitative error correction (for example arising from sequencing error and PCR amplification heterogeneity). Then, the
'CDR3Translator’ algorithm converted the output files of Decombinitator into full DNA sequences, next translated into protein sequences, from which the CDR3 sequences were derived (with their frequency)\textsuperscript{190}. 

RNA extractions from both pre- and post-vaccination peripheral blood samples were performed serially for participants TCV001 to TCV082. For 68/82 participants in whom at least 500ng RNA was obtained in pre-and post-vaccination samples, TCR library preparations were performed by laboratory colleagues Imran Uddin and Gayathri Nageswaran and sequencing was performed across four different NextSeq runs. Subsequently decombinitator algorithms were run by departmental colleague Dr Tahel Ronel. One participant was removed from further analysis as the sequencing step failed and so 67 participants were included in the final analysis. 

2.12.4 Repertoire Level Statistical Analysis and In-Depth Analysis of Pooled Expanded T Cell Receptors

The next analyses were performed in R (version 4.0.0) by departmental colleague Dr Tahel Ronel. Clustering analyses were performed to identify sets of TCRs with similar CDR3 sequences within and between the expanded TCRs of individuals for both $\alpha$ and $\beta$ chains.
First the TCRs which expanded post-vaccination for each individual were defined. The analysis of expansion was carried out on the assumption that the sampling of TCRs from the blood could be reasonably modelled by a Poisson process (in other words, that sequential samples were independent, and the mean frequency of most TCRs remained constant and small over the timeframe of the study). The mean frequency (m) for each TCR was estimated as the number of times a TCR was observed at timepoint one (pre-vaccination) (the count), normalised to the number of TCRs observed in the sample. The mean count of TCRs which were not observed at time point one, but were observed at time point two (post-vaccination), was estimated as one, since this was the median count for all TCRs in all samples. The probability of being observed n times at timepoint two could then be estimated by the Poisson distribution:

\[ P(x=n) = \frac{m^n e^{-m}}{n!} \]

where expanded TCRs were defined as all TCRs for whom \( p < 0.0001 \).

For each of the 67 participants, scatter plots were drawn showing the normalised count at timepoint two versus the normalised count at time point one for each TCR. The confidence boundaries for the Poisson distribution calculated as discussed above were also plotted.
Having statistically defined a set of expanded TCRs, the k-mer (triplet) sequence similarity between these CDR3s was calculated as described in Joshi et al\textsuperscript{174}. The normalised similarity was calculated using the string kernel function stringdot (with parameters stringdottype=‘spectrum’, length = 3, normalized=TRUE) from the Kernlab package\textsuperscript{194}. The iGraph package in R was used to transform the TCR similarity matrix into a similarity graphs (cluster plots), two TCRs (nodes) deemed to cluster (connected by an edge) if they share a pre-determined level of homology\textsuperscript{195}. Varying homology thresholds were trialled and the minimum threshold which consistently generated clusters with >x nodes using random samples of TCRs from the study was selected. Similar analyses from other studies have previously been published by our group\textsuperscript{174,192}.

Three sets of CDR3-clustering analyses were performed, each for the TCR $\alpha$ and $\beta$ chains. First, TCR clustering within individual participants was examined. In each individual, the TCRs determined to have expanded by Poisson analyses post-BCG-vaccination were combined with 1500 further TCR $\alpha$- or $\beta$-chains from each of the 67 individual’s own post-vaccination samples. These additional 1500 TCRs had not expanded, but the highest degree of similarity of triplet kernels to those which had expanded and these were used as ‘bait’ for the cluster plot. This option was selected instead of including all of the TCRs in the plots, which visually would have made them indecipherable. Further included in the same analyses were 1000 of approximately 5000 $\alpha$- and $\beta$-CDR3 sequences previously annotated in the
manually curated databases such as McPAS-TCR as specific for Mtb-epitopes, selecting those most similar to the TCRs identified in the study participants\textsuperscript{196,197}. To aid with visualisation of TCR clustering within individual participants, clustering diagrams were generated for both $\alpha$- and $\beta$-chains in each individual with cluster lines (edges) connecting homologous TCRs (nodes).

Second, all the TCRs found to have expanded in the post-vaccination samples (from the pre-vaccination samples by the Poisson distribution) were combined to form a single set of TCRs. These pooled, expanded TCRs were then analysed for clustering, whilst keeping track from which individual each TCR originated, and making note of how many individuals shared any public TCRs. In this set of analyses, TCRs were deemed to cluster if the CDR3 regions shared $\geq 0.7$ homology in $\geq 4$ TCRs.

Thirdly, the TCRs found to have expanded in the post-vaccination samples compared to the pre-vaccination samples by Poisson distribution from all participants, were once again pooled together to form single sets of expanded TCRs. Once again, combined with these were 1000 external TCRs previously annotated as Mtb-specific in manually curated databases most similar with the pool of expanded TCRs\textsuperscript{196,197}. Clustering plots were then generated, with TCRs deemed to cluster if the CDR3 regions shared $\geq 0.78$ homology, requiring $\geq 4$ TCRs per node.
2.13 Genomic sequencing

There are no current plans to perform genomic sequencing. However, two aliquots of 1ml pre-vaccination peripheral blood in EDTA were collected and are stored at -80°C to enable, for example GWAS future studies given appropriate ethical approval and participant consent.
Chapter 3 Preliminary Experiments to Optimise the Pre-vaccination Naïve T Cell Stimulation Assay

3.1 Introduction

The present study sought to identify if there is inherent variation in the responsiveness of different individuals’ NTCs and if this responsiveness is associated with the subsequent ability of that individual’s pre-primed memory T-cells to generate an antigen-specific response. Therefore, the first arm of the present study sought to quantify the phenotypic response of the NTCs of individuals to standard, non-antigen-specific stimulation. However, on embarking on the present study, it was unknown how best to deliver uniform and polyclonal T cell stimulation with reproducible measurable responses and which elicited any underlying inter-individual variation in NTC responsiveness. Therefore, prior to embarking on experimentation on the cells of enrolled participants, a series of preliminary assays were undertaken using laboratory donor (LD) cells, to ascertain the optimal experimental assay for NTC stimulation.

It was decided from the outset that, in order to best replicate physiological stimulation of the T cell by antigen and the subsequent T cell signalling pathways, the experimental non-antigen-specific stimulation needed to target the TCR, rather than less exact sites of action. Therefore, the monoclonal antibody anti-CD3 was
selected as the activating condition, as opposed to mitogens such as Phytohemagglutinin (PHA) or Concanavalin A (ConA), given anti-CD3 explicitly acts via the TCR-CD3 complex. PHA-L is a lectin extract from legumes such as the red kidney bean and binds to sugars on glycosylated surface proteins of T cells. The specific sites of PHA-T cell interaction remain poorly defined, but the CD2 receptor may be involved\textsuperscript{198,199}. In T cell stimulation by ConA, calcium ions may enter T cells via alternative calcium channels than occurs in CD3-specific signalling pathways\textsuperscript{200}. In addition to anti-CD3, CD28 co-stimulation was identified as necessary, given proliferation in response to ligation of CD3 alone may be followed by premature T cell apoptosis or anergy and highly purified human cells may fail to proliferate at all\textsuperscript{52,201}.

Having determined that anti-CD3 and anti-CD28 were to be used to stimulate the NTCs, it was considered how best to present these monoclonal antibodies to the NTCs and at which concentrations, in what ratio and over what time course so as to effectively stimulate them in a manner that was both reproducible and able elicit any inter-individual variation in responsiveness. Conditions evaluated included presenting NTC monocultures with variable concentrations of anti-CD3 bound immobilised to the surface of tissue culture plates or beads with variable concentrations co-stimulatory anti-CD28 either in solution or similarly immobilised\textsuperscript{49,51}. 
Of interest, at the outset of the present study, it was unknown if response to anti-CD3/CD28 stimulation of NTCs would be binary (all of an individual’s cells with the ability to activate do so at a threshold concentration of stimulant) or if varying the concentration of the antibodies would result in dose responses, with fewer cells responding at lower concentrations and more doing so at greater concentrations. Further, it was unknown if such thresholds or abilities to activate varied between individuals and whether different individuals’ NTCs had the same or differing maximal activation-potential.

Due to practical constraints, as is the case in most vaccinology studies, testing of the real participant samples had to be undertaken using frozen/thawed preparations, rather than fresh cells, so to the preliminary assays were developed on frozen/thawed cells.

3.2 Objectives

(1) To establish a consistent and uniform method of presenting anti-CD3 and anti-CD28 to NTC monocultures with resultant effective, measurable and reproducible NTC stimulation.

(2) To establish whether variable concentrations of anti-CD3 and anti-CD28 result in binary or dose-response effects in the expression of Ki67 by NTCs.
(3) To establish the most suitable concentrations of and ratio of anti-CD3 and anti-CD28 to elicit inter-individual variation in expression of Ki67 by NTCs.

(4) To establish the most suitable incubation duration with anti-CD3 and anti-CD28 to elicit inter-individual variation in expression of Ki67 by NTCs.

3.3. Results

3.3.1 Plate-immobilised Anti-CD3 and Anti-CD28

Initial NTC stimulation experiments employed anti-CD3 immobilised by adsorption to the plastic surface of the culture plates, as described in numerous previous publications\textsuperscript{202,203}. As expected, flow cytometry of both CTV-stained fresh and frozen/thawed unfractionated PBMC showed strong CD3+ T cell proliferation when presented with a range of densities of plate-immobilised anti-CD3 with fixed concentration soluble anti-CD28, see Fig 3.1.
Figure 3.1 Three lab donors (Lab Donor 1 (LD1), LD2 and LD3) demonstrating successful CD3+ T cell proliferation following five days incubation of fresh, whole PBMC with serial dilutions 0 to 1μg/ml of plate-immobilised anti-CD3 and soluble 1μg/ml anti-CD28, modelled by CTV-histograms. Upstream gating was performed as previously (size, singlet, live/dead, CD3+). Detectable cellular CTV diminishes from right to left along the x-axis with each division. Raw data are indicated by black line, proliferation model is indicated by red line, and component generations illustrated in green.
However, when the conditions were repeated with frozen/thawed monocultures of purified NTCs there was little evidence of CD3+ T cell commitment to proliferation, measured either by Ki67 or CTV dilution. These were repeated multiple times, using a range of conditions, but overall proliferation of the NTCs was both weak and inconsistent between experiments even with the same donor T cells. An example of this is illustrated in Fig 3.2, where the cells of three laboratory donors were thawed, enriched for NTCs and stimulated with serial dilutions 0 to 10μg/ml of plate-immobilised anti-CD3 (with a fixed concentration of soluble anti-CD28 at 2μg/ml), and repeated for one of the donors (LD4) on six separate occasions. The failure of purified NTCs to proliferate, in contrast to the T cells within unfractionated PBMC, was unexpected, and has not been previously reported or investigated. However, in these frozen/thawed NTC-enriched experiments, the positive control (commercial magnetic Dynabeads coated with anti-CD3 and anti-CD28) demonstrated more marked and reproducible NTC proliferation (see Fig. 3.3). An alternative method to immobilise the anti-CD3 and anti-CD28 stimulating antibodies on beads, rather than the plastic surface of the culture plates, was therefore explored in the next series of experiments.
Figure 3.2 An example of a preliminary assay where the frozen/thawed naïve T cell (NTC)-enriched cells of three laboratory donors (LDs) were incubated with serial dilutions of plate-immobilised anti-CD3 (presented on logarithmic scale on the x-axis) in combination with 2μg/ml soluble anti-CD28 in each well, and the CD3+ T cell expression of Ki67 was measured after three days. For laboratory donor 4 (LD4) the experiment was repeated on six separate occasions. Each data point represents mean of duplicate wells.
Figure 3.3 Expression of Ki67 by CD3+ T cells following three days incubation with commercial anti-CD3/CD28 Dynabeads in four laboratory donors, derived from the positive control wells of multiple experiments of frozen/thawed NTC-enriched monocultures. Each data point represents the value for a single well, columns indicate median and error bars the IQR.
3.3.2 Bead-immobilised Anti-CD3 and Anti-CD28, Including Density and Ratios of Immobilised Antibodies

Using streptavidin Dynabeads and biotin-labelled antibodies, it was possible to manufacture anti-CD3/CD28 beads holding pre-determined concentrations of immobilised anti-CD3 and anti-CD28 (as detailed in Chapter 2). The streptavidin beads provide a known antibody-binding capacity. Using this information, beads were incubated with variable concentrations of a 1:5 mixture of anti-CD3 and anti-CD28 biotinylated antibodies to achieve saturated, half saturated, quarter saturated and eighth saturated antibody-coated beads. Using manufacturer information, the beads were estimated to have bound approximately 42, 21, 10 and 5 µg/ml anti-CD3 antibody respectively. Additional fully-saturated beads were also produced with variable ratios of anti-CD3 to anti-CD28 (1:20 and 1:40, as well as 1:5).

The frozen/thawed CTV-labelled-NCTs of nine LDs were incubated with each strength of bead and subsequently stained (panel in Fig. 2.4) and run on flow cytometry for markers of activation, differentiation and proliferation.

Reproducibility was examined, both with intra-experimental technical repeats and by repeating the experiment for each of the donors on up to three separate occasions (using PBMC obtained from a single bleeding point, but thawed on three separate occasions).
Negligible difference in the CD3 T cell expression of Ki67 was found between the fully saturated beads holding different ratios of anti-CD3 to anti-CD28 (results not shown). Given that previous groups have found lower ratios to provide optimal stimulation, all further assays used the 1:5 ratio\textsuperscript{188}.

The dose responses of all the donors to the different ‘strengths’ of beads are shown in Fig. 3.4. Maximal response to stimulation was observed using half-saturated beads, above which the responses plateaued and fully-saturated beads did not further increase Ki67 expression. In most donors, maximum response corresponded to approximately 80 – 90% of CD3+ cells expressing Ki67, although in Laboratory Donor 18 (LD18; Fig 3.4H) there is the suggestion of a lower maximal response, with approximately 50% CD3+ cells expressing Ki67 on all repeats.
Figure 3.4 – Dose response, measured as percentage of CD3+ T cells expressing Ki67, in nine laboratory donors (LD11 – LD19) following three days incubation with anti-CD3/CD28 beads coated with variable concentrations of anti-CD3 and anti-CD28 (in a 1:5 ratio). Replicate wells for all conditions plotted and up to three experimental repeats (i-iii) per donor. Downward pointing triangles represent positive control (commercial anti-CD3/CD28 Dynabeads).
Preliminary estimates on the contributions of inter-individual and experimental variation towards the observed variation between donors were made by calculating the coefficient of variation ($C_V$) of Ki67 expression across donors and within repeat experiments on the same donors (see Fig. 3.5).

The intra-individual, intra-experiment $C_V$’s are illustrated in Fig. 3.5A. For all strengths of anti-CD3/CD28 beads, the intra-individual, intra-experiment $C_V$ for replicate wells was relatively low at around 10% or less (fully saturated median $C_V$ 1% (IQR 5%), half saturated median $C_V$ 2% (IQR 6%), quarter saturated median $C_V$ 10% (IQR 11%) and commercial Dynabeads median $C_V$ 4% (IQR 9%). These values are suggestive that in all conditions, there was low measurable noise within experiments.

Next, intra-individual, inter-experiment $C_V$’s were calculated and are illustrated in Fig. 3.5B. For all strengths of anti-CD3/CD28 beads, the intra-individual, inter-experiment $C_V$ was again relatively low at around 10% or less (fully saturated median $C_V$ 8% (IQR 25%), half saturated median $C_V$ 8% (IQR 8%), quarter saturated median $C_V$ 12% (IQR 17%) and commercial Dynabeads median $C_V$ 6% (IQR 9%). These values are suggestive that in all conditions, there was low measurable noise between experiments.
Next, the magnitude of inter-individual variability in CD3+ T cell expression of Ki67 was found with the inter-individual, all experimental C_v between all donors, illustrated by Fig. 3.5C. The C_v between donors was 19% for the fully saturated anti-CD3/CD28 beads, 25% for the half-saturated anti-CD3/CD28 beads, 47% for quarter saturated anti-CD3/CD28 beads and 21% for the commercial anti-CD3/CD28 Dynabeads. These values are suggestive that of all the conditions, the greatest inter-individual variation (with respect to commitment to proliferation) was elicited in response to sub-optimal stimulation, the quarter saturated anti-CD3/CD28 beads (C_v =47%).

Finally, Fig. 3.5D compares the C_v’s of CD3+ T cell expression of Ki67 for intra-individual, intra-experiment repeats; intra-individual, inter-experiment repeats; and inter-individual, all experiment results for the four different conditions (fully, half and quarter saturated anti-CD3/CD28 beads and commercial anti-CD3/CD28 Dynabeads). C_v’s for intra-individual, intra-experiment repeats and intra-individual, inter-experiment repeats were compared using Mann-Whitney two-tailed unpaired analyses. The inter-individual, all experiment C_v was a single numerical value for each condition and so was compared to the intra-individual, intra-experiment and intra-individual, inter-experiment C_v non-parametric confidence bounds at p<0.05 and p <0.01 and deemed significant at that level if outside of those bounds. For all conditions (fully, half and quarter saturated anti-CD3/CD28 beads and commercial anti-CD3/CD28 Dynabeads) the overall measured inter-individual
variation was greater than the intra-individual, intra-experimental variation. For the half and quarter saturated anti-CD3/CD28 beads and commercial anti-CD3/CD28 Dynabeads the overall measured inter-individual variation was also greater than the intra-individual, inter-experimental variation. This was not the case for the fully saturated beads, where, for this small pilot set of experiments, there was no discernible difference between the intra-individual, inter-experimental variation and the inter-individual variation. For quarter saturated anti-CD3/CD28 beads and commercial anti-CD3/CD28 Dynabeads there were significant differences between the inter-individual variation and the intra-individual variation.

On the basis of these preliminary experiments, the optimal condition by which to measure inter-individual variation in T cell responsiveness to non-specific TCR stimulation was identified as with the quarter saturated anti-CD3/CD28 beads. Proceeding to the study participant assays, the fully saturated anti-CD3/CD28 beads were also to be utilised as a testing condition to provide a positive control and in order that dose response curves could be constructed for the expression of parameters in response to sub-optimal and maximal concentrations of anti-CD3/CD28.
Figure 3.5 Scatter plots of coefficient of variation (C\textsubscript{V}) for CD3+ T cell expression of Ki67 in (A) the intra-individual, intra-experimental technical replicate wells; (B) intra-individual, inter-experimental repeat plates; and (C) inter-individual, all experiments following the incubation of naïve T cells (NTCs) of nine laboratory donors (LDs) for three days with quarter, half or fully saturated anti-CD3/CD28 beads or commercial anti-CD3/CD28 Dynabeads. Individual values depicted by circles, median represented by columns and interquartile range (IQR) given by bars. In bar chart (D) the results are summarised for each condition and category, again with columns representing medians and bars representing IQRs.

Comparisons between intra-individual, intra-experimental C\textsubscript{Vs} and intra-individual, inter-experimental C\textsubscript{Vs} by Mann-Whitney two-tailed unpaired analyses. The inter-individual, all experiment C\textsubscript{V} was compared to the intra-individual, intra-experiment and intra-individual, inter-experiment C\textsubscript{V} non-parametric confidence bounds at \( p<0.05 \) and \( p<0.01 \) and deemed significant at that level if outside of those bounds.
3.3.3 Naïve T Cell Assay Duration

The preliminary experiments reported in section 3.3.2 were all conducted with an incubation period of 67 hour (three days). Next, an experiment was undertaken to establish if this was in fact the optimal duration for the assay. Pre-vaccination frozen PBMC from six of the BCG-study participants, for whom the PBMC preparations had yielded the greatest number of cells, were thawed and enriched for NTCs and then seeded with fully and quarter saturated anti-CD3/CD28 beads on four separate plates: one incubated for 21 hours, one for 42 hours, one for 67 hours and one for 78 hours. Figure 3.6A illustrates the percentage of CD3+ T cells expressing Ki67 for each of the six participants at the four timepoints on stimulation with the quarter-saturated beads and, 3.6B shows the same for the fully-saturated beads.

For the quarter saturated anti-CD3/CD28 beads condition, at Day 1 (21hrs), there was negligible detectable Ki67. At Day 2 (42hrs), 2 - 29% CD3+ T cells were found to express Ki67. At Day 3 (67hrs) (the same time point used in the previous preliminary assays), 26 - 73% CD3+ T cells expressed Ki67. At Day 4 (88hrs), responses appear to begin to plateau with 32 - 80% of the participant CD3+ T cells expressing Ki67.
For the NTCs incubated with fully saturated anti-CD3/CD28 beads, at Day 1 (21hrs), there was minimal expression of Ki67. At Day 2 (42hrs), 25 - 74% cells expressed Ki67. At Day 3 (67hrs), 76 - 94% cells expressed Ki67. Again, the responses appear to plateau by Day 4 (88hrs), where 83 - 94% cells expressed Ki67.

At quarter saturated anti-CD3/CD28 beads, sub-optimal stimulation, there was inadequate demonstration of molecular commitment to proliferation prior to Day 3. Meanwhile, at Day 3, Ki67 was detectable in all participants and levels of expression were wide-ranging between participants. At fully saturated anti-CD3/CD28 beads, maximal stimulation, peak molecular commitment to proliferation appeared to have been achieved by Day 3.

Fig. 3.6C illustrates that regardless of which day of stimulation is analysed (Day 2, Day 3 or Day 4), responsiveness of the participants correlates between time points and rank order from most to least responsive participant is approximately preserved – a better responder at Day 2 is a better responder at Days 3 and 4 and a poorer responder at Day 2 is a poorer responder at Days 3 and 4.
(A) CD3+ Ki67+

(B) CD3+ Ki67+

(C) Recruit UIN:
- TCV028
- TCV031
- TCV052
- TCV071
- TCV094
- TCV096

Rank (mean % marker Ki67*)

Day 2
Day 3
Day 4
Figure 3.6 CD3+T cell expression of Ki67 in six study participants, following incubation with (A) quarter saturated and (B) fully saturated anti-CD3/CD28 beads at Day 1 (21hrs), Day 2 (42hrs), Day 3 (67hrs) and Day 4 (88hrs). Each data point represents a single well, with triplicate wells performed for each condition. (C) plots the mean of triplicate repeat Ki67 expression by each participant in response to quarter saturated anti-CD3/28 beads for each day and illustrates that participant rank order of responsiveness is approximately preserved at each time point.
Fig. 3.7 presents FACS plots of CTV dilution plotted against expression of Ki67 in CD3+ T cells for the four timepoints following incubation with quarter saturated anti-CD3/CD28 beads. The plots illustrate that at Day 1 (21hrs), none of the donors’ T cells had undergone cellular division; at Day 2 (42hrs) there had been minimal cell division with no or only one cycle of division. Proliferation and consequent CTV dilution has occurred by Day 3 (67hrs), with each participant’s cells having undergone up to three series of divisions. In light of the findings of the preliminary NTC stimulation duration assay, a total incubation time of 67 hours (three days) was selected to proceed for testing the study participants.
Figure 3.7 Flow cytometry plots of CD3+ cell CellTrace Violet (CTV)-signal (x-axis), with detectable cellular CTV diminishing from right to left with each division, versus Ki67 (y-axis) in the naïve T cells (NTCs) of six participants after Day 1 (21hrs), Day 2 (42hrs), Day 3 (67hrs) and Day 4 (88hrs) incubation with quarter saturated anti-CD3/CD28 beads. One of the triplicate technical repeats shown for each participant/condition, as well as one negative control (media only).
3.4 Discussion

Preliminary assays were performed to guide which conditions should be selected to stimulate the NTCs of the enrolled study participants. Unexpectedly, stimulation of monocultures of NTCs using variable concentrations of plastic-bound anti-CD3 and immobilised or soluble anti-CD28 generated weak and inconsistent proliferation. Interestingly, unfractionated PBMC responded much more strongly to the plastic-bound antibodies, reflective of this assay being widely reported in the literature to polyclonally stimulate T cells. It could be postulated that the density of immobilised agonist antibody may be more critical for naïve T cell activation. Binding the antibody to beads, as in the commercial anti-CD3/CD28 Dynabeads, may result in higher and more reproducible concentrations of surface-bound antibody than is possible by the passive and non-specific binding of antibodies to the surface of tissue culture plastics.

The commercial anti-CD3/CD28 Dynabeads potently stimulated the NTCs, but it was not possible to modulate the strength of the stimulus using this reagent, and the maximal stimulation observed might not capture subtle nuances in inter-donor variation in NTC responses. Therefore, a method was developed to manufacture beads with varying concentrations of antibody in a reproducible manner, using streptavidin-coated beads bound to biotinylated antibodies.
As expected, NTCs responded to antibody-coated beads in a variable, dose-dependent manner. The dose response curves could be characterised by both their slope and plateau. In order to capture both of these parameters, the final NTC stimulation assay utilised both a sub-optimal and a maximal dose of anti-CD3 (experimentally determined to be quarter saturated anti-CD3/CD28 beads and fully saturated anti-CD3/CD28 beads respectively). The preliminary experiments reported in this chapter suggest that differences in response between individuals may be more often observed at the lower antibody dose, although differences in maximal response may also occur.

The time point of 67 hours was selected to proceed for the remaining subjects, as by this point, sufficient T cells had begun both to express Ki67 and undergo cellular divisions.

There are limitations to these preliminary results. The concentrations of bound antibody were estimated, derived from manufacturer’s information, but it did not prove possible to experimentally measure the amount of bound antibody. Furthermore, to fully delineate the dose responses would have required measurements at many more different concentrations, and potentially at multiple time points. The decision to choose two representative concentrations, at one time point were driven largely by the practicalities of setting up the NTC stimulation assays at scale. A further limitation was that the only negative control employed
was by incubating the cells with media alone. Ideally a proportion of assays could have included the further negative control of beads coated with biotinylated IgG1 isotype. Finally, one LD appeared to generate a maximum response (of T cell expression of Ki67) considerably lower than the other LDs to both fully saturated anti-CD3/CD28 beads and commercial anti-CD3/CD28 Dynabeads, which was reproduced on further experiments using cells from the same batch of frozen PBMC. It is unclear whether this reflects real biology (that that LD’s cells are incapable of a greater response) or if it reflects a poor PBMC preparation. Ideally the experiments would have been repeated with the LD being re-bled to examine for reproducibility.

However, the preliminary assays did provide a wealth of information instrumental in designing the NTC-stimulation assay for the study participants, the subject of the next chapter, developed with confidence that it directly addressed the first research question: are some individuals’ NTCs inherently more responsive to activation. Subsequent to these preliminary experiments, the NTC assay was conducted on the pre-vaccination frozen/thawed NTCs of the 107 study participants.
Chapter 4 Inter-individual Variation in Naïve T Cell Response to Non-specific T Cell Receptor Stimulation

4.1 Introduction

Genetic, environmental and intrinsic factors generate substantial natural variation between the immunological systems of individuals, both in the molecular and cellular landscapes, before and also after any examinable perturbation. Accurate incorporation of this inter-individual variability into biological systems is crucial if functional relationships among the system components are to be inferred. The present study sought to measure the magnitude of inherent variation in the responsiveness is associated with the subsequent ability of that individual’s pre-primed memory T-cells to generate an antigen-specific response. Therefore, the first arm of the present study seeks to answer the question ‘Are some individuals’ T cells inherently more responsive to stimulation?’ by quantifying the phenotypic response of the NTCs of individuals to standard, non-antigen-specific stimulation. As recounted in Chapter 3, preliminary experiments enabled the development of an assay by which uniform, polyclonal stimulation was delivered to pre-vaccination NTC TCRs using two sets of bespoke anti-CD3/CD28 beads (providing sub-optimal and maximal concentrations of immobilised antibody), prior to flow cytometric measurement of the activation, differentiation and proliferation parameters listed in Table 4.1.
Table 4.1 Parameters utilised in the present study and measured by flow cytometry to quantify pre-vaccination naïve T cell (NTC) activation, proliferation and differentiation in response to stimulation by anti-CD3/CD28 beads.

<table>
<thead>
<tr>
<th>Phenotypic response</th>
<th>Measured parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation</td>
<td>PD-1&lt;br&gt;CD25</td>
</tr>
<tr>
<td>Proliferation</td>
<td>Ki67&lt;br&gt;Tracking dye dilution (Cell Trace Violet, CTV)</td>
</tr>
<tr>
<td>Differentiation</td>
<td>CD45RA&lt;br&gt;CCR7</td>
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Total observed variation in any measured parameters may be attributed to (i) technical or measurement noise, (ii) variation within a person over time and (iii) subject-to-subject variation. When seeking to establish individual heterogeneity in the immune systems of a population, it is crucial to delineate the relative proportions of such technical, temporal and inter-individual variations. We may seek to estimate the contribution from the first category with technical repeats and the second category by repeating the experimental for the individual at a different time point.\textsuperscript{64}

The heritable, intrinsic and/or environmental factors driving any inter-individual variation is beyond the scope of this study, with the assays developed to look downstream of the causative factors and instead at the functionality of the T cells. Any variation elicited may potentially reflect variation in TCR or co-stimulatory intracellular signalling machinery.

4.2 Hypothesis

There is inter-individual variation in the functional cellular responsiveness of NTCs to non-specific stimulation via the TCR.

4.3 Objectives
(1) To measure the expression of Ki67 by the pre-vaccination CD4+ and CD8+ NTCs of 107 study participants following stimulation by anti-CD3/CD28 beads at sub-optimal and maximal strengths.

(2) To measure the range of Ki67 expression by individuals’ pre-vaccination CD4+ NTCs in response to stimulation by sub-optimal strength anti-CD3/CD28 beads and estimate the contributions of inter-individual biological variation and technical noise.

(3) To measure the range of inter-individual variation in additional markers of activation, differentiation and proliferation expressed by pre-vaccination CD4+ and CD8+ NTCs in response to sub-optimal anti-CD3/CD28 beads stimulation.

(4) To identify correlations between markers of activation, differentiation and proliferation expressed by pre-vaccination CD4+ and CD8+ NTCs in response to stimulation by sub-optimal strength anti-CD3/CD28 beads.

4.4 Results

4.4.1 Expression of Ki67 by Pre-vaccination CD4+ Naïve T Cells Varies in Accordance with the Concentration of Anti-CD3/CD28 Stimulation

107 participants were bled immediately prior to BCG-vaccination and multiple aliquots of PBMC were prepared and frozen for each participant. As described in Chapter 2, frozen aliquots of PBMC were then thawed in batches, enriched for
NTCs and incubated for 67 hours with media alone (negative control), or with quarter saturated anti-CD3/CD28 beads (10μg/ml anti-CD3, 52μg/ml anti-CD28) or with fully saturated anti-CD3/CD28 beads (42μg/ml anti-CD3 and anti-CD28 208μg/ml), before staining using the panel in Fig. 2.4 for flow cytometry.

A low cell viability (<50% of cells alive by L/D stain on FACS) and failure of the positive control on repeated attempts at the assay was observed for frozen aliquots for three individuals and these participants were not included in any further pre-vaccination analysis. Viable PBMC were successfully retrieved from frozen aliquots for 104 participants. Fig 4.1 displays the dose response curves for CD4+ and CD8+ T cell expression of Ki67 as a function of the antibody concentrations presented by the anti-CD3/CD28 beads. In unstimulated cells, there was negligible measurable Ki67 expression. For NTCs incubated with quarter saturated anti-CD3/CD28 beads, a median of 37% CD4+ T cells expressed Ki67 (IQR 28%) and a median of 46% CD8+ T cells expressed Ki67 (IQR 33%). For NTCs incubated with fully saturated anti-CD3/CD28 beads, a median of 89% CD4+ T cells expressed Ki67 (IQR 9%) and a median of 93% CD8+ T cells expressed Ki67+ (IQR 8%).

In approximately one quarter of participants (25/104 participants), <85% of their CD4+ T cells expressed Ki67 by Day 3 of incubation with fully saturated anti-CD3/CD28 beads, indicative that the NTCs of these individuals have a lower peak
responsiveness than in the majority of individuals in the given experimental conditions. Similarly, in approximately one in five participants (19/104 participants), <85% of their CD8+ T cells expressed Ki67 by Day 3 at maximal stimulation.

There is a considerable range of NTC Ki67 expression between individuals and this inter-individual variation is more evident at sub-optimal stimulation with quarter saturated anti-CD3/CD28 beads (Cv 50% for CD4+Ki67+ and Cv 44% for CD8+Ki67+) than at maximal stimulation with fully saturated anti-CD3/CD28 beads (Cv 11% for CD4+Ki67+ and Cv 10% for CD8+Ki67+).
Figure 4.1. Dose response curves for (A) CD4+ and (B) CD8+ Naïve T cell (NTC) %Ki67 expression as a function of the antibody concentrations presented by anti-CD3/CD28 beads in 104 participants. Each point represents the mean of two or three replicate cultures with the standard deviation.
The expression of Ki67 by pre-vaccination CD4+ NTCs of 104 participants in response to stimulation with quarter saturated anti-CD3/CD28 beads are again displayed in Fig. 4.2. The time the data are displayed in a bar chart format, with individuals in rank order (the participants with the minimum, median and maximum values are indicated), and as a frequency distribution histogram, to demonstrate the range and shape of the distribution of parameter values amongst the participants, approximated by a lognormal distribution as shown.
Figure 4.2 Ki67 expression in the pre-vaccination CD4+ naïve T cells (NTCs) of 104 participants stimulated with quarter saturated anti-CD3/CD28 beads. (i) Bar chart displays results for each participant in rank order. Each bar represents the mean of up to triplicate technical repeats for each participant and the error bars represent the standard deviation. The blue bar represents the minimum value (TCV055, CD4+Ki67+=3%), the green bar represents the median (TCV101, CD4+Ki67+=37%), the red bar represents the maximum value (TCV014, CD4+Ki67+=90%), the dashed cyan line represents the median, the magenta line the first quartile and the dashed orange line the third quartile. (ii) Frequency distribution histogram, the fitted non-linear lognormal regression is illustrated and the R squared goodness of fit value is given.
4.4.2 Quantifying Sources of Variation in Ki67+ Expression in CD4+ T Cells in Response to Quarter Saturated anti-CD3/CD28 Beads

The extensive inter-individual variation observed in the pre-vaccination NTC anti-CD3/CD28 beads stimulation assay is due to a combination of biological variation between T cells of different individuals and technical inter-experiment variation. In order to evaluate the relative contribution of these two sources of variation, Ki67 expression in CD4+ T cells were repeated for a sub-sample of participants (11 individuals, 10% of the participants) in replicate experiments. For the replicate experiments, participants’ cells were sourced from different aliquots of the same PBMC preparations, but thawed and established for incubation on separate days and with anti-CD3/CD28 beads sourced from alternate batches. The data are again plotted in bar chart format with individuals in rank order (see Fig. 4.3), but experimental repeats are shown in a colour unique for each of the sub-sampled participants. Fig. 4.3 shows that each individual’s performance is to a large extent reproducible in repeat experiments, with the rank order of the response preserved in 8 of the 11 individuals.
Figure 4.3 (i) Ki67 expression in the pre-vaccination CD4+ naïve T cells (NTCs) of 104 participants stimulated with quarter saturated anti-CD3/CD28 beads in rank order, with the inclusion of eleven participants in which the cultures were set up in duplicate on different days, indicated by a pair of coloured bars. Each bar represents the mean of up to triplicate technical repeats for each participant and error bars represent the standard deviation. (ii) A graphic demonstrating the rank orders of the eleven participants for the replicate experiments.
To further estimate the contributions of biological and technical sources of variation between the expression of Ki67+ by CD4+ T cells in 104 participants in response to quarter saturated anti-CD3/CD28 beads in the NTC stimulation assay, CV values were calculated for technical repeats in the same experiment; replicate experiments of the same individual on different days; and between different individuals, the results of which are summarised in Fig. 4.4.

The intra-individual, intra-experiment technical repeats median CV was low (median CV=8%, IQR 11%), suggestive that there was low intra-individual technical noise within experiments. The intra-individual, inter-experiment replicates median CV was also low (median CV=15%, IQR 16%). There was no statistically significant difference between the intra-individual, intra-experimental and the intra-individual, inter-experimental coefficients of variance (Mann-Whitney U=485, \( n_1=104, n_2=11, p=0.42 \) two tailed), suggestive that experimental variables such as thawing of different aliquots on different days; the setting-up of different experiments; and using anti-CD3/CD28 beads preparations from different batches did not contribute much to variability.

In contrast, the CV for all pairwise individual comparisons of expression of Ki67+ by CD4+ T cells in response to quarter saturated anti-CD3/CD28 beads within a single experiment, the inter-individual, intra-experiment was much higher (median CV 38%, IQR 20%) than in either technical replicates (median CV=8%, Mann-
Whitney $U=90$, $n_1=104$, $n_2=22$, $p<0.0001$ two tailed) or replicate experiments (median $C_V=15\%$, Mann-Whitney $U=27$, $n_1=11$, $n_2=22$, $p=0.0001$ two tailed).

Finally, the coefficient of variation between all the different participants’ mean expression of Ki67+ by CD4+ T cells, the inter-individual, all experiment variation, was calculated at 50%. As this final $C_V$ represented a single value, it was not possible to use a nonparametric test to compare this all experiment inter-individual $C_V$ to the other measures of $C_V$ (intra-individual, intra-experiment ($n=104$); intra-individual, inter-experiment ($n=11$); inter-individual, intra-experiment ($n=22$)). However, this value of $C_V=50\%$ is outside of the 99% confidence interval (CI) of the intra-individual, intra-experiment $C_V$ median (median=8%, 99% CI 6 to 10%) and the 99% CI of the intra-individual, inter-experiment $C_V$ median (median=15%, 99% CI 1 to 48%), and is therefore significantly different from both of these sets of values at $p<0.01$. The all experiment inter-individual $C_V$ at 50% falls within the 95% CI of the inter-individual, intra-experiment $C_V$ median (median=38%, 95% CI 31 to 52%) and so is not significantly different from these measures.
Figure 4.4 Coefficient of variation (C_v) for Ki67 expression by CD4+ T cells following incubation with quarter saturated ant-CD3/CD28 beads for intra-individual, intra-experimental replicates (blue); intra-individual, inter-experimental repeats (red); inter-individual intra-experimental comparisons (green); and inter-individual inter-experimental comparisons (purple). Individual values illustrated by symbols, median represented by column and interquartile range represented by bars.
4.4.3 Expression of Phenotypic Parameters of Activation, Proliferation and Differentiation by Pre-vaccination CD4+ NTCs in Response to Sub-Optimal Anti-CD3/CD28 Stimulation

Having established that stimulation of NTCs by quarter saturated anti-CD3/CD28 beads for three days resulted in inter-individual variation in the expression of Ki67+ by CD4+ T cells, not attributable solely to experimental factors, further analysis was performed to ascertain inter-individual variation in expression of the remaining parameters of activation, proliferation and differentiation by CD4+ and CD8+ NTCs under the same conditions (see Fig. 4.5). Absolute, rather than change from baseline/unstimulated (delta values) are given for all parameters as there was negligible expression of these in the unstimulated negative control wells of the enriched NTCs cultures.

Once more, in response to the experimental conditions, a broad range of expression between participants was observed in the parameters. The median expression of activation marker CD25hi by CD4+ T cells was 59% (IQR 25%) (Fig. 4.5A) and by CD8+ T cells was 65% (IQR 27%) (Fig. 4.5B). The median expression of activation marker PD-1hi by CD4+ T cells was 23% (IQR 17%) (Fig. 4.5C) and by CD8+ T cells was 30% (IQR 27%) (Fig. 4.5D).

The median expression of proliferation marker Ki67+ by CD4+ T cells was 36% (IQR 28%) (Fig. 4.5E) and by CD8+ T cells was 46% (IQR 33%) (Fig. 4.5F).
Cellular proliferation was measured by the serial dilution of the cell tracer dye CTV and modelled by FlowJo software. For CD4+ T cells, a median of 11% cells entered division (IQR 15%) (Fig. 4.5G) and in CD8+ T cells it was 13% (IQR 17%) (Fig. 4.5H). The Division Index (DI, a measure of the average number of divisions undergone by cells) in CD4+ T cells had a median of 0.15 (IQR 0.21) (Fig. 4.5I) and in CD8+ T cells it was 0.17 (IQR 0.25) (Fig. 4.5J). The Proliferation Index (PI, a measure of the average number of divisions undergone by cells, excluding the undivided cells) for CD4+ T cells had a median of 1.3 (IQR 0.2) (Fig. 4.5K) and for CD8+ T cells it was 1.4 (IQR 0.2) (Fig. 4.5L). The Replication Index (RI, a measure of the fold-expansion of responding cells) for CD4+ T cells had a median of 2.6 (IQR 0.4) (Fig. 4.5M) and for CD8+ T cells it was 2.9 (IQR 0.6) (Fig. 4.5N).

Differentiation from naïve CD45RA+CCR7+ NTCs to CD45RA-CCR7+ central memory (T\text{CM}) cells occurred in a median of 12% CD4+ T cells (IQR 7%) (Fig. 4.5O) and 5% CD8+ T cells (IQR 5%) (Fig. 4.5P). Differentiated from CD45RA+CCR7+ NTCs to CD45RA-CCR7- effector memory (T\text{EM}) cells occurred in a median of 5% CD4+ T cells (IQR 5%) (Fig. 4.5Q) and 5% CD8+ T cells (IQR 7%) (Fig. 4.5R).

Within each bar chart, participants are delineated in whom the Ki67+ expression by T cells was at the minimum (participant TCV055, blue bars), median Ki67
(TCV101, green bars) and maximum Ki67 (TCV014, red bars) to provide visual reference of how three participants performed relative to themselves and the other participants for the different parameters.
Figure 4.5 Summary bar charts (denoted by (i)) and corresponding frequency distribution histograms (denoted by (ii)) for expression of parameters of activation (A - D), proliferation (E - N) and differentiation (O - R) by the CD4+ and CD8+ naïve T cells (NTCs) of 104 participants in response to three days incubation with quarter saturated anti-CD3/CD28 beads. Figures are given for expression of (A) CD4+CD25hi; (B) CD8+CD25hi; (C) CD4+PD-1hi; (D) CD8+PD-1hi; (E) CD4+Ki67; and (F) CD8+Ki67 by T cells. Derived from cell trace CTV dilution and modelling analysis, the next set of figures are for (G) CD4+ Percentage Divided; (H) CD8+ Percentage Divided; (I) CD4+ Division Index (DI, a measure of the average number of divisions undergone by all cells); (J) CD8+ DI; (K) CD4+ Proliferation Index (PI, a measure of the average number of divisions undergone by cells, excluding the undivided cells); (L) CD8+ PI; (M) CD4+ Replication Index (RI, a measure of the fold-expansion of responding cells); and (N) CD8+ RI. The final graphs pertain to the percentages of NTCs which have differentiated from CCR7+CD45RA+ NTCs to (O) CD4+CCR7+CD45RA- Central Memory T cells (T_{CM}); (P) CD8+CCR7+CD45RA- T_{CM}; (Q) CD4+CCR7-CD45RA- Effector Memory T cells (T_{EM}); and (R) CD8+CCR7-CD45RA- T_{EM}. Bar charts display results for that parameter in rank order along the x-axis from least responsive to most responsive. Each bar represents mean of up to triplicate technical repeats for each participant and error bars represent standard deviation. Within each bar chart, participants are delineated in whom the Ki67+ expression by T cells was at the minimum (participant TCV055, blue bars), median Ki67
(TCV101, green bars) and maximum Ki67 (TCV014, red bars) to provide visual reference of how three participants performed relative to themselves and the other participants for the different parameters. In each bar chart the dashed cyan line provides the median for that parameter, the dashed magenta line the first quartile and the dashed orange line the third quartile. For each frequency distribution histogram, the fitted non-linear lognormal regression is illustrated and the R squared goodness of fit value is given.
Fig. 4.6 presents a summary graph for the expression of all the measured parameters by CD4+ and CD8+ T cells in response to incubation with quarter saturated anti-CD3/CD28 beads for three days, which further demonstrate the inter-individual variation in responsiveness identified between the participants across the parameters.
Figure 4.6 A summary graph for the percentage of CD4+ and CD8+ T cells expressing each of the measured parameters in response to incubation with quarter saturated anti-CD3/CD28 beads for three days. Each dot represents the expression of that parameter by an individual (mean of up to three replicate repeats). The black bar represents the median and error bars represent the interquartile ranges.
4.4.4 Coefficient of Variation of T Cell Expression of All Parameters of Activation, Differentiation and Proliferation in Response to Quarter Saturated Anti-CD3/CD28 Beads

Fig. 4.7 provides a summary graph of the inter-individual C_V’s, in rank order, for the CD4+ and CD8+ T cell expression of parameters of activation (CD25hi and PD-1hi), proliferation (Ki67, percentage divided, DI, PI and RI) and differentiation into subsets T_CM (CCR7-CD45RA+) and T_EM (CCR7-CD45RA-) between the 104 participants in response to three days incubation with quarter saturated anti-CD3/CD28 beads.

For the given experimental conditions, four of the measured parameters, all pertaining to cellular proliferation derived from CTV modelling, exhibited less than 20% variation between the participants: the PIs’ C_V’s were 10% in both CD4+ and CD8+ T cells; and the RIs’ C_V’s were 13% in CD4+ T cells and 14% in CD8+ T cells 14%. All of the other parameters demonstrated greater than 20% variation between participants, and so may be considered to be more discriminative measures of relative responsiveness to uniform and sub-optimal stimulation. The parameters with the greatest measured variation between participants (C_V > 70%) were percentage divided (C_V in CD4+ T cells 72% and C_V in CD8+ T cells 73%); percentage of cells differentiated from CCR7+CD45RA+ NTCs to CCR7-
CD45RA- $T_{EM}$ cells ($C_v$ in CD4+ T cells 80% and $C_v$ in CD8+ T cells 109%); and DI ($C_v$ in CD4+ T cells 83% and $C_v$ in CD8+ T cells 85%).
Figure 4.7 Summary graph of the inter-individual coefficients of variation (C_V) of expression of parameters of activation (CD25hi and PD-1hi), proliferation (Ki67, percentage divided, division index (DI), proliferation index (PI) and replication index (RI) and differentiation (to CCR7-CD45RA+ T central memory cells (T_{CM}) and CCR7-CD45RA- T effector memory cells (T_{EM}), given in rank order, in 104 participants following incubation of their naïve T cells (NTCs) with quarter saturated anti-CD3/CD28 beads for three days.
4.4.5 Correlations Between T Cell Expression of Parameters of Activation, Proliferation and Differentiation in Response to Quarter Saturated Anti-CD3/CD28 Beads

A correlation matrix was constructed to establish if there are relationships between the T cell expression of parameters of activation, differentiation and proliferation in response to the uniform, non-antigen-specific stimulation in the pre-vaccination NTC assay, see Fig. 4.8. The values given within the grid represent the Spearman’s rank correlation coefficient (Spearman’s rho, \( \rho \)), a nonparametric measure of statistical dependence between each set of measured parameters. A value of \( \rho = +1 \) indicates a perfect positive correlation (indicated by red on the colour mapping), \( +0.7 \) suggest a strong positive correlation (paler red), \( +0.5 \) a moderate positive correlation (deep pink), \( +0.3 \) a weak positive correlation (pale pink), and 0 indicates no correlation (white). The graphic illustrates that many of the markers are strongly (approaching perfectly) positively correlated, for example Ki67 expression by CD4+ T cells and CD4+ T cells percentage divided (\( \rho = 0.95, p<0.0001 \)); and Ki67 and PD-1hi expression by CD8+ T cells (\( \rho = 0.96, p<0.0001 \)). Presumably, in such cases where there is high correlation, one may hypothesise that the inter-individual variation is acting at a common step in the mechanism leading to those parameters being upregulated (as proposed in the schema in Fig. 4.9A and B). However, for other markers, there is little or no correlation within the participants for expression of parameters. For example, CD4+ T cell expression of
CD25hi or PD-1hi and CD4+ T cell differentiation into $T_{CM}$ ($\rho=0.09$, $p=0.14$ and $\rho=0.06$, $p=0.11$ respectively). Here it may be hypothesised that there may be inter-individual variation in a step which is specific for some parameters but not others (as proposed in the schema in Fig. 4.9C and D).
Figure 4.8 Correlation matrix of the measured T cell parameters of activation (CD25hi and PD-1hi), proliferation (Ki67, percentage divided, division index (DI), proliferation index (PI) and replication index (RI) and differentiation (to CCR7-CD45RA+ T central memory cells (T<sub>CM</sub>) and CCR7-CD45RA- T effector memory cells (T<sub>EM</sub>), given in rank order, in 104 participants following incubation of their naïve T cells (NTCs) with quarter saturated anti-CD3/CD28 beads for three days. Each numerical value and depth of shade represents the Spearman’s rank correlation coefficient (Spearman’s rho, ρ) between the two parameters.
Figure 4.9 Proposed schema for sites of inter-individual variation when naïve T cells (NTCs) are stimulated via the T cell receptor (TCR) and upregulate markers of activation and initiate differentiation and/or proliferation.
4.5 Discussion

The results of the first arm of the present study, in which phenotypic responses of the NTCs of individuals to standard, non-antigen-specific TCR engagement were quantified, add considerable new depth to current understanding regarding variation between the functionality of different individuals’ NTCs. As described in Section 1.4, this has hitherto only had a cursory examination in the literature. In the Patel study, CD8+ T cells from a small number of different donors were found to demonstrate between 1.2- to 100-fold increases in expression of functional markers in response to incubation with commercial anti-CD3/CD28 Dynabeads\textsuperscript{53}. Using the ImmVar cohort, Ye et al observed a high degree of reproducible, inter-individual variation between cytokine production and gene expression by CD4+ T cells in response to anti-CD3/CD28 stimulation. The study found variation between individuals in their general immune ‘responsiveness’, with a widespread correlation between all induced cytokines\textsuperscript{54}.

The findings presented in the current chapter further support that the NTCs of some individuals are inherently more responsive to stimulation than the NTCs of other individuals. The CD4+ and CD8+ NTCs of each individual generated unique, non-linear, dose-dependent response (expression of intranuclear Ki67, indicative of commitment to proliferation) to variable concentrations of surface-fixed anti-CD3 and anti-CD28, with differential maximal responses attained for the
given conditions. The greatest inter-individual variation for the given experimental conditions was achieved using the sub-optimal stimulation of quarter saturated anti-CD3/CD28 beads. Results were found to be reproducible and the measurable inter-individual variation surpassed any technical noise. In response to the given experimental conditions, there were unimodal and continual ranges of expression in all measured CD4+ and CD8+ T cell parameters of activation, proliferation and differentiation, with some parameters demonstrating more inter-individual variation than others. There were positive correlations between most of the NTC stimulation assay parameters suggestive that an individual’s NTCs will respond to non-antigen-specific stimulation via the TCR by upregulating multiple modalities of incitement to comparable extents. Many of the pre-vaccination NTC stimulation assay parameters were strongly positively correlated. One may hypothesise that the inter-individual variation is acting at a common step in the mechanism leading to those parameters being upregulated. However, for other markers, there is less or no correlation within the participants for expression of those parameters. Here it may be hypothesised that there may be inter-individual variation in a step which is specific for some parameters but not others.

The present study has limitations. Given only two strengths of anti-CD3/CD28 beads were investigated in this series of experiments, more detailed dose-response profiles could not be generated. It is possible that in the approximately one-fifth to one-quarter of participants in which the maximal response measured
with fully saturated anti-CD3/CD28 beads) demonstrated <85% cells responsive is because in these participants the cells are not capable of greater maximal response (their maximal capacity for responsiveness is less than other participants) due to molecular or cellular factors. However, there are insufficient data to plot sigmoidal dose response curves with confidence. Further, it is plausible that experimental conditions might be further optimised (for example greater number of beads, longer incubation period, addition of exogenous IL-2) to increase the maximal response achieved by the participants in whom the apparent ceiling response to stimulation is <85% cells. Finally, it is plausible that in the less responsive individuals, an element of the PBMC preparation/freeze/thaw process was disruptive to their viable cell future function. Therefore, ideally, the assays would be repeated with a greater range of stimulation conditions to be able to construct dose-response curves to further define individual maximal responses and that in a proportion of participants the experiments are repeated on freeze/thawed PBMC from a separate bleeding point to ascertain true intra-individual reproducibility. However, the data obtained using the given experimental conditions are indicative that the NTCs of different healthy humans have variable biological capacities to generate non-linear, dose-dependent responses to standard stimulation, with the maximal achievable response lower in sizable minority of participants than in others.
Any variation in biological capacity between the NTCs of different individuals may be due to natural variability in an aspect or aspects of the T cell intracellular pathways. Any hypothesised model of the responsible component of an intracellular pathway determining the variation in NTC phenotypic functionality may be validated by undertaking the same NTC stimulation assay either *in vitro* with chemical blockade of the proposed pathway component or by using cells harvested from an animal genetic knock-out model or human with a monogenetic immunodeficiency specific for the candidate pathways.

The next chapter will examine if there is measurable, reproducible, inter-individual variation in T cell activation, cytokine production and proliferative response to cognate antigen (PPD) following an *in vivo* priming event (BCG-vaccination) in the same participants. This is with the aim of subsequently examining if there are relationships between how an individual’s NTCs respond to standard, non-antigen-specific stimulation and aspects of ‘trained’ immune response to secondary exposure to antigen. An immunological deconstruction of mechanisms responsible for the immunogenicity of a vaccine which provides variable protection in recipients would provide invaluable insights for the empirical design of future, improved vaccines.
Chapter 5 Inter-Individual Variation in Adaptive T Cell Response Following Secondary Exposure to Antigen

5.1 Introduction

The preceding chapter documented the characteristics of inter-individual variation in NTC phenotypic responses to standard, non-antigen-specific stimulation. This present chapter seeks to characterise inter-individual variation in vaccine-primed T cell responses to cognate antigen in the same participants. This is with the subsequent aim to establish if the pre-perturbation NTC baseline ‘responsiveness’ bears a relationship to vaccine immunogenicity. The present study hypothesises that there are intrinsic biological differences between the NTCs of healthy individuals, which is a determinant of their responsiveness to primary challenge and resultant differentiation, and therefore aspects of ‘trained’ immune response to secondary exposure to antigen.

As already described in depth in the introductory chapter, it is already known that there is considerable inter-individual variation in the response to BCG-vaccination in humans, both in terms of its immunogenicity and protection afforded against tuberculous disease. The variable response to BCG has been documented both between human vaccine trials in different country settings, but also within match case control studies, and remains much discussed but hitherto unexplained\textsuperscript{88,105,107,118}. An improved understanding of the T cell contribution to
variable BCG vaccine immunogenicity may aid the development of the next generation of vaccines against tuberculosis, especially given many candidate vaccines have been designed to boost the protection provided by BCG\textsuperscript{204}.

The second arm of the present study seeks to quantify the inter-individual variation in T cell priming by BCG vaccination in healthy, UK-resident adults using an assortment of surrogate activation, cytokine and proliferation markers for vaccine potency. As reviewed in the introductory chapter, these immunological parameters are not without limitations, but have been adopted by the vaccine community as standard parameters for BCG immunogenicity, have been validated experimentally and/or are biologically plausible for gauging BCG-response. As outlined in full in the materials and methods chapter, three separate tissue culture assays were developed in which the eight-week, post-vaccine frozen-thawed PBMC from the 107 participants were incubated with PPD for variable durations. These were subsequently stained and examined for activation induced markers, cytokines and measures of proliferation using multichromatic flow cytometry to construct multiparametric matrices of BCG-primed T cell responsiveness to cognate antigen, listed in Table 5.1.
Table 5.1 Parameters measured using multichromatic flow cytometry in the current study to construct multiparametric matrices of BCG-primed T cell responsiveness to PPD.

<table>
<thead>
<tr>
<th>Phenotypic response</th>
<th>T cell subtype</th>
<th>Measured parameters</th>
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</thead>
<tbody>
<tr>
<td>Early activation (activation induced markers, AIM)</td>
<td>CD4+</td>
<td>OX40 &amp; CD25</td>
</tr>
<tr>
<td></td>
<td>CD4+</td>
<td>CD40L</td>
</tr>
<tr>
<td></td>
<td>CD8+</td>
<td>4-1BB &amp; CD69</td>
</tr>
<tr>
<td>Late activation</td>
<td>CD4+ &amp; CD8+</td>
<td>PD-1</td>
</tr>
<tr>
<td>Cytokine production</td>
<td>CD4+</td>
<td>IFN-γ</td>
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<td></td>
<td>CD4+</td>
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<td></td>
<td>CD4+</td>
<td>IL-2</td>
</tr>
<tr>
<td>Proliferation</td>
<td>CD4+ &amp; CD8+</td>
<td>Ki67</td>
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<tr>
<td></td>
<td>CD4+ &amp; CD8+</td>
<td>Tracking dye dilution</td>
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5.2 Hypotheses

(1) There is inter-individual variation in pre-primed T-cell functional cellular response to antigen.

(2) An individual’s NTC response to non-specific TCR stimulation bears relationships to their pre-primed T-cell response to antigen.

5.3 Objectives

(1) To establish the range of inter-individual variation of Ki67 expression in post-BCG PBMC CD4+ T cells of 107 study participants following incubation with PPD and identify the contribution of technical noise to intra-individual variation.

(2) To establish the range of inter-individual variation in additional parameters of activation, cytokines and proliferation in CD4+ and CD8+ T cells from vaccinated individuals in response to PPD stimulation.

(3) To establish the correlations between markers of activation, cytokines and proliferation expressed by post-BCG PBMC CD4+ and CD8+ T cells following PPD stimulation.

(4) To establish the correlations between markers of activation, differentiation and proliferation by pre-vaccination CD4+ and CD8+ NTCs in response to sub-optimal anti-CD3/CD28 stimulation and markers of activation, cytokines and
proliferation expressed by post-BCG PBMC CD4+ and CD8+ T cells following PPD stimulation in the same 107 participants.

5.4 Results

5.4.1 Expression of Ki67 by CD4+ T Cells Following PPD Stimulation

Varies Between BCG-Vaccinated Individuals

The proliferation & late activation assay was conducted over 12 experiments, with between eight and 16 participants tested in each experiment. In this assay, the thawed post-vaccination PBMC were cultured for five days without addition (negative control), with 10μg/ml PPD (in duplicate or triplicate repeat) and with commercial anti-CD3/CD28 Dynabeads (positive control) and subsequently stained using the panel shown in Fig. 2.5C. Cultures of PBMC were set up for all individuals for the proliferation & late activation assay, but only those in which the cell viability post PPD stimulation was >20% and for whom there was a satisfactory positive control (as was the case in 92/107 participants) were analysed further.

The results of the Ki67 expression by post-vaccination CD4+ T cells after stimulation with PPD are summarised in Fig. 5.1. There was a statistically highly significant difference between the expression of Ki67 by CD4+ T cell following
the incubation of participants’ PBMC with PPD when compared with that of the participants' unstimulated PBMC (Wilcoxon Signed Rank Test p<0.0001, n=92), see Fig. 5.1i.

Fig 5.1ii gives in participant rank order, the delta values of Ki67 expression by the participant CD4+ T cells (expression after incubation with PPD minus that in unstimulated PBMC). The figure illustrates both the unimodal distribution and wide range amongst individuals of CD4+ T cell Ki67 expression in response to cognate antigen. The blue dots represent the individual with the minimum delta CD4+ T cell Ki67 expression (0%, TCV038), the green dots represent the individual with the median delta CD4+ T cell Ki67 expression (12%, TCV093) and the red dots represent the maximum delta CD4+ T cell Ki67 expression (59%, TCV078). The median delta Ki67 expression in CD4+ T cells after five days incubation with PPD was 12% (IQR=16%), with a minimum value of 0% and maximum value of 59%. The CV of Ki67 expression in CD4+ T cells between all individuals in all experiments was 86%.
(ii) CD4+ Ki67+

Δ %marker+

Recruit UIN

(iii) CD4+ Ki67+

Frequency

Δ %marker+

r^2 = 0.80
Figure 5.1 Ki67 expression in the CD4+ T cells of 92 participants after incubation with PPD (i) Scatter plot of Ki67 expression in CD4+ T cells for each participant both unstimulated and after incubation with PPD, where individual dots represent the mean of duplicate or triplicate wells, the columns represent the overall participant medians and the error bars the IQR. The blue dots represent the individual with the minimum delta CD4+ T cell Ki67 expression (0%, TCV038), the green dots represent the individual with the median delta CD4+ T cell Ki67 expression (12%, TCV093) and the red dots represent the maximum delta CD4+ T cell Ki67 expression (59%, TCV078). (ii) Bar chart displays the delta value for each participant (mean of duplicate or triplicate wells with PPD stimulation minus the unstimulated well) in rank order. The blue cross represents the minimum value, the green bar represents the median value, the red bar represents the maximum value, the dashed cyan line represents the median, the magenta line the first quartile and the dashed orange line the third quartile. (iii) Frequency distribution histogram for delta Ki67 expression in CD4+ T cells in all participants, the fitted non-linear lognormal regression is illustrated and the R squared goodness of fit value is given.
5.4.2. Quantifying Sources of Variation in Ki67 Expression in Post-Bacillus Calmette–Guérin CD4+ T cells Following Incubation with Purified Protein Derivative

The broad variation in Ki67 expression in CD4+ T cells demonstrable in Fig. 5.1 may represent both biological variation and technical noise between the different participants’ measured PBMC response to PPD. As a means to estimate the relative contribution of these two sources of variation, a sub-sample of the participants (seven participants, 8%) was re-examined for CD4+ T cell expression of Ki67 in response to PPD in replicate cultures (same blood draw, but different aliquots of frozen cells thawed and established for culture on a later date). In Fig. 5.2, the data are again plotted in scatter plot and bar chart format (the latter with individuals in rank order) but now with experimental repeats for the sub-sample of seven participants included and delineated by a colour unique for each of the sub-sampled participants. The plots illustrate that each individual’s performance is largely preserved in repeat experiments, with the rank order of the response maintained in 6 out of 7 individuals.
Figure 5.2 (i) Scatter plot of Ki67 expression in CD4+ T cells for 92/107 participants in the proliferation & late activation assay, both unstimulated and following incubation with purified protein derivative (PPD) for five days. Individual dots represent the mean of duplicate or triplicate wells, the columns represent the all participant medians and the error bars the interquartile range (IQR). Seven participants are included in which the cultures were set up in duplicate on different days, indicated by pairs of uniquely coloured spots. (ii) Bar chart displays the delta value of Ki67 expression in CD4+ T cells for each participant (mean of duplicate or triplicate wells with PPD stimulation minus the unstimulated well) in rank order and the seven participants for whom there were replicate experiments are indicated by pairs of the same uniquely coloured bars. (iii) Graphic demonstrating the rank orders of the seven participants for the replicate experiments.
To further estimate the contribution of biological and technical sources of variation between the participants in the post-BCG CD4+ T cell expression of Ki67+ in response to PPD, the C_v’s were calculated for technical repeats in the same experiment; repeats of the same individual on different days; and between individuals, the results of which are summarised in Fig. 5.3.

For intra-individual, intra-experimental, technical replicate wells within the same experiment, the median C_v was 12% (IQR=16%), suggestive that there was low intra-individual technical noise within experiments.

For the seven participants in whom intra-individual, inter-experimental replicate cultures were performed, the CD4+ T cell expression of Ki67 had a median C_v of 22% (IQR=17%). There was no statistically significant difference between the intra-individual, intra-experimental and the intra-individual, inter-experimental coefficients of variation (Mann-Whitney U=260, n₁=83, n₂=7, p=0.66 two tailed), suggestive that all of the thawing of different aliquots on different days and the setting-up of different experiments did not contribute much to variability.

The modest, intra-individual coefficients of variation values are in contrast to the inter-individual C_v values. The inter-individual, intra-experimental CD4+ T cell Ki67 expression after incubation with PPD median C_v was 77% (C_v = standard deviation between individuals’ mean delta Ki67 expression/mean of all individuals’
delta Ki67 expression x 100%, IQR=20%, n=12 experiments). This inter-individual, intra-experimental CV was much higher than in either technical replicates (median CV=12%, Mann-Whitney U=12, n₁=83, n₂=12, p<0.0001, two tailed) or replicate experiments (median CV=22%, Mann-Whitney U=0, n₁=7, n₂=12, p<0.0001, two tailed).

Finally, the CV between all the different participants’ mean post-BCG CD4+ T cell expression of Ki67 following incubation with PPD (the inter-individual, all experiment variation) was calculated at 86%. As this final all experiment inter-individual CV represents a single value, it is not possible to use a nonparametric test to compare it to the other measures of CV. However, this value of CV=86% is outside of both the 99% confidence interval (CI) of the intra-individual, intra-experiment CV median (median=12%, 99% CI 9 to 17%) and the 99% CI of the intra-individual, inter-experiment CV median (median=22%, 99% CI 1 to 34%), and is therefore significantly different from both of these sets of values at at least p=0.01. The all experiment inter-individual CV at 86% falls within the 95% CI of the inter-individual, intra-experiment CV median (median=77%, 95% CI 60 to 86%) and so is not significantly different from these measures.
Fig 5.3 Coefficients of variation (CV) for post-Bacillus Calmette–Guérin (BCG) CD4+ T cell expression of Ki67 after incubation with PPD for intra-individual intra-experimental replicates (blue); intra-individual inter-experimental repeats (red); inter-individual intra-experimental comparisons (green); and all individuals in all experiments (purple). The columns show the medians and error bars the interquartile range, with individual values depicted by symbols.
5.4.3 Inter-Individual Variation in Post-Bacillus Calmette–Guérin T Cell Expression of Activation Markers After Incubation with Purified Protein Derivative

Further analysis was performed to ascertain the extent of inter-individual variation in expression of cellular markers of activation by CD4+ and CD8+ T cells after incubation with PPD, displayed in Fig. 5.4. Parameters of T cell activation in response to incubation with PPD were measured across the three post-vaccination assays: CD4+ AIM (OX40 and CD25 co-expression) and CD8+ AIM (4-1BB and CD69 co-expression) in the AIM assay; CD40L upregulation in the ICS assay; and PD-1 expression in the proliferation & late activation assay. Experimental conditions were as described in the Chapter 2. Due to measurable background expression of the parameters of interest in unstimulated PBMC in most individuals, delta values of expression (percentage of cells expressing the marker in wells incubated with PPD minus percentage of cells expressing the marker in unstimulated PBMC) are given as well as absolute values.

For the 24-hour AIM assay, viable PBMC were cultured for all 107 participants over ten experiments, with the inclusion of six to 16 participants per experiment. The upregulation of AIM (co-expression of OX40 and CD25) by CD4+ T cells in response to PPD is depicted in Fig. 5.4A. Upregulation of OX40+CD25+ co-
expression was highly significant in CD4+ T cells incubated with PPD when compared with unstimulated cells (Wilcoxon Signed Rank Test p<0.0001, n=107). The delta percentage of CD4+ T cells co-expressing AIM after incubation with PPD median was 0.47% (IQR 0.60%). In 8/107 (7%) participants there was negligible (<0.05%) up-regulation of OX40+CD25+ in CD4+ T cells in response to incubation to PPD.

Fig. 5.4B demonstrates the upregulation of AIM (co-expression of 4-1BB and CD69), by CD8+ T cells in response to incubation with PPD. Upregulation of 4-1BB+CD69+ co-expression was highly significant in CD8+ T cells incubated with PPD compared with unstimulated cells (Wilcoxon Signed Rank Test p<0.0001, n=107). The delta percentage of CD4+ T cells co-expressing AIM after incubation with PPD median was 0.44% (IQR 0.73%). In 15/107 (14%) CD8+ T cells expression of AIM+ (co-expression of 4-1BB and CD69) was <0.05%. In 3/107 (3%) participants, expression of AIM after incubation with PPD in both post-BCG CD4+ and CD8+ T cells was negligible.

For the ICS assay (18 hours incubation with PPD, with the addition of BFA for the final 16 hours), viable PBMC with a satisfactory positive control were achieved for 102 of the 107 participants over ten experiments in total, with the inclusion of six to 16 participants per experiment. The detection of CD40L in post-BCG CD4+ T cells in response to PPD is depicted in Fig. 5.4C. Upregulation of CD40L
was highly significant in CD4+ T cells incubated with PPD compared with unstimulated cells (Wilcoxon Signed Rank Test p<0.0001, n=102). The median delta percentage of CD4+ T cells expressing CD40L after incubation with PPD was 0.15% (IQR 0.16%). In 11/102 (11%), post-BCG CD4+ T cells upregulation of CD40L was negligible (<0.01%) after incubation with PPD.

For the proliferation & late activation assay (incubated over five days), viable PBMC with a satisfactory positive control were achieved for 92 of the 107 participants over 12 experiments in total, with six to 16 participants per experiment. Upregulation of PD-1hi was highly significant in post-BCG CD4+ T cells following incubation with PPD when compared with unstimulated cells (Wilcoxon Signed Rank Test p<0.0001, n=92) (Fig. 5.4D). The median delta percentage of post-BCG CD4+ T cells expressing PD-1hi after incubation with PPD was 9.0% (IQR 11%). In 3/92 (3%) participants there was negligible (<1%) up-regulation of PD-1hi in CD4+ T cells in response to incubation to PPD. Expression of PD-1hi in CD8+ T cells in response to PPD is depicted in Fig. 5.4E. Upregulation of PD-1hi was highly significant in CD8+ T cells incubated with PPD compared with unstimulated cells (Wilcoxon Signed Rank Test p<0.0001, n=92). The delta percentage of CD8+ T cells expressing PD-1hi after incubation with PPD median was 1.5% (IQR 1.8%). In 6/92 (7%) CD8+ T cell expression of PD-1hi was <0.1%.
Within each bar chart and scatter plot, participants are highlighted in whom the Ki67 expression in CD4+ T cells was at the minimum (participant TCV038 in blue), median Ki67 (TCV093 in green) and maximum Ki67 (TCV078 in red) to provide visual reference of how three participants performed relative to themselves and the other participants for the different parameters.

Amongst the participants, all the measured parameters of T cell activation were upregulated in a highly significant manner following incubation of post-BCG PBMC with PPD compared when compared to no stimulation. There were wide ranges amongst the individuals in the post-BCG expression of measured parameters of activation in response to PPD, with all exhibiting unimodal distributions. Of further note is that there were ‘non-responders’ where minority fractions of the participants failed to upregulate T cells parameters of activation in response to PPD despite being primed with BCG vaccine eight weeks earlier.
Figure 5.4 (i) Scatter plots, (ii) summary bar charts and (iii) corresponding frequency distribution histograms for post-Bacillus Calmette–Guérin (BCG) CD4+ and CD8+ T cell expression of parameters of activation by 92 to 107 participants in response to incubation with purified protein derivative (PPD). Figures are given for (A) CD4+ T cell upregulation of AIM+ (co-expression of OX40+CD25+); (B) CD8+ T cell upregulation of AIM+ (co-expression of 4-1BB+CD69+); (C) CD4+ T cell upregulated, intracellular CD40L; (D) CD4+ T cell expression of PD-1hi; and (E) CD8+ T cell expression of PD-1hi. Scatter plots display T cell expression of activation markers for participants, both unstimulated and after incubation with PPD. Individual dots represent the mean of duplicate or triplicate wells, the columns represent the all participant medians, and the error bars the interquartile range (IQR). Bar charts display delta values (mean of duplicate or triplicate wells with PPD stimulation minus the unstimulated well) for that parameter in rank order along the x-axis from least responsive to most responsive. Within each scatter plot and bar chart, participants are highlighted in whom the Ki67 expression in CD4+ T cells was at the minimum (participant TCV038 in blue), median Ki67 (TCV093 in green) and maximum Ki67 (TCV078 in red) to provide visual reference of how three participants performed relative to themselves and the other participants for the different parameters. In each bar chart the dashed cyan line provides the median for that parameter, the dashed magenta line the first quartile and the dashed orange line the third quartile. For
each frequency distribution histogram, the fitted non-linear lognormal regression is illustrated and the R squared goodness of fit value is given.
5.4.4 Inter-Individual Variation in Post-Bacillus Calmette–Guérin CD4+ T Cell Expression of Th1 cytokines Following Incubation with Purified Protein Derivative

Upregulation of Th1 cytokines by CD4+ T cells in response to incubation with PPD were measured in the ICS assay (18 hours incubation with PPD, with the addition of BFA for the final 16 hours) and are displayed in Fig. 5.5. Experimental conditions are described in Chapter 2. Viable PBMC with a satisfactory positive control were achieved for 102 of the 107 participants over ten experiments in total, with the inclusion of six to 16 participants per experiment. There was some measurable production of cytokines by unstimulated PBMC in most individuals, so delta values for expression above the negative control (% of cells with cytokine detected in PPD-stimulated PBMC minus % of cells with cytokine detected in unstimulated PBMC) are given, as well as absolute values.

The detection of IFN-γ by CD4+ T cells in response to PPD is depicted in Fig. 5.5A. Upregulation of IFN-γ was highly significant in post-BCG CD4+ T cells incubated with PPD when compared with unstimulated cells (Wilcoxon Signed Rank Test p<0.0001, n=102). The delta percentage of CD4+ T cells expressing IFN-γ after incubation with PPD median was 0.09% (IQR 0.12%). In 22/102 (22%), post-BCG CD4+ T cells upregulation of IFN-γ was negligible (<0.01%).
The detection of TNF-α by CD4+ T cells in response to PPD is depicted in Fig. 5.5B. Upregulation of TNF-α was highly significant in post-BCG CD4+ T cells incubated with PPD when compared with unstimulated cells (Wilcoxon Signed Rank Test p<0.0001, n=102). The delta percentage of CD4+ T cells expressing TNF-α after incubation with PPD median was 0.09% (IQR 0.10%). In 19/102 (19%), post-BCG CD4+ T cells upregulation of TNF-α was negligible (<0.01%) after incubation with PPD.

The detection of IL-2 by CD4+ T cells in response to PPD is depicted in Fig. 5.5C. Upregulation of IL-2 was highly significant in post-BCG CD4+ T cells incubated with PPD when compared with unstimulated cells (Wilcoxon Signed Rank Test p<0.0001, n=102). The delta percentage of CD4+ T cells expressing IL-2 after incubation with PPD median was 0.03% (IQR 0.06%). In 18/102 (18%), post-BCG CD4+ T cells upregulation of IL-2 was negligible (<0.001%) after incubation with PPD.

Again, within each scatter plot and bar chart, participants are highlighted in whom the CD4+Ki67+ expression was at the minimum (participant TCV038, in blue, median Ki67 (TCV093, in green) and maximum Ki67 (TCV078, in red) to provide visual reference of how three participants performed relative to themselves and the other participants for the different parameters.
Amongst the participants, the measured Th1 cytokines were upregulated in a highly significant manner following incubation of post-BCG PBMC with PPD compared when compared to no stimulation. There were wide ranges amongst the individuals in the post-BCG expression of measured parameters of activation in response to PPD, with all exhibiting unimodal distributions. There were ‘non-responders’ where minority fractions of the participants failed to upregulate T cells parameters of activation in response to PPD despite being primed with BCG vaccine eight weeks earlier.
(Ci) CD4+ IL-2+

(i) CD4+ IL2+

(ii) Recruit UIN

(iii) CD4+ IL2+

$\Delta \% \text{cytokine}^+$

$\Delta \% \text{marker}^+$

$F\%$

$r^2 = 0.32$
Figure 5.5 (i) Scatter plots, (ii) summary bar charts, (iii) and corresponding frequency distribution histograms for post- Bacillus Calmette–Guérin (BCG) CD4+ T cell upregulation of Th1 cytokines, which were measured intracellularly by 102 participants in response to incubation with purified protein derivative (PPD) for 18 hours (with the addition of Brefeldin A (BFA) for final 16 hours). Figures are given for expression of CD4+ T cell expression of (A) IFN-γ, (B) TNF-α and (C) IL-2. Scatter plots display T cell expression of intracellular cytokines for participants, both unstimulated and following incubation with PPD. Individual dots represent the mean of duplicate or triplicate wells, the columns represent the all participant medians, and the error bars the interquartile range (IQR). Bar charts display delta values (mean of duplicate or triplicate wells with PPD stimulation minus the unstimulated well) for that parameter in rank order along the x-axis from least responsive to most responsive. Again, within each scatter plot and bar chart, participants are highlighted in whom the CD4+Ki67+ expression was at the minimum (participant TCV038, in blue, median Ki67 (TCV093, in green) and maximum Ki67 (TCV078, in red) to provide visual reference of how three participants performed relative to themselves and the other participants for the different parameters. In each bar chart the dashed cyan line provides the median for that parameter, the dashed magenta line the first quartile and the dashed orange line the third quartile. For each frequency distribution histogram, the fitted non-linear lognormal regression is illustrated and the R squared goodness of fit value is given.
5.4.5 Inter-Individual Variation in Post-Bacillus Calmette–Guérin T Cell Proliferative Response to Incubation with Purified Protein Derivative

Post-BCG T cell proliferative response to incubation with PPD for five days was quantified in participants by their expression of intra-nuclear Ki67 and by modelling of proliferation derived from dilution of cell tracer labelling, displayed in Figs. 5.6 and 5.7. Experimental conditions are described in the Chapter 2. Viable PBMC with a satisfactory positive control were achieved for 92 of the 107 participants over twelve experiments in total, with the inclusion of six to 16 participants per experiment and CTV staining was adequate in 73 of those 92 participants for use in the proliferation modelling software. There was some measurable expression of Ki67 in the unstimulated PBMC of some individuals, so for this parameter delta values above the negative control are given as well as absolute values (% of cells with Ki67 detected in PPD-stimulated PBMC minus % of cells with Ki67 detected in unstimulated PBMC). For the cell trace model-generated data, absolute values are given. There was often some modest background CTV dilution in the negative controls, but since the proliferation parameters are derived from the data by a model-based process, the parameters from negative wells could not simply be subtracted from the parameters of the positives.
The detection of Ki67 by post-BCG CD4+ T cells in response to PPD is presented in Fig. 5.6A. Upregulation of Ki67 was highly significant in post-BCG CD4+ T cells incubated with PPD when compared with unstimulated cells (Wilcoxon Signed Rank Test p<0.0001, n=92). The median delta percentage of post-BCG CD4+ T cells expressing Ki67 after incubation with PPD was 12% (IQR 16%). In 4/92 (4%) individuals, the post-BCG CD4+ T cells upregulation of Ki67 following incubation with PPD was negligible (<1% of cells). The detection of Ki67 by post-BCG CD8+ T cells in response to PPD is depicted in Fig. 5.6B. Upregulation of Ki67 was highly significant in post-BCG CD8+ T cells incubated with PPD when compared with unstimulated cells (Wilcoxon Signed Rank Test p<0.0001, n=92). The median delta percentage of CD8+ T cells expressing Ki67 following incubation with PPD was 3% (IQR 4%). In 15/92 (16%) individuals, post-BCG CD8+ T cells upregulation of Ki67 following incubation with PPD was negligible (<1% of cells).

Within each scatter plot and bar chart, participants are highlighted in whom the CD4+Ki67+ expression was at the minimum (participant TCV038, in blue), median Ki67 (TCV093, in green) and maximum Ki67 (TCV078, in red) to provide visual reference of how three participants performed relative to themselves and the other participants for the different parameters.
Figure 5.6 (i) Scatter plots, (ii) summary bar charts, (iii) and corresponding frequency distribution histograms for post- Bacillus Calmette–Guérin (BCG) (A) CD4+ T cell and (B) CD8+ T cell upregulation of Ki67 in 92 participants in response to incubation with purified protein derivative (PPD) for five days. Scatter plots display percentage of each participant’s T cells with detectable Ki67, both unstimulated and after incubation with PPD. Individual dots represent the mean of duplicate or triplicate wells, the columns represent the all participant medians, and the error bars the interquartile range (IQR). Bar charts display delta values (mean of duplicate or triplicate wells with PPD stimulation minus the unstimulated well) for that parameter in rank order along the x-axis from least responsive to most responsive. Within each scatter plot and bar chart, participants are highlighted in whom the CD4+Ki67+ expression was at the minimum (participant TCV038, in blue), median Ki67 (TCV093, in green) and maximum Ki67 (TCV078, in red) to provide visual reference of how three participants performed relative to themselves and the other participants for the different parameters. In each bar chart the dashed cyan line provides the median for that parameter, the dashed magenta line the first quartile and the dashed orange line the third quartile. For each frequency distribution histogram, the best fit lognormal distribution is illustrated and the R squared goodness of fit value is given.
Fig. 5.7 presents a selection of proliferation modelling indexes data derived from the cell tracer dilution. Percentage Divided indicated the percentage of that subset of cells that entered division (same as precursor frequency); Division Index (DI), a measure of the average number of divisions undergone by the cells (including the undivided cells); Proliferation Index (PI), a measure of the average number of divisions by dividing cells (excludes the undivided cells); and Replication Index (RI), a measure of the fold-expansion of only the responding cells (excludes the undivided cells). Of the post-BCG CD4+ T cells following incubation with PPD, a median 3% had undergone division by day five (IQR 4%); the DI median was 0.05 (IQR 0.06); the PI median was 1.8 (IQR 0.8); and the RI median was 9.5 (IQR 10.0). With regard to the CD8+ T cells, a median 0.8% had undergone division by day five (IQR 1.0%); the DI median was 0.01 (IQR 0.03); PI median was 1.6 (IQR 0.6); and RI median was 5.5 (IQR 4.1).

Amongst the participants, Ki67, an intra-nuclear molecule signifying commitment to proliferation, was upregulated in a highly significant manner following incubation of post-BCG PBMC with PPD compared when compared to no stimulation, more so in CD4+ T cells than in CD8+ T cells. There were wide ranges amongst the individuals in the post-BCG T cell expression of Ki67 in response to PPD, with unimodal distributions. Of further note is that there were ‘non-responders’ where minority fractions of the participants failed to upregulate T cells parameters of activation in response to PPD despite being primed with BCG.
vaccine eight weeks earlier. There was observed variation between the different participants in the degree to which their post-BCG T cells responded to PPD as measured by the cell tracer-derived proliferation indexes, all exhibiting unimodal distributions.
Figure 5.7 (i) Summary bar charts, (ii) and corresponding frequency distribution histograms, (iii) for post-Bacillus Calmette–Guérin (BCG) T cell proliferative response to five days incubation with purified protein derivative (PPD) in 73 participants derived from cell tracer dilution. (A) CD4+ T cell percentage divided; (B) CD8+ T cell percentage divided; (C) CD4+ T cell Replication Index (RI); and (D) CD8+ T cell RI. Bar charts display absolute values (mean of duplicate or triplicate wells with PPD stimulation) for that parameter in rank order along the x-axis from least responsive to most responsive. Participants are delineated in whom the CD4+Ki67+ expression was at the minimum (participant TCV038, blue bars) and maximum Ki67 (TCV078, red bars). The participant with median CD4+Ki67+ expression (participant TCV93) is not illustrated in this figure as the CTV did not stain adequately in the experiment for this participant. In each bar chart the dashed cyan line provides the median for that parameter, the dashed magenta line the first quartile and the dashed orange line the third quartile. For each frequency distribution histogram, the best fit lognormal frequency distribution is illustrated and the R squared goodness of fit value is given.
Fig. 5.8 is a summary graph for the expression of the measured parameters by post-BCG CD4+ and CD8+ T cells in response to incubation with PPD to demonstrate the spectrum of responsiveness identified between the participants across the parameters.
Figure 5.8 Summary graphs for the post-Bacillus Calmette–Guérin (BCG) CD4+ and CD8+ T cells responses to incubation with purified protein derivative (PPD) with regard to expression of (A) activation induced markers (AIM), (B) intracellular cytokines (ICS) and (C) proliferation & late activation parameters. Each dot represents the expression of that parameter by an individual (mean of up to three replicate repeats). The black bars represent the all participant medians and error bars represent the interquartile ranges (IQR).
5.4.6 Coefficients of Variation in Post-Bacillus Calmette–Guérin T Cell Expression of Activation Induced Markers, T Helper 1 Cell Cytokines and Proliferation & Late Activation Parameters in Response to Purified Protein Derivative

Fig. 5.9 provides a summary graph of the inter-individual C<sub>V</sub>’s, given in rank order, of the post-BCG T cell measured parameters (AIM, Th1 cytokines, proliferation & late activation) between the participants in response to incubation with PPD. For the given experimental conditions, all the measured parameters exhibited more than 20% variation between the participants, suggestive of inter-individual variation in all modalities of BCG-primed CD4+ and CD8+ T cell response to cognate antigen (PPD). The parameters with the greatest measured variation between participants (C<sub>V</sub>&gt;110%) were the upregulation of Th1 cytokines (C<sub>V</sub> for IL-2 181%; C<sub>V</sub> for IFN-γ 133%; and C<sub>V</sub> for TNFα 133%) and the cell tracer-derived DI (C<sub>V</sub> in CD4+ T cells 116% and in CD8+ T cells 193%).
Figure 5.9 Summary graph of the inter-individual coefficients of variation (Cv) of upregulation of activation induced markers (AIM, co-expression of OX40+CD25+ and expression of CD40L by CD4+ T cells, co-expression of 4-1BB+CD69+ by CD8+ T cells), intracellularly captured Th1 cytokines (IFN-γ, TNFα and IL-2), indexes of proliferation (upregulation of Ki67, percentage divided, division index (DI), proliferation index (PI) and replication index (RI) and a parameter of late activation (PD-1)), given in rank order, in 72 to 107 participants following incubation of post-Bacillus Calmette–Guérin (BCG) peripheral blood mononuclear cells (PBMC) with purified protein derivative (PPD) for 18 hours to five days.

5.4.7 Correlations Between the T Cell Upregulation of Activation Induced Markers, Th1 cytokines and Parameters of Proliferation & Late Activation and in Bacillus Calmette–Guérin-Primed T Cells in Response to Purified Protein Derivative

A correlation matrix was constructed to establish if there are relationships between the upregulation of AIM, Th1 cytokines and parameters of proliferation & late activation in BCG-primed T cells in response to incubation with cognate antigens (PPD), see Fig. 5.10. Once again, the values given within the grid represent the Spearman’s rho, a nonparametric measure of statistical dependence between each set of measured parameters. The figure illustrates that one pair of parameters
(CD4+ T cell expression of Ki67 and CD4+ T cell expression of PD-1) measured in the same assay (the proliferation & late activation assay) but not derived from the same measured data (i.e. not both derived from CTV detection) are strongly (approaching perfectly) positively correlated ($\rho=0.94$, <0.0001). Presumably, in such cases where there is such high correlation, one may hypothesise that the inter-individual variation is acting at a common step in the mechanism leading to those parameters being upregulated (as proposed in the schema Fig. 5.11A and B).

Further pairs of parameters had strong positive correlations (for example, CD4+ T cell co-expression of OX40+CD25+ and CD4+ T cell DI, $\rho=0.65$, <0.0001) and moderate positive correlations (for example CD4+ T cell co-expression of OX40+CD25+ and CD4+ T cell expression of PD-1, $\rho=0.55$, <0.0001; and CD4+ T cell co-expression of OX40+CD25+ and CD4+ T cell expression of Ki67, $\rho=0.54$, <0.0001). There were pairs of post-BCG T cell PPD-response parameters with only weakly positively correlation, for example CD4+ T cell upregulation of AIM (OX40+CD25+ co-expression) with the upregulation of Th1 cytokines (IFN-$\gamma$ $\rho=0.29$, p<0.001; TNF$\alpha$ $\rho=0.36$, p<0.0001; and IL-2 $\rho=0.21$, p<0.05). For the markers where the correlation is lower, one may hypothesise that in primed T cells encountering cognate antigen, there may be inter-individual variation in an intracellular pathway signalling step specific for one marker but not the other (Fig. 5.11C) or in two separate steps, each specific for their respective marker(s) (Fig. 5.11D).
Figure 5.10 Correlation matrix of activation induced markers (AIM), T helper 1 cells (Th1) cytokines and parameters of proliferation & late activation in Bacillus Calmette–Guérin BCG-primed T cells in response to incubation with cognate antigens (purified protein derivative, PPD). Each numerical value and depth of shade represents the Spearman’s rank correlation coefficient (Spearman’s rho) between the two parameters.
Figure 5.11 Proposed schema for sites of inter-individual variation when Bacillus Calmette–Guérin (BCG) primed T cells encounter cognate antigens (purified protein derivative, PPD) and upregulate markers of activation, generate cytokines and initiate proliferation.
5.4.8 Correlations Between the Pre-Vaccination and Post-Bacillus Calmette–Guérin Vaccination Parameters

A further correlation matrix was constructed (Fig. 5.12), as an initial approach to establish if there are relationships within the cohort between the parameters upregulated following non-specific anti-CD3/CD28 stimulation of pre-vaccination NTC and the parameters upregulated on subsequent PPD stimulation of post-BCG T cells. Should positive correlations be identified, it would be suggestive that more sophisticated modelling may identify pre-vaccination parameters predictive of BCG immunogenicity.

The initial univariate analysis is suggestive of relationships between pre-vaccination naïve T cell activation parameters and post-BCG immunogenicity. For example, there are weak correlations between pre-vaccination NTCs differentiation to T_{EM} cells following non-specific stimulation and subsequent post-BCG response to incubation with PPD CD4+ T cell upregulation of AIM (co-expression of OX40+CD25+) (r=0.29, p<0.01), CD4+ T cell upregulation of the late activation marker PD-1 (r=0.25, p<0.05) and CD4+ T cell upregulation of the proliferation marker Ki67 (r=0.21, p=0.06). However, the cursory inspection is also suggestive that some pre-vaccination parameters bear no relation to some other parameters generally considered as valuable in identifying response to BCG. For example, the
analysis did not substantiate associations between any of the NTC pre-vaccination parameters and subsequent post-BCG CD4+ T cell IFNγ response to PPD.
Figure 5.12 Correlation matrix between the parameters upregulated following non-specific anti-CD3/CD28 stimulation of pre-vaccination naïve T cells (NTCs) and the parameters upregulated on subsequent purified protein derivative (PPD) stimulation of post-Bacillus Calmette–Guérin (BCG) T cells. The values and depth of shade given within the grid represent the Spearman’s rank correlation coefficient (Spearman’s rho).
5.5 Discussion

In the second arm of the present study, PBMC was harvested from 107 young adults eight weeks post-BCG-vaccination, incubated with PPD and the phenotypic responses of the T cells quantified. The post-BCG assays were designed to measure the ‘strength’ of each participant’s T cell response to BCG-vaccination, through testing multiple parameters of the T cell adaptive immune response on secondary exposure to mycobacterial antigens. All of the measured T cell parameters of activation, Th1-cytokine production and proliferation in the post-BCG assays were upregulated in a highly significant manner following incubation of post-BCG PMBC with PPD.

The findings of this chapter are supportive that within the same-study cohort there is a measurable, unimodal, broad distribution of several parameters of the T cell response to BCG. The results are suggestive that BCG has a greater T cell immunogenicity in some young adults than in others. Some parameters demonstrated more inter-individual variation than others. Results were found to be reproducible and the measurable inter-individual variation surpassed any technical noise. There were variable ‘strengths’ of correlations between the post-vaccination parameters suggestive that inter-individual variation is occurring in steps unique to the parameters.
The data provides valuable additional information to the mycobacterial vaccine field. Prior studies have either focused on the variation in T cell response to non-mycobacterial vaccines (for example, to YF17D); have compared BCG efficacy between, rather than within, BCG trials (for example, in the Mangtani systematic review); have described inter-individual variation in immunogenicity within a BCG cohort utilising narrower range of parameters than is employed in the present study (for example, Finan et al described inter-individual variation Th1 and Th2-cytokine responses to mycobacterial antigens in infants after BCG-vaccination); or have examined inter-individual variation in BCG response in murine models\textsuperscript{16,17,19,88,118,124}.

One might speculate that variable BCG immunogenicity between individuals may be indicative of BCG efficacy in those recipients, but such conjecture would remain speculative without further studies such as concordance of immunogenicity and the ability of those cells to limit \textit{in vitro} mycobacterial growth or prevent \textit{in vivo} tubercular disease. The assays cannot represent definitive measures of protection against mycobacterial diseases, as there is currently no proven gold-standard measure of BCG efficacy. However, the present study utilised multiple parameters which are both supported by previously published evidence and are biologically plausible in an effort to measure BCG-response in lieu of a gold-standard measure of vaccine efficacy.
A preliminary univariate analysis was undertaken to explore if there were relationships between the parameters upregulated following non-specific anti-CD3/CD28 stimulation of pre-vaccination NTC and the parameters upregulated on subsequent PPD stimulation of post-BCG T cells in the same participants. It is suggestive that some pre-vaccination parameters bear relation to parameters of post-BCG immunogenicity. The one pre-vaccination and three post-BCG assays have generated thousands of data points for approximately a hundred participants. A future study is planned to incorporate these data into a multivariate model, to ascertain if there are pre-vaccination NTC parameters predicative of BCG T cell immunogenicity.

The present study has limitations. It was not comprehensively excluded that, prior to their BCG-vaccination in the study, the participants were all immunologically naïve to mycobacteria and had not had previous sensitisation to the genus, which may have had an impact on measured T cell responses in the post-vaccination assays. Extensive effort was made to reduce the risk of recruiting individuals with previous BCG and TB sensitisation, through the seeking of a history of previous BCG, TB or TB treatment, including asking participants to check with their families, GPs and health records; checking for previous BCG scars prior to vaccination; and through performing QuantiFERON testing. Definitive confirmation of any prior sensitisation to mycobacteria could have been made by performing enzyme-linked immunosorbent assays (ELISAs) to PPD (akin to the
TB ELISpot) on pre-vaccination PBMCs and any participant with a pre-BCG IFNγ-response to PPD, excluded from further analysis.

Further limitations also include that the measurements made do not alone reflect how ‘well’ participants’ T-cells have been primed by BCG-vaccination, as each individual’s T cells will have different proliferative abilities and variations in activation-induced death, all of which will contribute to measured outcomes. In addition, T cells are not the only variable determinant of protective immunity to mycobacteria within the cultured PBMC. Results will have also been influenced by, for example, the number and functional capacity of each individual’s antigen presenting cells to process and present antigen to the T cells. Some of the measured variation will reflect inter-individual variation between the non-T-cell components of PBMC, so any signal from T-cell variation will have to be of sufficient signal to be detected by the modelling. A further potential confounder is that different formulations of Tuberculin PPD with different potencies are available and they do not represent a pure, homologous antigen but contain variable combination of proteins isolated from culture media filtrates of a human strain of *Mycobacterium tuberculosis*²⁰⁵. However, for the present study all PPD was sourced from the same batch and vial.
In the next chapter, the final results chapter, high-throughput molecular TCR-sequencing is utilised to seek both qualitative and quantitative T cell response to vaccination with BCG.
Chapter 6 Characterisation of Peripheral Blood T Cell Receptors Pre- and Post-Bacillus Calmette–Guérin Vaccination

6.1 Introduction

An additional dimension to the current study and presented in the current chapter is a set of experiments and analyses examining peripheral blood TCRs pre and post-BCG-vaccination. From a sub-group of 67 participants, TCRs from peripheral blood immediately prior to- and eight-weeks post-BCG-vaccination were PCR-amplified, sequenced and analysed by bioinformatic pathways using TCR-annotation software. These data were explored to identify the dynamic impact of that perturbation on the host’s TCR repertoire171.

The TCR α- and β-chains have three hypervariable regions: complementary determining regions 1, 2 and 3 (CDR1, 2 and 3). The CDR3 regions of TCR α- and β-chains display the highest variability of the TCR and are the regions which come into closest contact with epitopes and are therefore the focus for these analyses206. Two unrelated, healthy individuals typically share only approximately 1-2% per cent of their TCR repertoire35–37. Homology in sequences of the CDR3 region of different T-cell clones found both within an individual and between individuals are likely to be cognizant of the same antigen35,159,173,207. β-chain sequences display more diversity between individuals than α-chains, hence TCR
analyses focus primarily on $\beta$-chain findings. If, post-immunological challenge, a set of TCRs are found to have expanded in number and which share homology (cluster) within and between individuals, those TCRs are likely to be reactive for that perturbing antigen(s).

Short motifs of amino acids in the CDR3 have been shown to play a role in determining the antigen specificity of a TCR. Identifying homologous clusters of similar CDR3s is a useful tool in characterising an individual’s immune response to antigen. Although there are several other metrics of TCR similarity in the literature (for example, TCRdist, which combines local and global similarity distance metrics), amino acid triplet sharing between CDR3 regions has been used extensively in our research group, as it is reflects the importance of small amino acid motifs in antigen recognition. Clustering algorithms (and others which are similar) sort TCR $\alpha$- or $\beta$-chains with a high likelihood of a shared specificity on account of conserved motifs and overall conformity of the CDR3 sequences. In the present study, participant TCR CDR3 sequences were divided into amino acid triplets and submitted to pairwise comparisons for sharing, in order that those with similar sequences be grouped together. This metric, called a triplet kernel, and ranges from 0 (no similarity) to 1.0 (identity). Sequences are represented by vertices on the clustering diagram, and the sequences that share a pre-determined level of similarity are connected by an edge.
A lower triplet kernel homology tends to be employed for clustering analyses between individuals compared to when looking for clustering within individuals as TCRs tend to be more homologous within individuals than between them. In the following analyses, ≥0.85 triplet kernel homology was used as the threshold for within individual clustering and ≥0.7 for between individuals. The similarity threshold is decided by comparison with an appropriate control set, accounting for the background similarity level.\(^{174}\)

In TCR repertoire analyses, antigen-reactive TCRs may be identified both in the TCRs found to have expanded post-perturbation, but also in TCRs not found to have expanded but which cluster with expanded TCRs. Antigens are complex and T cell responses to them are polyclonal. When TCRs are sequenced from biological samples such as peripheral blood, only a small number (perhaps 50,000 to 100,000 TCRs) of the entire repertoire is identified. Therefore, such sampling is only a representation of, rather than a catalogue of, the entire repertoire. It is therefore possible that antigen-specific T cells have expanded in the circulation in response to a challenge but only one of that clone and TCR is identified in the sampling. Any clustering analyses using expanded TCRs only is unlikely to be sufficient to demonstrate meaningful clustering. Should TCRs be found to be present and not expanded in the sample but exhibit clustering, it is possible that they have expanded overall in the host and are antigen-specific. Therefore, in such analyses, it is conventional to still include the ‘neighbourhood’ of the expanded
TCRs (the TCRs which have not expanded but share sequence homology with those that have expanded) in analyses and to contrast this with control cluster plots from the same pool. If the clustering is more extensive in the former than the latter, it is suggestive of antigen-specific TCR-response\textsuperscript{174}.

Should there be an expansion of TCRs detectable in peripheral blood in post-BCG peripheral blood compared with pre-vaccination peripheral blood and should these expanded TCRs cluster, including between individuals and including with TCRs from the same pool not found to have expanded, this would be suggestive of their being BCG-specific. As a validation exercise, further clustering analyses were performed to ascertain if TCRs found in participants post-BCG were similar to TCRs identified as specific for Mtb-epitopes in public catalogues (for example confirmed as Mtb-specific using tetramer-sorting experiments).

The pre- and post-BCG TCR data are of interest and importance on their own merit, as the analyses seek to identify, in principle, if peripheral blood may be utilised to identify antigen-specific host T cell response signatures to vaccination. Further, these data are pertinent to the present study as they too may be incorporated into any multivariable model developed to seek pre-vaccination parameters predictive of BCG-immunogenicity.

6.2 Hypotheses
It is hypothesised that there is expansion of TCR CDR3 sequences post-BCG that significantly exceed sampling variation, cluster together at random, including with non-expanded TCRs and show significant overlap with published Mtb-reactive TCRs.

6.3 Objectives

To identify a set of BCG-antigen specific TCRs by
(1) Identifying TCRs which have expanded in the peripheral blood of 67 individuals post-BCG-vaccination;
(2) Identifying TCR homology within and between the post-BCG-vaccination TCRs of 67 individuals;
(3) Identifying homology between the post-BCG-vaccination TCRs of 67 individuals and public Mtb-annotated TCRs.

6.4 Results

RNA extractions from peripheral blood samples were performed serially for participants TCV001 to TCV082. For the 68/82 participants in whom at least 500ng RNA was obtained in pre-and post-vaccination samples on quantification by Qubit, TCR libraries were generated and sequenced across four different NextSeq
runs (as described in detail in Chapter 2). The Fastq files were processed to produce annotated TCR files using the Decombinator package. One participant was removed from further analysis due to failure at the sequencing step and so 67 participants were included in the final analysis.

6.4.1 Expansion of Peripheral Blood T Cell Clones Between Pre- and Post-Bacillus Calmette–Guérin Vaccination

The population of expanded TCR α- and β-chains for each individual in post-BCG blood, when compared with pre-BCG blood, was defined as follows. Since the total number of TCR α- and β-chains was variable between participants, the abundance of TCRs were normalised per sample as counts per million. TCR α- and β-chains were considered expanded (or contracted) if the probability of the observed abundance in the post vaccination sample, given its abundance in the pre-vaccination sample, could have occurred by chance was p<0.0001 using a Poisson distribution model. TCR α- and β-chains which were absent pre-vaccination, but detected post-vaccination, were assumed to have been missed in the pre-vaccination samples and assigned an abundance of 1 (rather than 0) pre-vaccination, which was the median abundance of TCR within the samples.

Variability was identified amongst the participants between the numbers of TCR α- and β-chains found to have expanded between normalised pre- and post-BCG
samples. Fig. 6.1 gives example scatter plots for three individual participants, depicting expansion and contraction for their α- and β-chains between sample-taking. Fig 6.2 gives summary plots for the number of expanded α- and β-chains for each individual in the post-BCG samples from the pre-BCG samples following normalisation. The number of TCR β-chains which had expanded from pre- to post-vaccination in each individual ranged from 1 to 237 β-chains (median 14 β-chains, IQR 19 β-chains). These expanded β-chains represented 0.002 to 0.32% of the individual’s total sampled post-vaccination TCR β-chains (median 0.027%, IQR 0.030%). The number of TCR α-chains which had expanded from pre- to post-vaccination in each individual ranged from 0 to 219 α-chains (median 10 α-chains, IQR 12 α-chains). These expanded α-chains represented 0 to 0.44% of the individual’s total post-vaccination sampled TCR α-chains (median 0.026%, IQR 0.025%). Fig. 6.3 gives a correlation plot for the number of β-chains versus α-chains expanded in each individual in the post-BCG samples from pre-BCG samples, where there was a strong positive correlation between the values, Spearman’s rho ρ=0.82, p<0.0001, n=67 pairs.
Figure 6.1 Scatter plots to depict the expansion and contraction of TCR $\alpha$- and $\beta$-chain between pre- and post-BCG-vaccination peripheral blood samples in three example individuals. Each dot represents the frequency of a particular TCR $\alpha$- or $\beta$-chain when numbers have been normalised in the pre-vaccination and post-vaccination samples for that individual. The $x$-axis presents the normalised count of a TCR in the pre-vaccination samples. The $y$-axis presents the normalised count of a TCR in the post-vaccination samples. For any example in which equal numbers of a particular TCR $\alpha$- or $\beta$-chain were identified in both pre- and post-vaccination samples (without change in its abundance between samples), the dot falls on the diagonal $x=y$. TCR $\alpha$- and $\beta$-chains were deemed expanded or contracted in accordance with a Poisson distribution model if the probability of the observed abundance in the post vaccination sample, given its abundance in the pre-vaccination sample, could have occurred by chance was $p<0.0001$. The dashed blue lines represent the Poisson distributions: the TCRs that did not change in abundance significantly by this definition fall within the two blue lines; any dots below the lower right blue lines represent TCR $\alpha$- or $\beta$-chains present in the pre-vaccination sample and then significantly contracted in the post-vaccination sample; any dots above the upper left blue lines represent TCR $\alpha$- or $\beta$-chains present in the post-vaccination sample significantly expanded from the pre-vaccination sample. Examples of both TCR $\alpha$- and $\beta$-chains expansion/retraction are given from three participants: (A) TCV078, the individual with the least
measured β-chain expansion from normalised pre- to post-vaccination blood samples (1 expanded β-chain, 0.003% of the sample); (B) TCV038, the individual with the median measured β-chain expansion from normalised pre- to post-vaccination blood samples (14 expanded β-chains, 0.034% of the sample); and (C) TCV047, an individual with a large number of expanded β-chains in post-vaccination samples when compared to pre-vaccination samples (187 expanded β-chains, 0.32% of the sample).
Figure 6.2 Summary plots of the normalised number of expanded T cell receptors (TCRs) post-Bacillus Calmette–Guérin (BCG) vaccination for each individual (i) β-chains and (ii) α-chains
Figure 6.3 A correlation plot for the number of $\beta$-chains versus $\alpha$-chains expanded in each individual after normalisation from pre- to post-Bacillus Calmette–Guérin (BCG) peripheral blood samples.
6.4.2 Individual TCR Clustering Analyses

In the first set of clustering analyses, TCR clustering within individual participants was examined. In each individual, the TCRs determined to have expanded by Poisson analyses post-BCG-vaccination were combined with 1500 further TCR α- or β-chains from each of the 67 individual’s own post-vaccination samples. These additional 1500 TCRs had not expanded, but the highest degree of similarity of triplet kernels to those which had expanded and these were used as ‘bait’ for the cluster plot. This option was selected instead of including all of the TCRs in the plots, which visually would have made them indecipherable. Further included in the same analyses were 1000 of approximately 5000 α- and β-CDR3 sequences previously annotated in the manually curated databases such as McPAS-TCR as specific for Mtb-epitopes, selecting those most similar to the TCRs identified in the study participants\textsuperscript{196,197}. The clustering data are summarised in Table 6.1 and selective summary plots are presented in Fig. 6.4. To aid with visualisation of TCR clustering within individual participants, clustering diagrams were generated for both α- and β-chains in each individual with cluster lines (edges) connecting homologous TCRs (nodes).
Table 6.1 Summary table of key results from the individual post-Bacillus Calmette–Guérin (BCG) T cell receptor (TCR) clustering analyses (i) $\beta$-chains and (ii) $\alpha$-chains.

<table>
<thead>
<tr>
<th></th>
<th>Minimum Value</th>
<th>Median value</th>
<th>Maximum Value</th>
<th>Interquartile range (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(i)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total no. of $\beta$-chains clustering</td>
<td>53 [TCV016]</td>
<td>216 [TCV030]</td>
<td>420 [TCV060]</td>
<td>86</td>
</tr>
<tr>
<td>Total no. of $\beta$-chain clusters</td>
<td>24 [TCV016]</td>
<td>79 [4 recruits]</td>
<td>115 [TCV060]</td>
<td>32</td>
</tr>
<tr>
<td>Max. no. of $\beta$-chains in a cluster</td>
<td>3 [TCV016]</td>
<td>11 [4 recruits]</td>
<td>44 [TCV072]</td>
<td>13</td>
</tr>
<tr>
<td>Max. no. of $\beta$-chains clustering to a single $\beta$-chain</td>
<td>2 [TCV016]</td>
<td>6 [6 recruits]</td>
<td>16 [TCV060]</td>
<td>4</td>
</tr>
<tr>
<td>No. of expanded $\beta$-chains incorporated in clusters</td>
<td>0 [20 recruits]</td>
<td>1 [18 recruits]</td>
<td>36 [TCV068]</td>
<td>3</td>
</tr>
<tr>
<td><strong>(ii)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total no. of $\alpha$-chains clustering</td>
<td>167 [TCV044]</td>
<td>319 [TCV055]</td>
<td>508 [TCV072]</td>
<td>132</td>
</tr>
<tr>
<td>Total no. of $\alpha$-chain clusters</td>
<td>65 [TCV082]</td>
<td>111 [4 recruits]</td>
<td>152 [TCV057]</td>
<td>21</td>
</tr>
<tr>
<td>Max. no. of $\alpha$-chains in a cluster</td>
<td>5 [TCV026]</td>
<td>17 [TCV046]</td>
<td>156 [TCV042]</td>
<td>21</td>
</tr>
<tr>
<td>Max. no. of $\alpha$-chains clustering to a single $\alpha$-chain</td>
<td>3 [TCV044]</td>
<td>8 [7 recruits]</td>
<td>71 [TCV021]</td>
<td>8</td>
</tr>
<tr>
<td>No. of expanded $\alpha$-chains incorporated in clusters</td>
<td>0 [12 recruits]</td>
<td>2 [7 recruits]</td>
<td>60 [TCV068]</td>
<td>4</td>
</tr>
</tbody>
</table>
Figure 6.4 Summary plots for the individual pre-vaccination to post-Bacillus Calmette–Guérin (BCG) T cell receptor (TCR) clustering analyses for (A) $\beta$-chains and (B) $\alpha$-chains for (i) total number of CDR3s clustering and (ii) total number of CDR3 clusters for each participant. Participants are highlighted in whom the total number of $\beta$-chains clustering was at the minimum (participant TCV016, blue bars), median (TCV030, green bars) and maximum (TCV060, red bars).
The TCRs identified as expanded in the post-vaccination samples demonstrated the characteristics one would expect of antigen response, with clustering in many of the participants. However, there was a large degree of variability in the individual response as measured by the clustering metrics and in some participants no expanded TCRs were found to cluster. For example, the total number of TCRs clustering in each individual for the given clustering analyses ranged from 53 to 420 β-chains (median 216 β-chains, IQR 86 β-chains) and 167 to 508 α-chains (median 319 α-chains, IQR 132 α-chains). The total number of clusters for the given analyses ranged from 24 to 115 clusters (median 79 clusters, IQR 32 clusters) in β-chains and 65 to 152 clusters (median 111 clusters, IQR 21 clusters) in α-chains. In 20 of the 67 participants, no expanded β-chains post-vaccination demonstrated clustering. In 10 of the 67 participants, no expanded α-chains post-vaccination demonstrated clustering. By way of contrast, controls showed a significantly lower degree of clustering at the same similarity threshold, producing fewer and smaller clusters than was seen in the seen in the post-vaccination expanded-TCR analysis. The wide ranges in clustering metrics found between individuals may be reflective of a variable range of immunogenicity to BCG in different individuals including cases of non-response (akin to the findings of the previous chapter where inter-individual variation in functional cellular responses to PPD by post-BCG T cells was described).
In all of the participants, there were post-BCG β-chains which were not necessarily found to have expanded, but clustered with TCRs previously annotated as Mtb-specific. In five individuals there were up to three β-chains post-BCG identified which fulfilled all of: expanded, clustered and previously been annotated as Mtb-reactive.

Fig. 6.5 gives examples of individuals’ clustering plots for three of the 67 participants’ α- and β-chains. Each TCR (node) appears on a plot if it clustered with at least one other TCR. TCR (nodes) were connected by cluster lines (edges) if the sequences shared ≥0.85 homology. Controls using random samples of 1500 TCRs from the post-vaccination samples of each individual were performed to determine the threshold homology, examples of which are given in Fig. 6.6. Controls showed a significantly lower degree of clustering at the same similarity threshold, producing fewer and smaller clusters than was seen in the seen in the post-vaccination expanded-TCR analysis. For β-chains, each individual’s control post-vaccination clustering analysis demonstrated 2 to 10 clustered TCRs in total (median 4 clustered TCRs) and 1 to 5 clusters in total (median 2 clusters). For α-chains, each individual’s control post-vaccination clustering analysis demonstrated 2 to 24 clustered TCRs in total (median 11 clustered TCRs) and 1 to 12 clusters in total (median 5 clusters).
Figure 6.5 Individual clustering plots for three of the 67 participants’ T cell receptor (TCR) α- and β-chains. Examples given for the individuals with (A) the fewest (TCV016), (B) the median (TCV030) and (C) the greatest total number (TCV060) of β-chain CDR3 regions found to cluster in the analyses. Each TCR (node) appears on a plot if it clustered with at least one other TCR. TCRs (nodes) were connected by lines (edges) if the sequences clustered with ≥0.85 homology. Both the placement of nodes within a cluster plot and the distance between unconnected nodes is arbitrary. Nodes not connected by lines share <0.85 triplet homology. TCRs not knowingly previously annotated as recognising a TB epitope are depicted as a circle. TCRs previously annotated as recognising a TB epitope are depicted as a square. TCRs coloured grey indicate that the TCR was found in the individual’s post-vaccination sample but it was not found to be expanded in relation to the pre-vaccination sample. Magenta indicates that the TCR was in the expanded set. Yellow TCR were unobserved in our data set (but have been annotated to be specific for Mtb and cluster with TCRs sequenced in the present study’s participants).
Figure 6.6 Individual control clustering plots for the post-vaccination T cell receptor (TCR) β- and α-chains for the same three participants depicted in Fig. 6.5 (TCV016, TCV030 and TCV060) by way of comparison. Controls were derived using random samples of 1500 TCRs from the post-vaccination samples of each individual. Each TCR (node) appears on a plot if it clustered with at least one other TCR. TCRs (nodes) were connected by lines (edges) if the sequences clustered with ≥0.85 homology.
6.4.3 Pooled T cell receptor (TCR) Clustering Analyses

In the second clustering analyses, all the TCRs found to have expanded in the post-vaccination samples (from the pre-vaccination samples by the Poisson distribution) were combined to form a single set of TCRs. These pooled, expanded TCRs were then analysed for clustering, whilst keeping track from which individual each TCR originated, and making note of how many individuals shared any public TCRs. Plots for these analyses are given in Fig. 6.7. These plots do not include unobserved TCRs previously annotated as recognising TB epitopes. However, TCRs both observed and expanded were analysed to see if they themselves had previously been annotated as specific for Mtb. In this set of analyses, TCRs were deemed to cluster if the CDR3 regions shared ≥0.7 homology in ≥4 TCRs.
Figure 6.7 Cluster plots for all TCR (A) β-chains and (C) α-chains found to have expanded in the post-vaccination samples, when compared to pre-vaccination samples by the Poisson analyses, then combined to form a single set of TCRs. Each individual’s TCRs are represented by a unique colour. Each TCR (node) appears on a plot if it was observed as expanded in at least one participant and clustered with at least four other TCRs. TCRs (nodes) were connected by lines (edges) if the sequences clustered with ≥0.70 homology. Both the placement of nodes within a cluster plot and the distance between unconnected nodes is arbitrary. Nodes not connected by lines bare no relation to each other.

(B) and (D) are the same plots but now colour-coded differently. The colour of the circle or square represents the number of individuals who share that TCR. If only one individual has contributed those expanded TCRs to the clustering plot it is depicted in pale blue, two individuals in yellow, three in lilac, four in pink.
Using the described analyses, clustering was identified between the post-vaccination expanded TCRs of 42 of the participants for TCR β-chains and 61 of the participants for TCR α-chains, inclusive of sequences previously annotated as specific for Mtb. Some participants were more represented in the plots than others. Up to three individuals were found to share the same β-chain sequences and four individuals shared same α-chain sequences. This was inclusive of an Mtb-specific β-chain sequence shared by two participants and an Mtb-specific α-chain sequence shared by four participants. Control plots, given in Fig. 6.8, demonstrated significantly lower clustering.

The clustering and publicity demonstrated between the expanded TCRs of different individuals in post-vaccination is suggestive of a there being a peripheral blood, BCG-specific measurable TCR response. This is further supported by some of identified expanded, clustering and public sequences being previously the identified as specific for mycobacterial (Mtb) antigens.
Figure 6.8 Control plots for the combined clustering analyses for T cell receptors (TCRs) identified pre- and post-Bacillus Calmette–Guérin (BCG): (A) β-chains pre-vaccination (n=1827) (B) β-chains post-vaccination (n=1827) (C) α-chains pre-vaccination (n=1436 TCRs) and (D) α-chains post-vaccination (n=1436 TCRs). As in combined clustering analyses, TCRs were deemed to cluster if the CDR3 regions shared ≥0.7 homology in ≥4 TCRs,
6.4.4 Pooled TCR Clustering Analyses Incorporating Unobserved MTB-Specific TCRs

In the third set of clustering analyses, the TCRs found to have expanded in the post-vaccination samples compared to the pre-vaccination samples by Poisson distribution from all participants, were once again pooled together to form single sets of expanded TCRs. Once again, combined with these were 1000 external TCRs previously annotated as Mtb-specific in manually curated databases most similar with the pool of expanded TCRs\textsuperscript{196,197}. Clustering plots were then generated, see Fig. 6.9, with TCRs deemed to cluster if the CDR3 regions shared $\geq 0.78$ homology, requiring $\geq 4$ TCRs per node.

Using the described analyses, clustering was identified between the post-vaccination expanded TCRs of 65/67 participants and annotated Mtb-specific TCRs for both TCR $\beta$-chains and for TCR $\alpha$-chains. Some participants were more represented in the plots than others. Up to five individuals were found to share the same $\beta$-chain sequences which clustered with Mtb-specific sequences.

The clustering and publicity demonstrated between the expanded TCRs of different individuals post-BCG-vaccination and TCRs previously identified as Mtb-specific is suggestive of a TCR response to mycobacterial antigens detectable in peripheral blood eight-weeks post-BCG-vaccination.
Figure 6.9 Cluster plots for all T cell receptors (TCR) (A) β-chains and (C) α-chains found to have expanded in the post-Bacillus Calmette–Guérin (BCG) samples, when compared to pre-vaccination samples by the Poisson analyses, pooled to form a single set of TCRs and combined with the 1000 TCRs previously annotated as *Mycobacterium tuberculosis* (Mtb)-specific most similar with the pool of expanded TCRs in manually curated databases\textsuperscript{196,197}. Each individual’s TCRs are represented by a unique colour. TCRs unaobserved in the participants and annotated as Mtb-specific are represented by black squares. Each TCR (node) appears on a plot if it was observed as expanded in at least one participant and clustered with at least four other TCRs. TCRs (nodes) were connected by lines (edges) if the sequences clustered with ≥0.78 homology. Both the placement of nodes within a cluster plot and the distance between unconnected nodes is arbitrary. Nodes not connected by lines bare no relation to each other. (B) and (D) are the same plots but the nodes representation of participant TCRs are now colour-coded differently. The colour of the circle or square represents the number of participants who share that TCR.
6.5 Discussion

The peripheral blood TCRs were sequenced immediately prior to and eight-week post-BCG-vaccination and analysed for expansion, clustering and publicity (including with annotated Mtb-specific TCRs) in 67 participants. The set of experiments were undertaken to explore if there is a peripherally detectable TCR signature response to BCG vaccination and if this was more prominent in some participants than others.

The analyses are suggestive of peripheral blood detection of antigen response. The \( \alpha \)-chain TCRs in all analyses demonstrated more clustering than was seen in the \( \beta \)-chain TCRs as would be expected as \( \alpha \)-chains contain more homologous regions to each other than \( \beta \)-chains. It is well-established that TCR CDR3 region sequences which demonstrate similarity and cluster, including those found between individuals, share antigen-specificity properties and are likely cognizant for the same epitopes\(^{35,159,190}\). In the participants, who were unrelated and of unselected HLA phenotype, post-BCG expanded sequences generated sizeable clusters of homologous sequences, when compared to unselected samples, and were shared between multiple participants. Further, public-database Mtb-specific TCRs, experimentally validated to bind Mtb epitopes, were identified in and/or clustered with participant TCRs. The observations aresuggestive that the live mycobacterial BCG-vaccination induces a polyclonal population of T cells detectable in
peripheral blood eight-weeks post vaccination and bearing TCRs with the ability to recognise mycobacterial antigens. This is supportive of a peripherally detectable TCR signature response to BCG vaccination. High throughput sequencing processing techniques are relatively novel and therefore there have been few well-sized studies on the effects of vaccine challenges on TCRs. Literature searches performed for the present study were unable to identify published data on the human TCR repertoire in response to BCG-vaccination. To date, there has only been a small handful of studies published on TCR sequencing in the context of vaccination (including in five recipients of the hepatitis B vaccine and seven recipients of the yellow fever vaccine)\textsuperscript{169,170}.

A limitation of TCR sequencing and clustering analyses in general are that they represent sampling of the participants’ TCRs and do not catalogue the entire repertoire. If TCRs are found to have expanded in a post-perturbation sample when compared to a pre-perturbation sample, it is suggestive that those TCRs represent sizeable clones of T cells but it is possible that TCRs have expanded in the participant but those have been missed in the sampling. In this way, TCR sequencing is not a comprehensive insight into the antigen-response but instead is a manner of characterising and visualising it. A limitation of the presented TCR data is that TCR sequencing was not performed on a separate control group (the controls used were from within the participants by performing clustering analyses on their pre- or post-vaccination samples). Ideally the study should have included
controls not receiving an intervention (BCG-vaccination), with TCR sequencing performed eight weeks apart to compare expansion and clustering to those who were BCG-vaccinated. An alternative control clustering analyses could be performed by incorporating non-observed, annotated TCRs for an alternate, non-mycobacterial pathogen to which the participants would not have been expected to have pre-existing immunity, in order to compare these plots with those generated in the participants to Mtb-specific TCRs.

The results described in this present chapter may begin to further inform the understanding of the T cell mechanisms of action of the BCG-vaccination and also provide groundwork for future projects. There was variable demonstrable expansion and clustering of TCR clones from pre- to post-BCG between the participants. Individual participant expansion and clustering values generated by the TCR analyses could be incorporated into future multivariant systems vaccinology modelling to explore if they are correlates for BCG-immunogenicity or aid in the identification of pre-vaccination predictors of BCG-response.

Studies have previously demonstrated the ability to use TCRs to sort individuals into whether or not they have encountered a pathogen. For example, Emerson et al demonstrated CMV carriers may be identified from those uninfected based on the cumulative frequency of particular TCR β-chain CDR3 regions\textsuperscript{211}. The current study provides a proof of concept that that peripheral blood TCRs may be used to
identify BCG-induced signatures by which recipients might be sorted as vaccine-responders or non-responders, with the potential for the development of clinically-useful mycobacterial-vaccination response testing.

Ideal next steps to the work presented in the current chapter includes validating the TCRs identified as expanded using the in-house triplet kernel metric using an independently derived clustering algorithm, for example GLIPH\textsuperscript{159}. Further validation could be sought by experimentally examining if clustering occurs between TCRs sequenced from AIM-selected, FACS-sorted single cells expanded in vitro from incubation of post-BCG PBMC with mycobacterial antigens and the TCRs sequenced from post-BCG peripheral blood in the same individuals.
Chapter 7 Discussion

7.1 Introduction

The current project is concerned with variation between the immune systems of humans in health, investigating the contribution variation between host T cells may make to outcomes following antigenic challenge.

The attenuated *M. Bovis* BCG vaccination against tuberculosis, provokes a predominantly T cell response in the recipient\textsuperscript{136-142}. There is considerable inter-trial and inter-individual variation in BCG-induced protection afforded against TB and BCG-induced immunogenicity, which remains unexplained\textsuperscript{88,118}. An improved understanding of why BCG vaccine efficacy varies to such a great extent, including any T-cell component to this, would be critical to informing the development of the next generation of vaccines against tuberculosis\textsuperscript{128,129,130}.

In the present study, the possibility that inter-individual variation in T cell functionality may contribute towards variation in BCG immunogenicity is introduced. It is hypothesised that there is heterogeneity between individuals in both the NTC responsiveness to non-specific stimulation and in the ‘trained’ immune response of BCG-vaccine-primed T cells to secondary exposure to antigen and that relationships exist between the two.
The peripheral blood TCRs before and after BCG-vaccination are also sequenced and analysed to identify any BCG-vaccine-induced signature. The ultimate aim of the current study is to identify factors by which T cell vaccine design may be improved.

7.2 Study Findings

The present study quantified integral inter-individual variation in CD4+ and CD8+ NTC responsiveness to non-specific standard stimulation by using a multiparametric matrix of participant T cell response to NTC anti-CD3/CD28 stimulation assays. Each individual’s CD4+ and CD8+ NTCs generated a unique, non-linear, dose-dependent response in expression of intranuclear Ki67, (indicative of commitment to proliferation) to variable concentrations of surface-fixed anti-CD3 and anti-CD28, with differential maximal responses attained by different participants for the given conditions. The greatest inter-individual variation for the given experimental conditions was achieved using sub-optimal stimulation. Results were found to be reproducible and the measurable inter-individual variation surpassed any technical noise. Some parameters demonstrated more inter-individual variation than others. Many of the pre-vaccination NTC stimulation assay parameters were strongly positively correlated.
Prior studies have predominantly explored inter-individual variation between the immune subset composition of ‘healthy’ individuals. The current study transcends this and concerns itself with the variations between individuals in cellular phenotype and functionality in responses to antigenic challenge, which is commonly engendered in studies but infrequently commented upon. Here, the present study builds on the findings of Ye et al who observed a high degree of reproducible, inter-individual variation between cytokine production and gene expression by CD4+ T cells in response to anti-CD3/CD28 stimulation in the ImmVar cohort.

The BCG vaccination was administered to participants and then eight weeks later their immune response to PPD antigens was quantified. Thereby, the inter-individual variation in response of memory T-cells to cognate antigen to which they have been pre-primed was quantified. There is currently no gold standard measure of BCG immunogenicity. The present study utilised several indirect *in vitro* mycobacterial antigen T cell-stimulation assays to capture a comprehensive set of CD4+ and CD8+ T cell antigen-induced phenotypic attributes in BCG-vaccinated individuals, to construct a multi-dimensional profile of vaccine-immunogenicity in each subject. Each of the measured T cell parameters of activation, Th1-cytokine production and proliferation in the post-BCG assays were upregulated in a highly significant manner following incubation of post-BCG PMBC with PPD and the results are suggestive that BCG has a greater T cell immunogenicity in some young
adults than in others. Some parameters demonstrated more inter-individual variation than others. Results were found to be reproducible and the measurable inter-individual variation surpassed any technical noise. There were variable ‘strengths’ of correlations between the post-vaccination parameters suggestive that inter-individual variation is occurring in steps unique to the expression of each of the parameters.

The data provides valuable additional information to the mycobacterial vaccine field. Prior studies have either focused on the variation in T cell response to non-mycobacterial vaccines; have compared BCG efficacy between, rather than within, BCG trials; have described inter-individual variation in immunogenicity within a BCG cohort utilising narrower range of parameters than is employed in the present study; or have examined inter-individual variation in BCG response in murine models\textsuperscript{16,17,19,88,118,124}.

The TCR analyses are suggestive of peripheral blood detection of antigen response. In the participants, post-BCG expanded sequences generated sizeable clusters of homologous sequences, when compared to unselected samples, and were shared between multiple participants. TCR CDR3 region sequences which demonstrate similarity and cluster, including those found between individuals, share antigen-specificity properties and are likely cognizant for the same epitopes\textsuperscript{35,159,190}. Further, public-database Mtb-specific TCRs, experimentally
validated to bind Mtb epitopes, were identified in and/or clustered with participant TCRs. The observations are suggestive that the live mycobacterial BCG-vaccination induces a polyclonal population of T cells detectable in peripheral blood eight-weeks post-vaccination and bearing TCRs with the ability to recognise mycobacterial antigens. This is supportive of a peripherally detectable TCR signature response to BCG vaccination. To date, there have only been a small handful of studies published on TCR sequencing in the context of vaccination (including in five recipients of the hepatitis B vaccine and seven recipients of the yellow fever vaccine).^{169,170}

### 7.3 Limitations

The present study has limitations. Only two strengths of anti-CD3/CD28 beads were investigated in pre-vaccination NTC stimulation assays, generating only rudimentary dose-response profiles. Next, it is plausible that in the individuals found to be less responsive, an element of the PBMC preparation, freeze and thaw process was disruptive to their viable cell future function. Therefore, ideally, the assays would be repeated with a greater range of stimulant conditions to be able to construct dose-response curves to further define individual maximal responses and that in a proportion of participants the experiments are repeated on freeze/thawed PBMC from a separate bleeding point to ascertain temporal intra-individual reproducibility.
It was not comprehensively excluded that, prior to their BCG-vaccination in the study, the participants were all completely immunologically naïve to mycobacteria and definitive confirmation of this could have been made by performing ELISpots on pre-vaccination PBMCs and any participant with a pre-BCG IFNγ-response to PPD, excluded from further analysis. Further limitations also include that the post-vaccination assays do not alone reflect how ‘well’ participants’ T-cells have been primed by BCG-vaccination and some of the measured variation will reflect inter-individual variation between the non-T-cell components of PBMC. A further potential confounder is that PPD does not represent a pure, homologous antigen but contain variable combination of proteins isolated from culture media filtrates of a human strain of Mtb\textsuperscript{205}.

Limitations of the TCR data include that TCR sequencing was not performed on a separate unvaccinated control group. Further, control clustering analyses could have been performed by incorporating non-observed, annotated TCRs for an alternate, non-mycobacterial pathogen to which the participants would not have been expected to have pre-existing immunity, in order to compare these plots with those generated in the participants to Mtb-specific TCRs.

7.4 Future Work
A preliminary univariate analysis was undertaken to explore if there are relationships between the parameters upregulated following non-specific anti-CD3/CD28 stimulation of pre-vaccination NTC and the parameters upregulated on subsequent PPD stimulation of post-BCG T cells in the same participants. This initial analysis is suggestive that some pre-vaccination parameters bear relation to parameters of post-BCG immunogenicity. The one pre-vaccination and three post-BCG assays have generated thousands of data points for approximately a hundred participants. A future study might progress to incorporate these data into a multivariate model, to ascertain if there are pre-vaccination NTC parameters predicative of BCG T cell immunogenicity.

To experimentally investigate molecular pathways and epigenetic factors underlying inter-individual variation in phenotypic T cell responses to stimulation was beyond the scope of the current project. Any hypothesised model of the responsible component of an intracellular pathway determining the variation in NTC phenotypic functionality may be validated by undertaking the same NTC stimulation assay either in vitro with chemical blockade of the proposed pathway component or by using cells harvested from an animal genetic knock-out model or human with a monogenetic immunodeficiency specific for the candidate pathways.

The substantial variation demonstrated in the ‘immunotypes’ of humans and their immune system responses to challenges is considered to be the result of a vast
array of heritable and non-heritable host factors. Given appropriate ethics approval, the EDTA blood stored for the participants could be utilised to identify the genetic contributions to inter-individual variation in T cell responsiveness and BCG immunogenicity using genome-wide association studies (GWAS) and epigenetic analyses, such as DNA methylation analysis.

One might speculate that variable BCG immunogenicity between individuals may be indicative of BCG efficacy in those recipients, but such conjecture would remain speculative without further studies such as concordance of immunogenicity and the ability of those cells to limit in vitro mycobacterial growth or prevent in vivo tubercular disease. A research priority is to identify a gold standard measure of mycobacterial immunogenicity.

With regards to the TCR analyses, next steps would include comparing how the TCRs identified as expanded using the triplet kernel metric feature using an independently derived clustering algorithm (GLIPH). Further validation of the TCR could be through the identification if clustering occurs between TCRs sequenced from AIM-selected, FACS-sorted single cells expanded in vitro from post-BCG PBMC by mycobacterial antigens and the TCRs sequenced from post-BCG peripheral blood in the same individuals. Studies have previously demonstrated the ability to use TCRs repertoires to sort individuals into whether or not they have encountered a pathogen such as CMV$^{211}$. The current study
provides a proof of concept that peripheral blood TCRs may be used to identify BCG-induced signatures with the potential for the development of clinically-useful mycobacterial-vaccination response testing.

7.5 Concluding Remarks

Human T-cell responsiveness with regards to overall efficacy and functional bias may have diversified through selection following exposure of different ancestries to different types of pathogens. One may surmise that whilst patients born with monogenic deficiencies may present more overtly in early life, with increased risk of severe infections, more subtle immune variation may contribute towards otherwise seemingly healthy individuals producing noteworthy immune responses including sub-optimal response to vaccination. Characterisation of baseline inter-individual variation between healthy-individuals pre-pathogen or vaccine challenge may identify correlates for post-perturbation responses and unlock critical molecular and cellular pathways for targeted development of future therapeutics and preventative strategies.
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46. ligation prevents T cell activation.
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44. IL effects on costimulation and cytokine

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42. 36

41. T cell activation and development.

40. therapy (Review).

39. abundant CDR3 sequences that are associated with self

38. Nature

37. life shapes th

36. Related immunity.

35. cell antigen receptor genes and T

34. Nature


32. 2016;

31. 2015;

30. 2014;

29. 2013;

28. 2012;

27. 2011;

26. 2010;

25. 2009;

24. 2008;

23. 2007;

22. 2006;

21. 2005;

20. 2004;

19. 2003;

18. 2002;

17. 2001;

16. 2000;

15. 1999;

14. 1998;

13. 1997;

12. 1996;

11. 1995;

10. 1994;

9. 1993;

8. 1992;

7. 1991;

6. 1990;

5. 1989;

4. 1988;

3. 1987;

2. 1986;


Mora T, Walczak AM. Quantifying lymphocyte receptor diversity. bioRxiv 2016; 046870.


Chemnitz JM, Parry RV, Nichols KE, June CH, Riley JL. SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. J Immunol 2004; 173(2): 945-54.


166. Lee HW, Park SJ, Choi BK, Kim HH, Nam KO, Kwon BS. 4-1BB promotes the survival of CD8+ T lymphocytes by increasing expression of Bcl-xL and Bfl-1. J Immunol 2002; 169(9): 4882-8.
177. Tuberculosis: the green book, chapter 32. UK Health Security Agency; Published 20 March 2013, last updated 3 August 2018


