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**A Study Of Factors Underlying BCG Immunogenicity Differences  
Across Countries: The Influence Of DNA Methylation Patterns And  
Antigen Presenting Cells**

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Thesis submitted in accordance with the requirements for the degree of

*Doctor of Philosophy*

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Department of Immunology and Infection

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London School Of Hygiene & Tropical Medicine

Funded by MRC Vaccine Research

## **Declaration**

I, Mateusz Jaroslaw Hasso-Agopsowicz, 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.

## **Abstract**

**Introduction:** The protective efficacy of the BCG vaccine against pulmonary tuberculosis varies across the globe and is lower in countries near the equator. Previous studies have shown disparate immunogenicity profiles after infant BCG vaccination in The United Kingdom, Malawi, The Gambia and Indonesia. Pre-exposure to environmental mycobacteria and maternal or infant co-infections are one of the mechanisms that could explain these variations. Exact molecular mechanisms that drive sustained differences in BCG immunogenicity are not known.

**Methods and Hypothesis:** In this study we aimed to investigate molecular mechanisms that drive differences in BCG immunogenicity. We hypothesised that differential DNA methylation or dynamic phenotypes of antigen presenting cells contribute to underlying differences in BCG immunogenicity. We used samples from South African, Ugandan and UK BCG vaccinated infants to investigate their BCG-induced immune responses. In South Africa we compared DNA methylation profiles between high and low BCG responders. In the UK and Uganda, we compared BCG specific immune responses and DNA methylation profiles between both countries. Lastly, in Uganda, we investigated longitudinal changes in dendritic cell phenotypes.

**Results:** We found that differential DNA methylation of immune pathways such as T cell activation, or potassium and calcium channel Signalling is associated with variation in BCG immunogenicity in South African infants. The



comparison of immune responses between UK and Ugandan infants revealed that Ugandan infants have a strong inflammatory response to BCG, but levels of TH1 and TH2 cytokines are similar. The analysis of DNA methylation between these countries revealed that genes of B cell activation pathway are more methylated in Uganda, whereas genes of TGF $\beta$  regulatory pathways are more methylated in the UK. The analysis of antigen presenting cells revealed that key dendritic cell populations are absent at birth and develop within the first year of life. We also highlight that cytomegalovirus (CMV), Epstein-Barr virus (EBV) and maternal latent tuberculosis infection (LTBI) have profound effects on dendritic cell phenotypes.

**Implications:** These results point towards a role for DNA methylation in the regulation of BCG immune responses, however, *in vitro* studies focusing on highlighted gene candidates and pathways should be conducted. Small differences in immunogenicity profiles after BCG vaccination in the UK and Uganda suggest that BCG efficacy in these countries should be similar, an area requiring a further investigation. The absence of dendritic cells at birth should be investigated and the kinetics of their development within the first week of life examined. Special attention should be paid to the development of vaccines or treatments for CMV, EBV and LTBI, as these pathogens modulate DC phenotypes.

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Herewith I would like to thank people that made this scientific journey possible, reminded me to stay focused and, despite pitfalls, helped me to concentrate on the positive side of science. Firstly, I would like to thank my supervisors Dr Steven Smith and Professor Hazel Dockrell for their continuous support, guidance, and encouragement. I especially would like to thank Steve for tolerating my involvement in all the extra-curricular activities.

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I would like to acknowledge mothers and infants who participated in this study. Without you, this project would not be possible.

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*“Only dead fish go with the flow”*

*Andy Hunt, an author*

## Abbreviations

5mC	5-methylcytosine
AGO	Argonaute complex
APCs	Antigen presenting cells
BCG	Bacillus Calmette–Guérin
bp	Base pair
C	Classical
C	Crude
cDCs	Classical Dendritic Cells
CDP	Common DC progenitor
CFP-10	Culture filtrate protein-10
CIITA	Class II transactivator gene
CLR	C-type lectin receptor
CM	Central memory
CMV	Cytomegalovirus
cmvIL-10	Cytomegalovirus encoded homologue of IL10
CRAC	Calcium-release-activated calcium
CTLs	Cytotoxic T cells
DC-SIGN	Dendritic cell-specific ICAM-3 grabbing non-integrin
DCs	Dendritic Cells
ddPCR	Droplet digital PCR
DMGs	Differentially methylated genes
DMRs	Differentially methylated regions
DNMTs	DNA methyltransferases
EBV	Epstein-Barr Virus
Eis	Enhanced survival protein
ELISA	Enzyme-linked immunoassay
ELISPOT	Enzyme-linked immunospot
EM	Effector memory
ER	Endoplasmic reticulum
ESAT-6	Early secretory antigen target-6
EU	European Union
FLT3	Fms-like tyrosine kinase 3
GMCSF	Granulocyte-macrophage colony-stimulating factor
GPCR	G Protein-coupled receptor
H3K4me	Histone 3, 4th lysine, monomethylation

HATs Histone acetyltransferase enzymes  
 HDAC Histone deacetylase  
 HDACs Histone deacetylases  
 HEV High endothelial venules  
 HHV-5 Human Herpesvirus-5  
 HIV Human Immunodeficiency Virus  
 HK2 Hexokinase-2  
 Hsp65 65 kDa heat-shock protein  
 ICS Intracellular cytokine staining  
 IFN Interferon  
 IgG1 Immunoglobulin G1  
 IGRA IFN $\gamma$  release assay  
 IL Interleukin  
 IP10 Interferon gamma-induced protein 10  
 ISGs Interferon stimulated genes  
 LAMB1 Laminin subunit beta 1  
 LCs Langheran cells  
 lncRNAs long non-coding RNAs  
 LPS Lipopolysaccharide  
 LSHTM London School of Hygiene and Tropical Medicine  
 LT Lymphoid Tissue  
 LTBI Latent Tuberculosis Infection  
 M-CSFR Macrophage colony-stimulating factor receptor  
 MA Mycobacterium avium  
 mAChRs Muscarinic acetylcholine receptor signalling pathway  
 ManLAM Mannosylated lipoarabinomannan  
 MAP Mitogen-activated protein  
 MBCs Mycobacteria-specific memory B cells  
 MCP1 Monocyte chemoattractant protein 1  
 MDDCs Monocyte-derived Dendritic Cells  
 MDR-TB Multi-drug resistant tuberculosis  
 MFI Median Fluorescence Intensity  
 MGMT O<sup>6</sup>-methylguanine-DNA-methyltransferase  
 MHC Major histocompatibility complex  
 MIP-1 $\alpha$  Macrophage Inflammatory protein 1 $\alpha$   
 miRNAs micro RNAs

Mtb	Mycobacterium tuberculosis
mTOR	Mammalian target of rapamycin
MVA85A	Modified Vaccinia virus Ankara expressing Ag85A
NF- $\kappa$ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFAT	Nuclear factor of activated T cells
NGO	Non-governmental organisation
NK	Natural Killer
NLT	Non-Lymphoid Tissue
NO	Nitric Oxide
NOD-SCID	Non-obese diabetic severe combined immune deficiency mice
NTM	Non-tuberculous mycobacteria
P.	Plasmodium
PAMPs	Pathogen-associated molecular patterns
PAX	Paired box protein
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PD1	Programmed cell death 1
pDCs	Plasmacytoid Dendritic Cells
PDL1	Programmed cell death ligand 1
PFA	Paraformaldehyde
PFKP	Platelet phosphofructokinase
PIM	Phosphatidylinositol mannoside
PM	Placental malaria
PPA	Panther Pathway Analysis
PPD	Purified Protein Derivative
PRRs	Pathogen recognition receptors
PY	Person-years
RISC	RNA-induced silencing complex
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RR	Risk ratio
RRBS	Reduced representation bisulphite sequencing
S.	Shistosoma
SATVI	South African Vaccine Initiative
SDG	Sustainable Development Goals
SEA	Schistosoma mansoni soluble egg antigen

SEB Staphylococcus Enterotoxin B  
 siRNA Small interfering RNA  
 SOCE Store-operated calcium channels  
 SPADE Spanning-tree Progression Analysis of Density-normalized Events  
 TB Tuberculosis  
 TBVI Tuberculosis Vaccine Initiative  
 TCR T cell receptor  
 TD Terminally differentiated  
 Tfh Follicular helper T cells  
 TGF $\beta$  Transforming growth factor  $\beta$   
 TGS Transcription gene silencing  
 Th T helper  
 Tk Thymidine Kinase  
 TLR Toll-like receptor  
 TNF $\alpha$  Tumour Necrosis Factor  $\alpha$   
 TP Triple positive  
 Tregs T regulatory cells  
 TSLP Thymic stromal lymphopoietin  
 tSNE t-Stochastic Neighbour Embedding  
 TST Tuberculin Skin Test  
 UK United Kingdom  
 UN United Nations  
 UNSCT Uganda National Council for Science and Technology  
 UPEC Uropathogenic *Escherichia Coli*  
 UV Ultraviolet  
 UVRI Uganda Virus Research Institute  
 VDR Vitamin D receptor  
 viSNE visual Stochastic Neighbour Embedding  
 WHO World Health Organization  
 Wks Weeks  
 X<sup>2</sup> Chi-squared test  
 XDR-TB Extensively drug-resistant tuberculosis

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## **1. Introduction**

### **1.1. Tuberculosis and the BCG vaccine: remaining challenges**

#### **1.1.1. An overview of tuberculosis**

Tuberculosis (TB) is an airborne infectious disease caused by bacillus *Mycobacterium tuberculosis* (Mtb), identified by Robert Koch in 1882<sup>1</sup>, and preventable with Bacillus Calmette–Guérin (BCG) vaccine. It is mainly a disease of the lung as 70% of cases are pulmonary; however, Mtb can spread to other organs such as meninges, lymph nodes and bones resulting in an extrapulmonary disease<sup>2</sup>.

##### **1.1.1.1. The burden of tuberculosis**

TB is the ninth leading cause of death worldwide and the primary cause due to a single infectious agent. In 2016 alone, there were 1.7 million deaths worldwide and an estimated 10.4 million new cases. The global incidence of TB is 140/100,000 persons and remains highest in countries of Sub-Saharan Africa (Central African Republic, South Africa, Lesotho, Mozambique) and South-East Asia (The Philippines, Democratic People's Republic of Korea)<sup>3</sup> (figure 1).

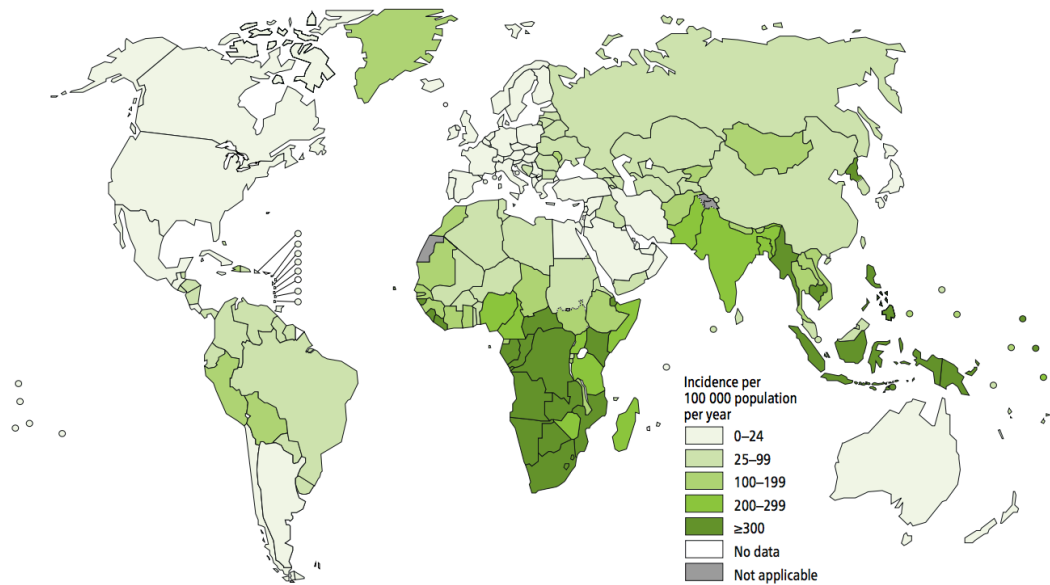


Figure 1. Global incidence of tuberculosis in 2016 per country. Source: Global Tuberculosis Report 2017, World Health Organization, Geneva, Switzerland<sup>3</sup>.

#### 1.1.1.2. The heterogeneity of tuberculosis infection and risk factors

TB is a complex disease and can manifest itself in the form of an active or latent infection. Individuals exposed to Mtb can become infected and rapidly progress to the active form of the disease. They could remain latently infected and might progress to active TB later in life. Exposed individuals could also be rapidly cleared of Mtb bacilli, resulting in a lack of adaptive immune responses, or the infection could be contained locally without detectable systemic responses (figure 2).

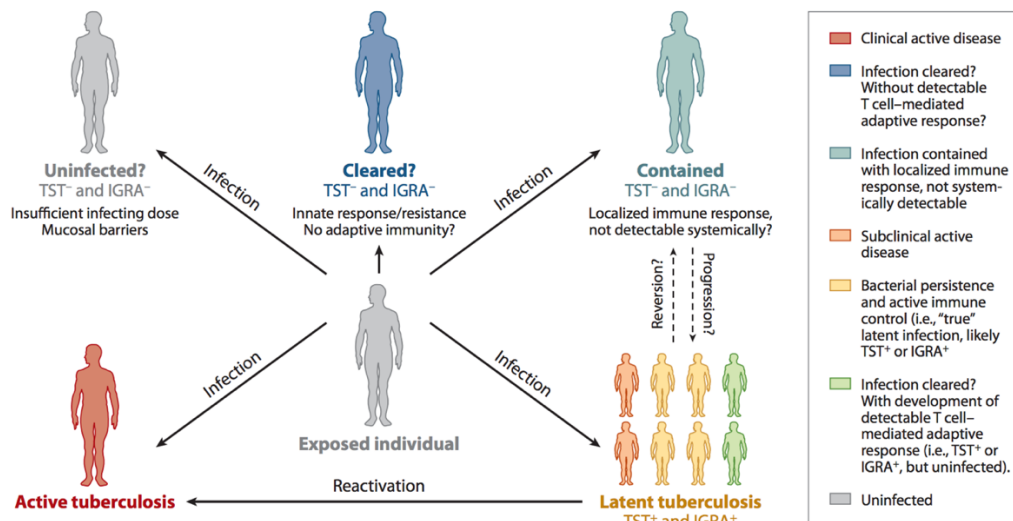


Figure 2. The heterogeneity of TB infection. Source: *The Immune response in tuberculosis*. Anne O'Garra et al.<sup>4</sup>

Typical symptoms of active TB include but are not limited to fever, weight loss, night sweats, cough (often with blood), lung cavities and densities<sup>5</sup>. Recent estimates suggest that 23% of the world's population is infected with latent TB, amounting to a total of 1.7 billion people<sup>6</sup>. Latently infected individuals have no clinical evidence of TB disease, and epidemiological studies suggest that 5-10% of them will develop the active form of TB<sup>7</sup>. The risk of progressing to the active form of TB is increased in childhood<sup>8</sup>. It also depends on numerous risk factors including HIV infection, diabetes, alcohol and drug use, poverty, malnutrition and overcrowding, limited or no access to health services, smoking and imprisonment<sup>3</sup>. These factors reduce overall well-being and are detrimental to health and the immune system. A weakened immune system is no longer able to control the latent TB infection, resulting in Mtb dissemination, and an active form of TB disease.

### 1.1.1.3. The landscape of TB diagnosis and treatment

A complex disease manifestation makes the diagnosis of TB difficult. The host's reactivity to Mtb antigens can be measured by the tuberculin skin test (TST), otherwise known as the Mantoux test. The patient is challenged intradermally with purified protein derivative (PPD), an extract of Mtb. Development of a hypersensitivity reaction indicates that a patient has an immune response to Mtb antigens. PPD antigens are present in Mtb, as well as in BCG and environmental mycobacteria; therefore the disadvantage of TST is its inability to distinguish between latent TB, active TB, BCG vaccination, or pre-exposure to environmental mycobacteria<sup>9</sup>. More recent assays were developed that measure the immune response to early secretory antigen target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10), two Mtb proteins that are not present in the BCG vaccine, and have a limited presence in the environment<sup>10</sup>. The reactivity to these antigens is examined by the production of interferon  $\gamma$  (IFN $\gamma$ ) in an IFN $\gamma$  release assay (IGRA), measured either by enzyme-linked immunoassay (ELISA) (Qiagen), or enzyme-linked immunospot (ELISPOT) (TSPOT.TB<sup>TM</sup>) assay. These assays can distinguish between Mtb infection and BCG vaccination or exposure to environmental mycobacteria but are unable to identify whether the disease is active or latent. Other diagnostic methods include culture-based methods, where patient sputum is cultured in a laboratory facility and examined for the presence of Mtb bacilli<sup>11</sup>. This diagnostic method is considered a reference standard; however Mtb is a slow growing mycobacterium, and the culture diagnosis can take up to 12 weeks. The culture-based techniques require category III containment facilities and highly trained laboratory staff, both of which are scarce in low-income countries where the TB burden is high. Another method is a direct microscopic examination of bacilli of sputum smear through the use of Ziehl-Neelsen staining. This is an inexpensive and rapid method, however, has poor sensitivity and is unable to distinguish between different species of mycobacteria<sup>11</sup>. Lastly, the

Xpert® MTB/RIF assay is a rapid molecular test for TB diagnosis that can provide results within two hours. It has a superior performance to microscopic smear examination, and studies report a sensitivity of 86.8% and specificity of 93.1%<sup>12</sup>. Xpert® MTB/RIF assay is particularly useful in the detection of multi-drug resistant TB (MDR-TB) (defined as resistance to rifampicin and isoniazid); however, it is the most costly diagnostic tool.

Once diagnosed, TB is a complicated disease to treat. Mtb is a slow-growing mycobacterium and requires a prolonged antibiotic treatment. The standard World Health Organization (WHO) recommended drug regimen in patients infected with drug-susceptible Mtb lasts for six months and contains two months of isoniazid, rifampicin, pyrazinamide and ethambutol treatment, followed by four months of isoniazid and rifampicin given on a daily basis<sup>13</sup>. The treatment of MDR-TB is extraordinarily complex and can last up to 12 months, with multiple oral and injectable drugs that induce severe side effects<sup>14</sup>.

#### **1.1.1.4. Tuberculosis in the United Kingdom and Uganda**

According to recent WHO estimates there were 6500 TB cases in the UK in 2016, with an average annual incidence rate of 9.9/100,000 persons<sup>3</sup>. The incidence of TB in the non-UK-born population (49.4/100,000) is 15 times higher than the UK-born population, and 74% of TB cases are born abroad. The number of new cases has been declining at a sustained level of 10% since 2012, but slowed down to 1% in 2016<sup>15</sup>. To address the incidence of TB and combat the disease, the UK has implemented ten critical areas for action in the *Collaborative TB Strategy for England 2015-2020* guideline. These recommendations include reducing active TB in new migrants through pre-entry screening programmes, preventing TB reactivations through LTBI testing, reducing delay and maintaining the quality of diagnostics, and

ensuring an integrated approach with a focus on social determinants<sup>15</sup>. BCG is not recommended nationally and used only in areas where TB incidence is  $\geq 40$  patients per 100,000 persons<sup>15</sup>.

Uganda is a Sub-Saharan African country with a high TB burden. WHO estimates suggest that in 2016 there were 83,000 new TB cases and the average incidence rate was 201/100,000 persons. Importantly, 42% of new TB cases were in HIV-positive individuals<sup>3</sup>. An estimated 49% of individuals are latently infected with TB<sup>16</sup>. Intermittent access to anti-TB drugs, challenges in drug delivery to remote and mobile communities, poor health-seeking behaviours and drug adherence, as well as inadequate funding of TB treatments, are some reasons that explain the high TB incidence in Uganda<sup>17</sup>.

#### **1.1.1.5. The role of the WHO and UN in fighting tuberculosis**

Eradicating TB and putting a stop to the TB epidemic requires a mobilised effort of international stakeholders. Multiple non-governmental organisations (NGO), as well as international agencies such WHO and United Nations (UN), are contributing to this global effort. WHO announced the End TB Strategy for 2016-2035, with the primary aim of reducing TB deaths by 90% and TB incidence by 80% by 2030, in comparison with 2015<sup>18</sup>. UN has published 17 sustainable development goals (SDG), including a goal to ensure healthy lives and promote well-being for all at all ages<sup>19</sup>. Both strategies outline many aims and objectives to end the TB epidemic. Some are TB specific (increase treatment coverage and success rate, reduce proportion of people that experience catastrophic costs due to TB, improve coverage of LTBI treatment) and some address risk factors specific for TB (reduce prevalence of HIV and diabetes, reduce alcohol and drug use, achieve universal healthcare coverage, decrease proportion of population living below poverty line and in slums). Importantly, both

strategies emphasise the need to develop new and improved vaccines that are effective against pulmonary TB, especially in countries where the burden is high.

### **1.1.2. TB vaccines in use and development**

BCG was introduced in 1921 and is the only licensed vaccine for tuberculosis. It consists of an attenuated strain of *Mycobacterium bovis*, the etiological agent of TB in cattle. Despite its widespread use, the efficacy of BCG against pulmonary TB varies, and BCG can disseminate in HIV-infected and immunocompromised individuals causing a disease called BCGosis. As a consequence, BCG is not recommended for these populations, resulting in a large number of neonates susceptible to TB<sup>20</sup>. Because of BCG limitations, novel vaccine candidates are under development. These fall into three main categories: priming, boosting and therapeutic vaccines. Priming vaccines are pre-exposure preventive vaccines and are typically administered before the first exposure to Mtb, often to neonates. These vaccines often contain antigens from stages of replication and metabolism of Mtb<sup>20</sup>. H1 and H4 vaccines, both in phase IIa clinical trials are examples of priming pre-exposure vaccines currently in development (figure 3)<sup>21</sup>. Boosting vaccines are preventive vaccines administered after previous BCG vaccination. They target adolescents or adults previously immunised with BCG or known LTBI status and often include fusion proteins, a combination of antigens from both active and latent stages of the infection. Examples of these vaccines include H56 (phase IIa, dormancy antigen H1 and Rv2660c), M72 (phase IIb, antigens Rv1196 and Rv0125) and ID93 (phase IIa; antigens Rv2608, Rv3619, Rv3620, Rv1813 ). Lastly, therapeutic vaccines are being developed to improve the outcome of active TB treatment. The development of therapeutic vaccines is particularly vital in MDR-TB and extensively drug-resistant TB (XDR-TB), where treatment success is less than 50%. Therapeutic vaccines aim to enhance the immune response to TB antigens, hoping to clear the infection quicker.

M. Vaccae (phase III) and RUTI (phase IIb) are examples of such vaccines (figure 3)<sup>20</sup>.

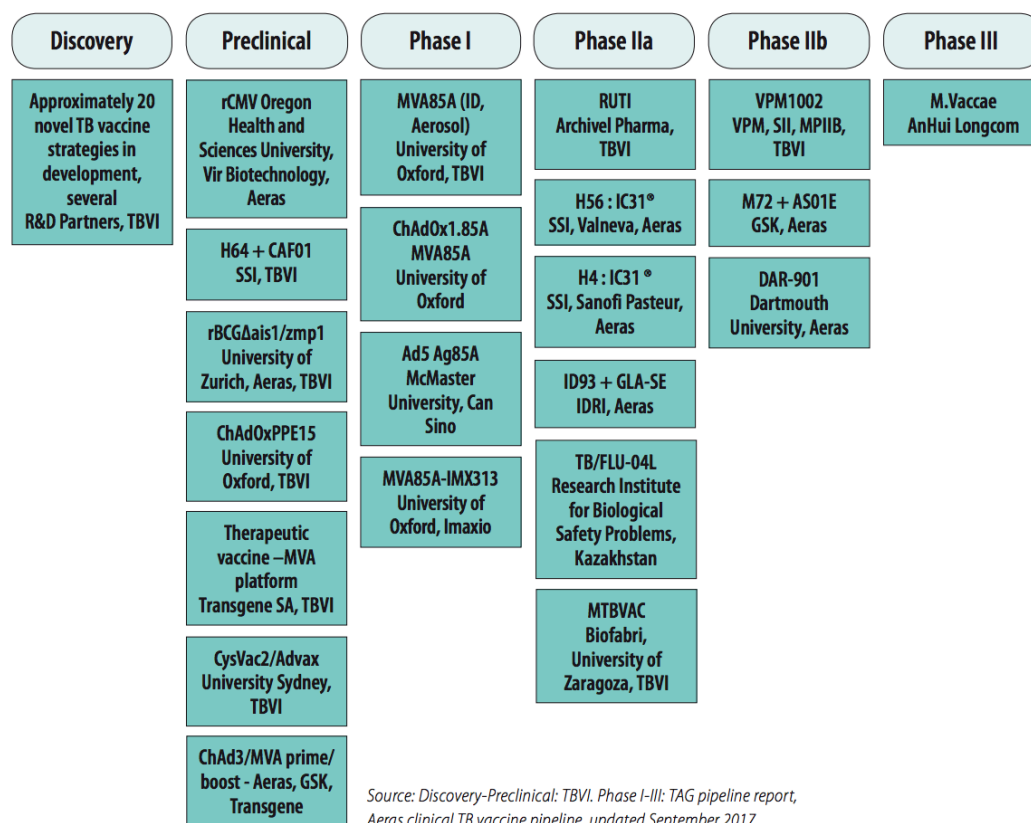


Figure 3. Clinical and preclinical TB vaccine pipeline. Source: Global Report on Tuberculosis Vaccines 2018<sup>21</sup>

The most advanced candidate vaccine in clinical trials is M. Vaccae. It is a heat-inactivated whole-cell vaccine derived from *Mycobacterium vaccae*. The efficacy trial started in 2013, aiming to enrol 10,000 individuals; however, there is no further information available<sup>20</sup>. The second most advanced vaccine is the recombinant BCG vaccine, VPM1002. Early clinical studies have shown a superior safety and efficacy profile to the BCG vaccine. VPM1002 is currently undergoing phase IIb trials in HIV exposed and unexposed infants and entering phase III efficacy trial in India<sup>22</sup>.



Recently, a BCG-boosting vaccine candidate, a recombinant strain of Modified Vaccinia Virus Ankara that expresses Mtb antigen 85A (MVA85A) was tested in a phase IIb trial in BCG vaccinated South African infants. Despite inducing a moderate IFN $\gamma$  producing and Ag85-specific CD4 T cell responses, the efficacy of the vaccine was 17.3% against tuberculosis disease and -3.8% against Mtb infection<sup>23</sup>. TB vaccine design remains challenging as there are no reliable correlates of protection, the progress of the disease is slow, disease endpoints are not well defined, and incidence is low, increasing the need for large and costly clinical trials. Possible explanations for MVA85A failure include targeting infants vs adults. The vaccine has previously shown much greater immunogenicity in adults, who are the primary source of TB transmission. Another possible explanation could be the route of delivery. MVA85A was received intradermally and induced a systemic immune response. However, pulmonary TB is the predominant manifestation of the disease, and perhaps new TB vaccines should be delivered via a mucosal route to induce local pulmonary immune responses.

Novel approaches to design TB vaccines are being explored. Mucosal vaccines aim to produce both humoral and cellular immune responses, both locally at mucosal surfaces and systemically<sup>24</sup>. As Mtb enters the body through mucosal surfaces of the airways, it is clear that mucosal vaccines that induce strong mucosal responses could be effective in TB prevention. Mucosal vaccines are easy to deliver, and are not associated with pain or fear of needles<sup>25</sup>. There are currently ten mucosal vaccines in development, out of which eight are delivered intranasally, one orally, and one nasally<sup>24</sup>.

The field of TB vaccine development has expanded rapidly in the past decade, thanks to European Union (EU)-funded international consortia led by Tuberculosis Vaccine Initiative (TBVI) and a coordinated effort of multiple stakeholders. Importantly, the

development of new TB vaccines is impaired without a thorough knowledge of how the current vaccine BCG works, why does it sometimes fail to work, and what are the protective immune responses it induces.

#### **1.1.2.1. The immune response to the BCG vaccine**

The immune response to the BCG vaccine is complex and overarches both innate and adaptive immune responses, both of which will be described here.

##### **1.1.2.1.1. The innate immune response to the BCG vaccine**

When administered intradermally, BCG induces a local inflammatory response. Pathogen-associated molecular patterns (PAMPs) (lipoproteins, glycoproteins such as mannosylated lipoarabinomannan (ManLAM) or phosphatidylinositol mannoside (PIM)) are recognised by pathogen recognition receptors (PRRs) including toll-like receptor (TLR) 2 & 4, complement receptors and mannose receptors of monocytes, neutrophils and dendritic cells<sup>26,27</sup>. TLR2 engagement, imperative for BCG recognition<sup>28</sup>, signals via myeloid differentiation primary response gene 88 (MYD88) and upregulates NF- $\kappa$ B leading to an increased production of: cytokines (interleukin 1 (IL1), IL6, tumour necrosis factor  $\alpha$  (TNF $\alpha$ )), chemokines (CXCL8, CCL2), costimulatory receptors (CD80 & CD86) and adhesion molecules (E-selectin)<sup>29</sup>. In macrophages, prolonged stimulation of TLR2 negatively affects major histocompatibility complex (MHC) class I antigen processing and downregulates the expression of MHC class II, reducing antigen presentation to T cells, and impairing the adaptive immune response<sup>30</sup>. Similarly, opsonisation of BCG with factor H, a regulatory protein of complement that inhibits the alternative complement pathway, has been shown to increase levels of pro-inflammatory cytokines IL6 and TNF $\alpha$ <sup>31</sup>. An acute and robust proinflammatory response activates macrophages and results in

quick and efficient killing of BCG via production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). The efficient killing limits the time to process BCG antigens and present them to T cells, potentially impairing the adaptive immune responses and subsequent efficacy of the BCG vaccine<sup>32</sup>. Therefore, a balanced inflammatory response is essential to effectively kill BCG while assuring the induction of an adaptive immune response sufficient to protect against future TB disease. Studies suggest that BCG persists for up to 4 weeks and the cellular filtrate from suction blisters reveal that CD3 lymphocytes, CD14 monocytes and CD15 neutrophils are predominant at the site of inoculation<sup>33</sup>.

Neutrophils compose up to 60% of white blood cells and secrete large amounts of cytokines and chemokines to prime phagocytosis<sup>34</sup>. Upon contact with BCG, neutrophils produce chemokines IL8, CCL2, CCL3; and cytokines such as IL1 $\alpha$ , IL1 $\beta$  and Transforming Growth Factor  $\beta$  (TGF $\beta$ ). BCG exposure in neutrophils also induces their surface markers such as CD11b, CD18, and Fc $\gamma$ Rs II and III<sup>35</sup>. BCG-containing neutrophils have a delayed apoptosis<sup>36</sup>, suggesting that BCG may use them as a vehicle to disseminate to lymph nodes into the proximity of T cells and dendritic cells (DCs). Interestingly, the interaction of BCG infected neutrophils with dendritic cells has shown to drive DC maturation and induce BCG antigen presentation in DCs for T cell priming<sup>37</sup>. Neutrophils have also proved to stimulate T cell activation in lymph nodes thereby activating adaptive immune responses<sup>38</sup>. They have also been shown to play a role in regulating the BCG infection by secreting large amounts of IL10<sup>39</sup>, and high frequencies of neutrophils are observed at sites of infection in the lungs of patients with TB<sup>40</sup>. Lastly, the role of neutrophils is highlighted in gene expression studies where neutrophil signatures are prominent in TB disease<sup>41</sup>.

Dendritic cells are major antigen presenting cells (APCs) and act as a bridge between innate and adaptive immune responses. Dendritic cell receptors that recognise BCG are TLR2 & 4, TLR9<sup>42</sup>, CR3 (CD11b/CD18), CR4 (CD11c/CD18), DEC-205 (CD205), and dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN, CD209)<sup>32</sup>. DC-SIGN is the most important phagocytic receptor as antibody neutralisation of DC-SIGN reduces BCG interaction with DCs by 80%<sup>43</sup>. Once phagocytic receptors recognise BCG PAMPs, they are internalised and presented on the surface of DCs in the context of MHC-II to CD4 T cells. *In vitro* studies suggest that the exposure of DCs to BCG PAMPs upregulates DC specific maturation markers *in vitro*: MHC-II, CD40, CD80, CD86<sup>44</sup> CD83, HLA-DR<sup>45</sup> and increases the secretion of IL12, which *in vivo* drives TH1 responses and induces IFN $\gamma$  production in T cells<sup>45</sup>.

#### **1.1.2.1.2. The adaptive immune response to the BCG vaccine**

BCG engages multiple arms of adaptive immune responses (figure 4). BCG-containing DCs *in vivo* traffic to draining lymph nodes where they present BCG antigens to CD4 T cells in the MHC II context. The presence of TH1 polarising cytokines such as IL12 and IL18 and antigen presentation activates CD4 T cells and makes them traffic to the site of infection, where they secrete IFN $\gamma$ , which stimulates macrophages to phagocytose BCG. Indeed, the secretion of IFN $\gamma$  and CD4 TH1 immune response is paramount for the effective killing of BCG<sup>46</sup>. A TH1 immune response is observed following BCG immunisation in adults and infants<sup>47</sup>. Mice studies suggest that CD4 T cells are the principal effector cells induced by BCG in the lung<sup>48</sup>.

Human *in vitro* and *in vivo* studies suggest that BCG induces a robust antigen-specific CD8 response with increased secretion of IFN $\gamma$  and cytotoxic proteins, cell

proliferation and degranulation<sup>32,49</sup>. In the periphery, CD8 T cells have shown to induce apoptosis of BCG infected cells via the FasL-Fas interaction, resulting in apoptosis of Fas-expressing cells<sup>50</sup>. This allows other immune cells to phagocytose apoptotic bodies and further drive the adaptive immune response. In contrast, in lungs, this mechanism limits the excessive tissue damage and pulmonary failure<sup>32</sup>. In addition to CD8 responses to BCG antigens presented in an MHC I context, there is evidence of CD1 restricted CD8 T cells after BCG immunisation in human<sup>51</sup> and guinea pigs<sup>52</sup>. These cells recognise mycobacterial lipid and have been suggested to play a role in maintaining the latent stage of the disease in the periphery or, prevent TB dissemination<sup>32</sup>.

The production of IL17A and IL22<sup>53</sup> characterises CD4 TH17 T cells. They play a dual role in protection and pathology. BCG vaccination induces CD4 TH17 cells to produce IL17A in lungs, which recruits neutrophils and granulocytes, resulting in an excessive immunopathology<sup>54</sup>. However, IL17A was shown to be important in maintaining CD4 IFN $\gamma$  responses in the lungs of mice<sup>55</sup>. TH17 cells have been shown to enhance vaccine-induced protection in mice models, following Mtb challenge<sup>56,57</sup>. A study in UK infants reports that BCG induces TH1 and TH17 responses in T cells isolated from peripheral blood<sup>58</sup>. Further evidence shows that in BCG vaccinated mice, robust TH1 lung responses are dependent on IL17A and IL23 from lung resident memory TH17 cells<sup>55</sup>.

The expression of FOXP3 and CD25<sup>59</sup> characterises T regulatory cells (Tregs). In BCG vaccinated mice, Treg depletion increases BCG efficacy leading to a decrease in the Mtb burden<sup>60</sup>. Human studies confirm that BCG vaccinated adults who develop mild local skin inflammation following BCG immunisation, have increased levels of CD8 Tregs. This finding is in contrast to adults who develop a strong skin inflammation and have predominantly multifunctional CD4 T cells<sup>61</sup>. Tregs have been

proposed as mediators of the limited efficacy of BCG, by appearing shortly after exposure to environmental mycobacteria and regulating the BCG induced immune response<sup>32</sup>. Tregs can thus be detrimental to BCG efficacy, but could also play an essential role in preventing excessive immunopathology.

Mtb and BCG are intracellular mycobacteria, which is perhaps why antibodies and B cell lymphocytes have not been extensively studied in the Mtb and BCG context. Recent evidence suggests that BCG vaccinated adults have an increased frequency of mycobacteria-specific memory B cells (MBCs) than non-vaccinated adults<sup>62</sup>; and that BCG induces antibody responses that peak 56 days post vaccination in adults<sup>63</sup>. Other studies highlight that patients with active TB or LTBI have an increased proportion of B cells with impaired proliferation, immunoglobulin and cytokine production; and that T cell activity was dependent on the presence of functionally competent B cells<sup>64</sup>, when compared to healthy controls. Other studies reveal a functional role of antibodies in tuberculosis. Antibodies from LTBI patients induce more phagolysosomal maturation, inflammasome activation and drive intracellular killing of Mtb when compared to antibodies from active TB patients, and these functions were associated with differential antibody glycosylation between TB and LTBI patients<sup>65</sup>. This suggests that the development of TB from latent to the active stage could be due to defects in antibody functionality.

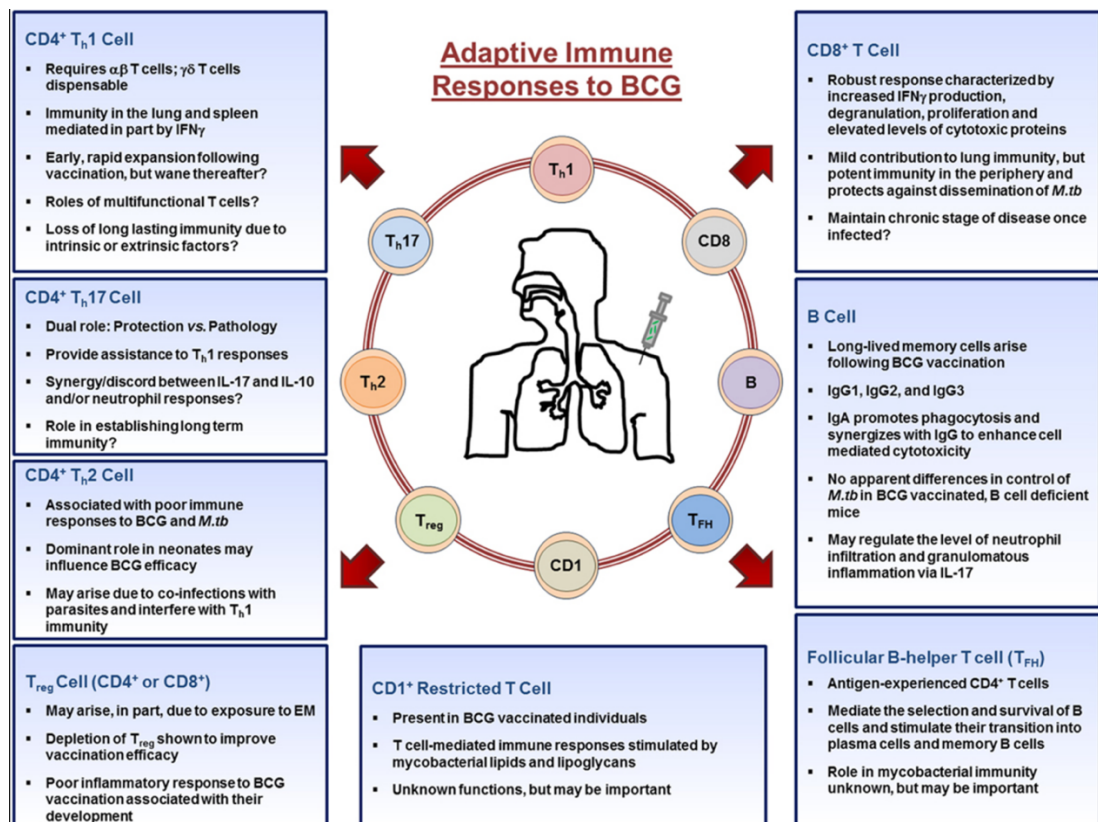


Figure 4. An overview of the adaptive arm of the BCG induced immune responses. Source: *Immune Responses to Bacillus Calmette–Guérin Vaccination: Why Do They Fail to Protect against Mycobacterium tuberculosis?*<sup>32</sup>

Taken together, both the innate and adaptive immune responses to BCG vaccination are complex, fragmented and multifaceted. While TH1 cytokines and specifically IFN $\gamma$  remain important, our understanding of BCG induced immunity must expand beyond TH1. Importantly, we should consider the immune response to BCG and *Mtb* as a delicate balance between the induction of BCG and *Mtb* killing and associated immunopathology vs the regulation of the immune system and prolonged antigen expression and activation of the adaptive arm of the immune system. Increasingly research highlights a growing role for multiple immune components including CD8, Tregs, B cells and other cells. Elements of the immune system do not work in

isolation, and future research should focus on trying to understand the multi-layered BCG immune response in a context of a system.

#### **1.1.2.2. Heterologous effects of BCG**

BCG is a complex vaccine, not only because of the immune responses it induces but also due to its varying efficacy against TB as well as protection against other diseases. BCG challenges the idea of the vaccine specificity as growing evidence suggests that BCG protects infants from pathogens other than *Mtb*, a concept known as heterologous or non-specific protection.

##### **1.1.2.2.1. Evidence for heterologous effects of BCG**

Multiple studies from West and East Africa and India have reported reduced all-cause infant mortality in BCG immunised infants, lasting up to 5 years. The efficacy of BCG in reducing all-cause infant mortality ranges between 6-72% in clinical trials to 2-95% in observational studies, giving strong evidence that BCG reduces infants' deaths for reasons other than TB<sup>66</sup>.

The reduction in infants' mortality could be partially explained by a reduction of overall infection rates in BCG immunised infants. Evidence suggests that BCG immunisation significantly reduces the number of infections in infants. The strongest evidence to date is the analysis of BCG immunised infants in 33 countries, which reports that BCG vaccinated infants have a lower incidence of respiratory infections by 17-37%<sup>67</sup> when compared to non-vaccinated peers. Aside from respiratory infections, BCG immunised infants had lower rates of neonatal sepsis in Guinea-Bissau<sup>68</sup> and Spain<sup>69</sup>, and lower rates of nematode infections in Uganda<sup>70</sup>. The heterologous effects of BCG



are more prominent in low-income countries where disease burden is high, as studies in Denmark failed to confirm that BCG reduces infants' rate of infections<sup>71</sup>.

#### **1.1.2.2.2. Potential mechanisms of BCG-induced heterologous effects.**

Multiple mechanisms have been proposed as potential mediators of BCG induced heterologous protection. BCG induces a TH1 response, which may go beyond mycobacterial specificity. Increased cytokine responses (both TH1 and TH2), or high lymphocyte proliferation, or CD4 T cell proliferation were observed in The Gambia<sup>72,73</sup>, Uganda<sup>74</sup>, South Africa<sup>75</sup>, Guinea-Bissau<sup>76</sup>, Denmark<sup>77</sup>, Philippines<sup>78</sup>, and the United Kingdom<sup>79</sup>, in response to multiple antigens and pathogens. Infant immune responses shift from TH17 to TH1 focused within the first year of life<sup>80</sup>. BCG was proposed to facilitate that process and in effect, enlarge a pool of heterologous TH1 cells.

There is conflicting evidence on humoral responses to non-mycobacterial stimuli. BCG immunisation increased antibody levels against influenza vaccine in UK adults<sup>81</sup>; increased antibody levels to HBsAg and polio antigens in The Gambia<sup>73</sup>; and increased antibody concentrations to pneumococcal antigens in Australian infants<sup>82</sup>. However studies in South Africa<sup>83</sup>, The Gambia<sup>72</sup> and Denmark<sup>84</sup> failed to confirm these findings. Importantly, BCG vaccine strain, the age of immunisation and the age at observation all varied between studies, perhaps helping to explain the disparity in observed results.

Aside from T cell and humoral mediated immunity, the most plausible mechanism to explain heterologous effects of BCG is innate immunity training. The concept refers to innate memory and the idea that following BCG stimulation (or other live vaccines

such as measles), innate cells will respond stronger and more rapidly upon secondary challenge with an unrelated pathogen. In mice, when macrophages were exposed to BCG, they demonstrated increased control of *Candida albicans* (*C. albicans*) infection *in vitro* and *in vivo*<sup>85</sup>. Similarly, BCG vaccination of Dutch adults increased expression of TLR4, CD11b and CD14 in monocytes up to one year, and increased their production of proinflammatory cytokines (IL1 $\beta$ , IL6, TNF $\alpha$ ) in response to *Staphylococcus aureus* or *C. albicans* when measured up to three months later<sup>86</sup>; as well as increasing NK cell production of IL1 $\beta$  and IL6 for up to three months<sup>87</sup>. These findings indicate that monocytes and NK cells could mediate the reduction in all-cause mortality following BCG vaccination.

Metabolic changes in cells facilitate the mechanisms of innate immune training. Treatment of monocytes with BCG or  $\beta$ -glucan (a component of *C. albicans* fungal cell wall and often used as a model for BCG training) shifts their metabolic pathway usage from oxidative phosphorylation to aerobic glycolysis, characterised by increased glucose consumption and lactate production, as well as increased phosphorylation of mammalian target of rapamycin (mTOR)<sup>88</sup>. Similarly, peripheral blood mononuclear cells (PBMC) from BCG vaccinated Dutch adults, have been shown to produce more lactate and express higher levels of glycolysis pathway components hexokinase-2 (HK2) and platelet phosphofructokinase (PFKP) after heterologous stimulation, at two weeks and three months after BCG immunisation when compared to pre-BCG immunisation PBMC<sup>89</sup>. Importantly, the role of glycolysis in innate immune training was further highlighted by inhibition of the mTOR pathway and subsequently reduced production of lactate, TNF $\alpha$  and IL6 in *ex vivo* BCG trained monocytes<sup>89</sup>.

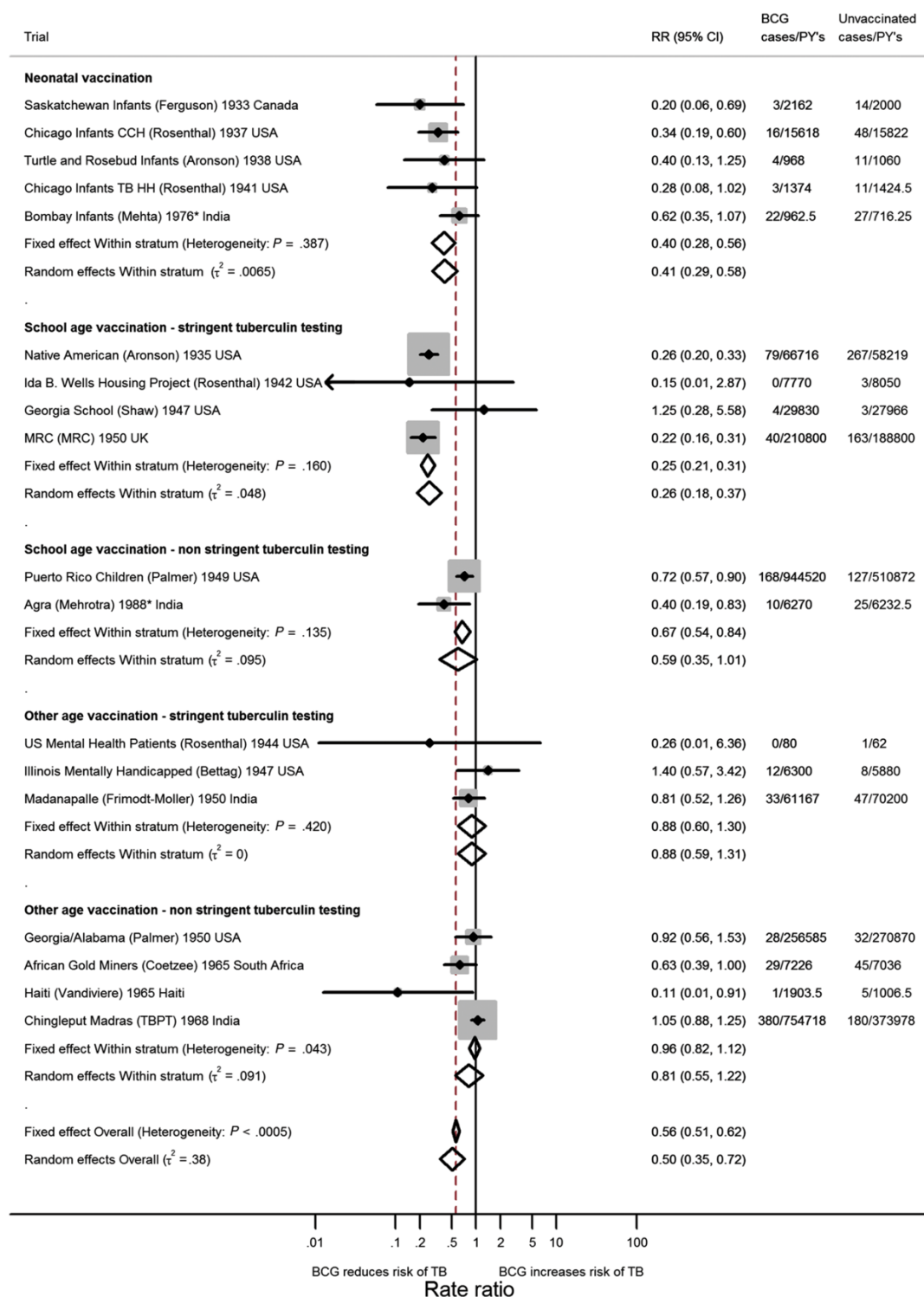
Epigenetic mechanisms and specifically histone modifications are another mechanism that can drive innate immune training. The aforementioned increased production of  $\text{TNF}\alpha$  and IL6, as well as increased expression of TLR4 after BCG vaccination, was associated with increased levels of trimethylation of lysine 4 at histone 3 (H3K4me3) marker, a histone modification associated with permissiveness and upregulation of protein transcription<sup>86</sup>. Besides,  $\beta$ -glucan training of monocytes increased their levels of H3K4me3 at promoters of glycolysis and mTOR pathways, as well as IL6 and  $\text{TNF}\alpha$  promoters, highlighting their role in trained innate immunity<sup>89</sup>. Aside from H3K4me3, BCG training has been shown to decrease levels of H3K9me3 (an inhibitory marker of gene transcription) at promoters of these pathways and genes<sup>89</sup>.

In summary, the heterologous effects of BCG are mediated by multiple pathways. Immune pathways such as TH1 polarisation and the role of antibodies are important, however, molecular pathways such as metabolic changes and modifications of the epigenetic profile are also highlighted. These studies help us to understand how BCG works on a molecular level. They emphasise a new mechanism such as epigenetic modifications that could play a role in explaining the variation in BCG efficacy.

#### **1.1.2.3. Variation in the efficacy of BCG vaccine**

The BCG vaccine against TB is notorious for giving rise to disparate host immune responses and uneven protection against pulmonary TB<sup>90</sup>. When BCG works well, it can induce long-lasting protection, extending up to 20 years in the UK<sup>91</sup>, 40 years in Norway<sup>92</sup>, and 60 years in Alaska<sup>93</sup>. A recent meta-analysis of BCG efficacy reported that BCG-induced protection against meningeal and miliary tuberculosis is high in infants (risk ratio (RR), 0.1) and children stringently tested for previous mycobacterial

exposure (RR, 0.08)<sup>90</sup>. However, the protection against pulmonary TB, the most common form of TB disease varies across the globe. The highest reported efficacy against pulmonary TB was observed in Indian infants in Canada (RR, 0.20)<sup>94</sup>, and lowest in Indian infants in India (RR, 0.62)<sup>95</sup>, and the pooled effect for neonatal BCG vaccination was RR 0.41 (figure 5). In older children, the efficacy of BCG vaccine is lower and depends on stringent testing to previous mycobacterial exposure. In stringently tested children, immunised at school age, the pooled efficacy of BCG vaccine against pulmonary TB is RR 0.26; however, this efficacy drops with an increased RR of 0.59 in non-stringently tested children<sup>90</sup>. The meta-analysis highlights three factors as primary contributors to observed heterogeneity in BCG efficacy studies: age at immunisation, latitude, and previous mycobacteria exposure. Other mechanisms have also been proposed. These include: differences in human genetics; exposure to ultraviolet (UV) light; levels of vitamin D; co-infections with helminths, malaria parasite and other pathogens; malnutrition; variation in mycobacteria genotypes; BCG strain used; composition of gut microbiota; disruption of vaccine cold chain supply; and administration of other vaccines. Some of these hypotheses will be addressed in the next chapter. It is possible that all of these factors influence BCG immunogenicity to some extent but it is unlikely that any one is exclusive or dominant. It is clear that to understand the lack of BCG efficacy in some countries we need to understand precise molecular mechanisms that drive its immunogenicity.



*Figure 5. An overview of the BCG efficacy. Rate ratios and 95% confidence intervals for pulmonary tuberculosis, stratified by age vaccinated and stringency of prevaccination tuberculin testing. Abbreviations: BCG, Bacille de Calmette et Guérin; CCH, Cook County Hospital; CI, confidence interval; D + L, DerSimonian and Laird method; M-H, Mantel-Haenszel method; MRC, Medical Research Council; PY, person-years; RR, rate ratio; TB, tuberculosis; TB HH, tuberculous households; TBPT, Tuberculosis Prevention Trial. Source: Protection by BCG Vaccine Against Tuberculosis: A Systematic Review of Randomized Controlled Trials<sup>90</sup>*

#### **1.1.2.4. Potential reasons for differences in the BCG immunogenicity**

##### **1.1.2.4.1. Genetic factors and the immune response to BCG**

The DNA sequence serves as a template for protein transcription, instructing which types of proteins to produce when the human body is combating pathogens. Therefore, it is plausible that variations in DNA sequences between populations would, in turn, determine the array of proteins that are produced in response to vaccines, including BCG. Marchant et al<sup>96</sup> have conducted a twin cohort study in The Gambia and measured the heritability of genes in response to vaccine antigens, including BCG specific antigens. They have found significant heritability for IL13 responses to PPD (46%) and 65 kDa heat-shock protein (Hsp65) (50%), while IFN $\gamma$  responses achieved a substantial heritability to PPD (41%) and Mtb (39%) antigens. Lower IFN $\gamma$  heritability was observed in Ugandan household contact study by Tao et al.: 23% for CFP-10, 16% for ESAT-6 and 12% for Ag85B<sup>97</sup>. Lastly, Randhawa et al. report that polymorphism of TLR1 and TLR6 in South African infants is associated with increased production of TH1-like cytokines IL-2 and IFN $\gamma$ <sup>98</sup>, further supporting the argument that human genetics play a role in the modulation of immune responses

to vaccines. All studies discuss that although we see intermediate levels of heritability in genes that are core to the immune system, it is unlikely that these gene variations can solely explain different patterns of BCG efficacy.

#### **1.1.2.4.2. Pre-exposure to environmental mycobacterium and the immune response to BCG and Mtb**

A growing body of evidence suggests that the exposure to environmental mycobacteria results in a cross-reactivity of immune responses among mycobacterial antigens<sup>99</sup>. Studies have reported IFN $\gamma$  responses to non-tuberculosis mycobacterial antigens before BCG vaccination, both in the UK<sup>100</sup> and Malawian<sup>101</sup> young adults suggesting exposure to mycobacteria. The role of this environmental exposure and its modulation of the immune response following BCG vaccination as well as susceptibility to TB has been studied in animal models<sup>102–108</sup> and humans<sup>99,109</sup>. Proposed mechanisms include masking, which suggests that the exposure to mycobacteria elicits an immune response that cannot be further elevated with BCG administration; and blocking, implying that previous immunity acquired to environmental mycobacteria prevents multiplication of BCG, which subsequently fails to augment a sufficient immune response<sup>99</sup>.

Numerous animal studies consistently inform that prior and post-exposure to non-tuberculous mycobacteria (NTM) can affect the immune response to the BCG vaccine and subsequently hinder the protection against tuberculosis disease<sup>102–107</sup>. Mice *in vivo* studies confirm that various strains of NTM give rise to an immune response that is recalled rapidly after the BCG vaccination, suggesting their potential ability to augment the BCG protection<sup>102,103</sup>. Mice exposed to *Mycobacterium avium* (MA) before BCG vaccination show decreased IFN $\gamma$  responses to *Mycobacterium bovis* derived PPD and increased immunoglobulin G1 (IgG1) titres against mycobacterial

antigens, characteristics of a TH2-like shift of the immune response<sup>105</sup>. Furthermore, infection with killed and systemically delivered MA post BCG vaccination resulted in increased TH1 and TH17 like responses and increased protection against Mtb infection. This was in contrast to when mice were exposed to oral, live and attenuated MA, which gave rise to TH2 like responses and decreased protection against Mtb infection<sup>104,106</sup>.

Studies in cattle further support the argument that the exposure to NTM affects the outcome of BCG vaccination. BCG vaccine failed to protect against *M. bovis* in calves that were shown to have elevated IFN $\gamma$  responses to MA PPD before BCG vaccination, suggesting an infection or environmental exposure to MA<sup>110</sup>. In contrast, one study suggests that pre-exposure to MA primes responses of the immune system and that the failure to detect BCG-specific responses is due to masking by immune responses to mycobacterial antigens present in MA<sup>107</sup>.

In conclusion, most of the studies support the idea of cross-reactivity of immune responses across mycobacterial species. This suggests that previous mycobacterial exposure can affect the outcome of BCG vaccination. However, most of the studies are performed in animal models, and more research in humans is needed in order to fully explain variations in immune patterns elicited by the BCG vaccine.

#### **1.1.2.4.3. Infant co-infections and BCG immune responses**

Another hypothesis that could explain variations in the BCG induced immune response is the impact of other infections prenatal, postnatal and at the time of or post-vaccination. Unlike the UK (or other developed countries), countries where BCG efficacy is poor are nearer the equator and the prevalence of infections such as malaria, human immunodeficiency virus (HIV), tetanus, helminths and others is



high<sup>111</sup>. One could hypothesise that these infections would shape the immune system and affect vaccine-induced immune responses resulting in variable immunisation efficacies.

Malaria is a dominant infectious disease caused by four species of *Plasmodium*: *Plasmodium falciparum* (*P. falciparum*), *P. vivax*, *P. ovale* and *P. malariae*. Its prevalence is common in tropical countries, close to the equator, where BCG induced protection against TB is low<sup>112</sup>. Research groups have investigated the potential outcome of postnatal malaria infection or in-utero exposure on the immunogenicity of BCG. Parra et al<sup>113</sup> found that *P. yoelli* infected mice vaccinated with BCG had a reduced number of IFN $\gamma$  and TNF $\alpha$  producing as well as reduced frequency of triple positive (TP) CD4 and CD8 antigen-specific T cells at 2 but not 7 and 10 weeks post-malaria infection<sup>113</sup>. This suggests that malarial modulation of the immune response is transient and unlikely to affect the immunisation profile of the BCG vaccine. In contrast, attenuated human CD4 T-cell responses were observed in infants that survived placental malaria (PM) vs control group, and lower IFN $\gamma$  responses were observed upon stimulation of PBMC with tuberculin PPD<sup>114</sup>. Lastly, Elliott et al<sup>115</sup> found an association of infant malaria with lower levels of IFN $\gamma$ , IL-5 and IL-13; and maternal malaria and number of malaria episodes in infancy with lower IL-5<sup>115</sup>.

Helminths are still the most common intestinal infection worldwide affecting the lowest income countries, where sanitation is inadequate<sup>116</sup>. Helminth infections are known to reshape the immune system by causing chronic infections, inducing strong TH2 immune responses, and activating Tregs<sup>117</sup>. These immune characteristics resemble aspects of the host immune response where BCG immunogenicity is low, giving substantial grounds to hypothesise that helminth infections could shift the BCG immune response to TH2, resulting in a loss of protection against TB. To test this, Elliott et al<sup>118</sup> conducted a large trial looking at the effect of single-dose anthelmintic

treatment during pregnancy on the infant immune response to BCG. They found no influence of either albendazole or praziquantel treatment, two common anthelmintic drugs, on the immune response to BCG<sup>118</sup>. The same group investigated whether treatment of children with albendazole affected their immune responses<sup>119</sup>. Five years post-BCG immunisation, Albendazole treated children had slightly lower IFN $\gamma$ , IL5 and IL13 immune responses when whole blood was stimulated with crude (c) CFP<sup>119</sup>. In contrast, infants exposed in utero to helminths showed lower IFN $\gamma$ <sup>120</sup> and higher IL5<sup>120</sup>, IL10<sup>115</sup> secretion upon stimulation of PBMC with PPD<sup>120</sup> or cCFP<sup>115</sup>. Similarly, helminth-infected children had lower secretion of IFN $\gamma$  and IL12 from PBMC stimulated with PPD<sup>121</sup> and albendazole treated children had increased PPD specific T cell proliferation and IFN $\gamma$  production<sup>122</sup>. Lastly, mice infected with low dose *Schistosoma mansoni* (*S. Mansoni*) and challenged with BCG markedly increased TH2 and suppressed TH1 responses<sup>123</sup>. Helminth-induced suppression of TH1 immune response to BCG was found to be driven by Tregs<sup>124</sup> and TGF $\beta$ <sup>121</sup>, and the newest study shows that infection with *Schistosoma haematobium* increases the Treg subset, and Tregs decrease post-treatment, increasing BCG specific cytokine production<sup>125</sup>.

HIV infection impairs the immune system and depletes the host of CD4 cells<sup>126</sup>, crucial for TB protection and effective responses following BCG<sup>127</sup>. As HIV infected and the disadvantaged immune system fails to control the vaccine-induced BCG infection, WHO's recommendation is not to vaccinate infants with symptomatic HIV<sup>128</sup>. This policy is supported by data suggesting a risk of local and disseminated BCG disease in HIV infants<sup>129</sup>. Moreover, the immune response to BCG is severely impaired with a lower secretion of IL5, 13, IFN $\gamma$ <sup>115</sup> and reduced number of polyfunctional CD4 T cells producing IFN $\gamma$ / IL2/ TNF $\alpha$ <sup>130</sup>. Lastly, infants exposed to HIV in-utero but who remain uninfected, show a transient increase of the CD4 subset of cells with no effect on BCG immunisation<sup>131</sup>.

It is clear that co-infections can modulate the immune system and affect the outcome of immunisation. However, as most vaccines (incl. BCG) are given at birth or within the first year of life when the prevalence of infectious diseases is low, it is highly unlikely that the lower efficacy of the BCG vaccine can be a consequence of co-infections alone.

We now know that immune responses to BCG can vary by regions and we are familiar with potential reasons for these variations. Aside from investigating cytokines, we must look at cells and their phenotypes that respond to BCG vaccination. In addition, we should consider molecular mechanisms such as epigenetics that drive the sustained differences in immune responses across populations.

### **1.2. Dendritic cells as an essential immune mechanism in the mediation of adaptive immune responses.**

Dendritic cells are specialised antigen-presenting cells that play a role in bridging innate and adaptive immunity. They comprise only 0.1% of all peripheral blood cells and their mapping and studying in human remains difficult<sup>132</sup>. There are three major types of dendritic cell: plasmacytoid DCs (pDCs), classical dendritic cells type 1 (cDC1) and type 2 (cDC2). Inside epidermis and epithelia, resident dendritic cells are called Langerhan cells (LCs), and monocytes are known to differentiate into monocyte-derived dendritic cells (MDDCs) during inflammation<sup>132</sup>. The majority of DC studies are conducted in mice, and recent technological advances allow for the translation of findings in mice to humans<sup>133</sup>.

### **1.2.1. The role of dendritic cells**

The central role of dendritic cells is to capture PAMPs in the periphery, internalise, process and present them to T cells for initiation of the adaptive arm of the immune response. DCs selectively express T cell co-stimulatory markers and secrete cytokines to precisely drive T cell differentiation into various subsets. By being able to steer T cell-mediated responses against pathogens and self-antigens, DCs are therefore master regulators of the adaptive immune system<sup>134</sup>.

#### **1.2.1.1. Presentation of antigens by dendritic cells**

The presentation of self and foreign antigens is the dominant role of dendritic cells. Foreign and self-antigens, as well as pathogen associated molecular patterns (PAMPs), are recognised by multiple cell surface receptors including Fc, Scavenger, C-type lectin family, macrophage mannose receptor, DEC205, and DC-SIGN<sup>134</sup>. The PAMP recognition by DC receptors initiates clathrin-dependent endocytosis or phagocytosis. A receptor-independent sampling of the extracellular environment is called micropinocytosis<sup>135</sup> (figure 6).

Clathrin-dependent endocytosis is an essential process in vesicular trafficking. After antigen recognition, a complex modulator protein machinery forms a clathrin-coated endocytic vesicle. Through actin rearrangement and multiple processes such as scission and uncoating, the clathrin-coated vesicle is internalised and fused with the endosome<sup>136</sup> (figure 6). In contrast, micropinocytosis is a clathrin-independent process that involves membrane ruffling and creation of large vacuoles that contain PAMPs, which are internalised and lysed by lysosomes. In DCs, micropinocytosis is a constitutive process that allows random sampling of extracellular fluid<sup>134</sup>. Lastly, phagocytosis is a receptor-mediated process that results in the creation of

phagosomes. Immature DCs are capable of phagocytosis; however, upon activation, they lose their phagocytic capability<sup>137</sup>. The main proteases found in DC phagosomes are cysteine proteases such as cathepsins, aspartate proteases and asparagine endopeptidase<sup>138</sup>; however, their proteolytic activity is reduced when compared with other phagocytic cells such as neutrophils<sup>139</sup>. It was proposed that DCs partially degrade proteins to present them in the MHCII context rather than fully, as is the case for neutrophils and macrophages<sup>140</sup>. Exogenous PAMPs are processed and loaded onto MHCII molecules in late endosomes, whereas endogenous antigens are loaded onto MHCI molecules in the endoplasmic reticulum (ER). Once DCs process antigens, they differentiate into their mature phenotype, increase expression of CD83, CD80, CD86, HLA-DR and CCR7, and travel to lymph nodes to present processed antigens to T cells<sup>141</sup>.

Exogenous antigens are internalised, processed and presented to T cells in the context of MHCII molecules whereas endogenous antigens are presented in an MHCI context. The role of MHCI presentation of antigens is to identify modified cells such as highly proliferating tumour cells. Antigens loaded onto MHCI molecules are recognised by naïve CD8 T cells that become activated and initiate cell death.

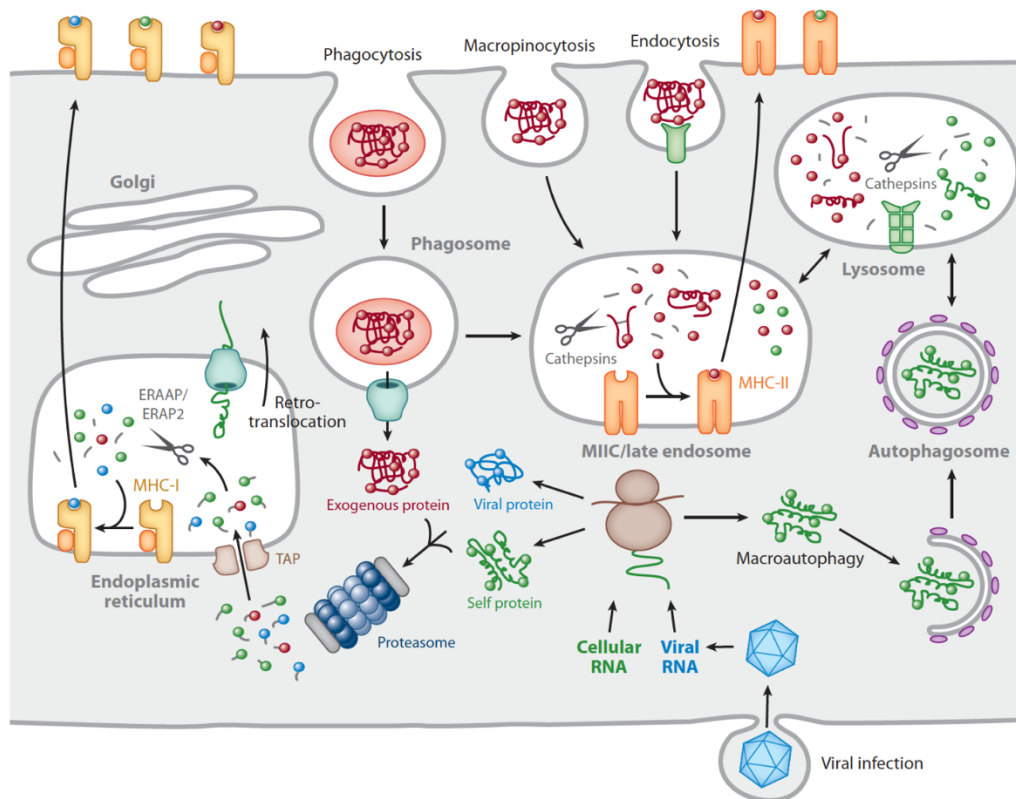


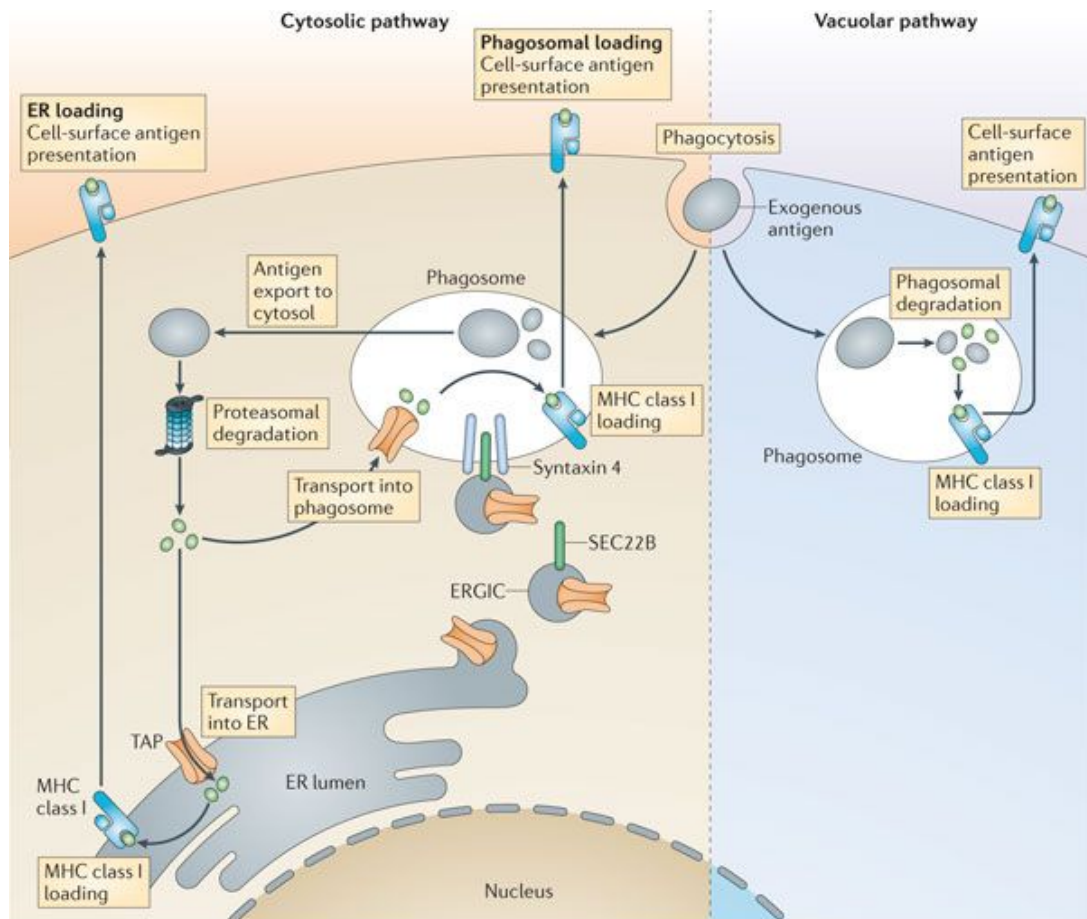
Figure 6. An overview of pathways involved in antigen internalisation, processing and presentation. Source: *Pathways of Antigen Processing*<sup>135</sup>.

#### 1.2.1.2. Cross-presentation of antigens by dendritic cells

In contrast to the presentation of self-antigens, cross-presentation is a process in which exogenous antigens (such as antigens derived from viral DNA) are presented in an MHC-I context. The cross-presentation is a mechanism that activates naïve antigen-specific CD8 T cells to become cytotoxic T cells (CTLs), which by releasing perforin and granzyme facilitate the killing of infected cells<sup>142</sup>.

The cytosolic pathway and the vacuolar pathway are two primary mechanisms described for cross-presentation. In the vacuolar pathway, phagocytosed exogenous PAMPs are degraded in the phagosome and loaded onto MHC-I, to be later transported and presented on the cell surface. In the cytosolic pathway, exogenous

peptides are degraded by proteasomes and transported to the ER lumen via TAP transporter proteins. In the ER, degraded antigens are loaded onto MHCI and transported to cell surface<sup>142</sup> (figure 7).



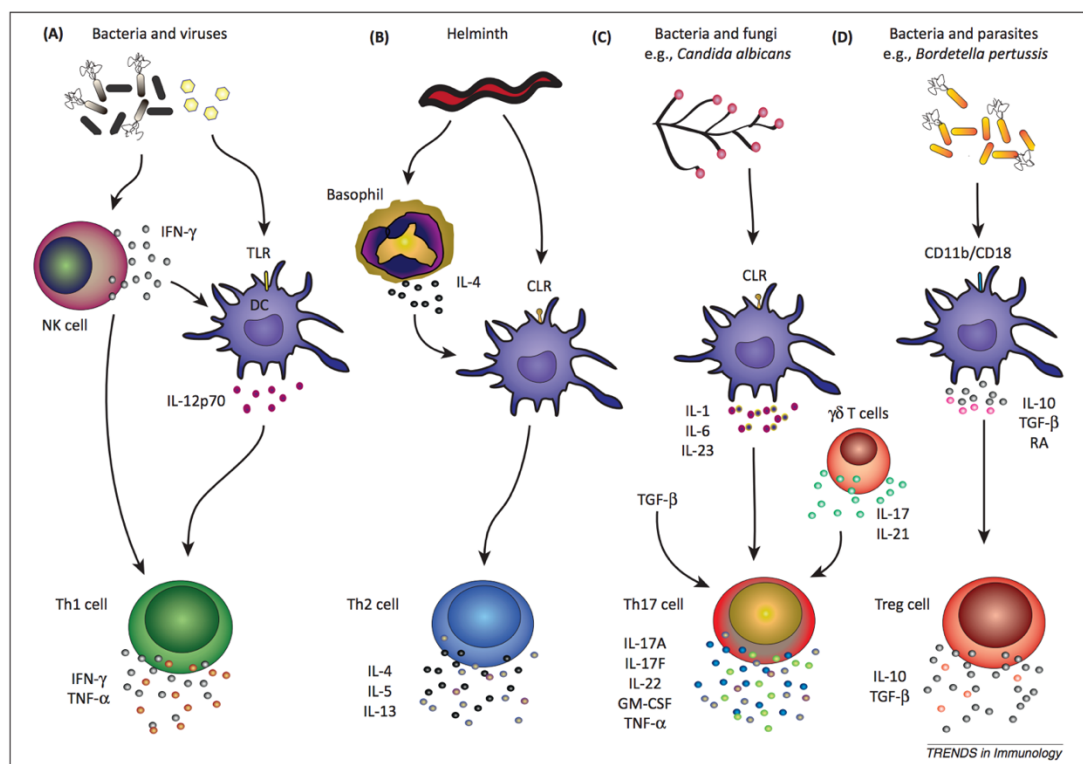
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Figure 7. An overview of cytosolic and vacuolar pathways used in antigen cross-presentation by dendritic cells. Source: Cross-presentation by dendritic cells<sup>142</sup>

### 1.2.1.3. The polarisation of T cells by dendritic cells

Dendritic cells present antigens to CD4 T cells to drive activation, proliferation and differentiation into an effector phenotype. A proportion of the effector T cell population will survive for years as an effector memory population, which can be quickly mobilised upon secondary antigen exposure. Depending on the type of antigens, DCs

can mediate T cell activation and drive them towards a specific phenotype, tailored for an efficient eradication or control of an antigen or pathogen. Three factors influence the type of T cell response that will develop after DC-T cell interaction. Firstly, the expression of co-stimulatory markers on a DC surface that drives CD4 T cell activation. Secondly, the type of antigen expressed by DCs in a context of MHCII molecule. Thirdly, the array of cytokines that DCs or cells within the vicinity of DC-T cell interaction produce<sup>143</sup>. There are four major types of TH subsets: TH1, TH2, TH17 and Tregs (figure 8) as well as TH9 and follicular helper T cells (Tfh).



*Figure 8. An overview of multiple pathways of T cell differentiation, stimuli and cytokines that induce them. Abbreviations: CLR, C-type lectin receptor; GM-CSF, granulocyte–macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; NK, natural killer; PAMPs, pathogen-associated molecular patterns; RA, retinoic acid; SEA, *Schistosoma mansoni* soluble egg antigen; TGF, transforming growth factor; Th, T helper cell; TLR, Toll-like receptor; TNF, tumor necrosis factor; Treg, regulatory*



*T cell. Source: Dendritic cells and other innate determinants of T helper cell polarisation*<sup>143</sup>.

A TH1 response is characterised by an elevated T cell production of IFN $\gamma$  and TNF $\alpha$ , which activate macrophages to induce nitric oxide (NO) mediated killing of pathogens. The differentiation of T cells to the TH1 phenotype requires a robust MHCII interaction with T cell receptor (TCR)<sup>144</sup>, DC-derived CXCL10 with T cell CXCR3 (in the lymph node)<sup>145</sup>, and ligation of mature DC molecules CD80 or CD86 to T cell CD28<sup>143</sup>. The TH1 response is often developed in response to viral, bacterial and protozoan infections, which stimulate membrane toll-like receptors (TLR) 4, TLR5, TLR12 and endosomal TLR3, TLR7 and TLR9<sup>146,147</sup>. TLR stimulation induces production of pro-inflammatory cytokines and IL12-p70, a crucial cytokine that drives TH1 differentiation<sup>143</sup> (figure 8). TH1 cells are characterised by the expression of T-bet, STAT3 and STAT1 transcription factors<sup>148</sup>.

A TH2 response is characterised by an increased T cell production of IL4, IL5 and IL13, and has evolved as a protective arm of immunity against large parasites such as helminths. TH2 cells are characterised by the expression of GATA3, STAT6, DEC2 and MAF transcription factors<sup>148</sup>. TH2 responses drive antibody class switching in B cells to IgG1 and IgE and alternatively activate macrophages to promote tissue repair<sup>149</sup>. A weak MHCII/TCR interaction<sup>144</sup>, as well as binding of the Notch receptor ligand, Jagged to Notch 1 and Notch 2 on CD4 cells, have been shown to contribute to TH2 differentiation<sup>150</sup>. The TH2 response is developed in response to parasitic antigens such as helminths or *Schistosoma mansoni* soluble egg antigens (SEA). These antigens engage C-type lectin-like receptors (CLRs) on the surface of DCs. DCs drive TH2 differentiation in cooperation with other cells. Following helminth exposure, epithelial cells produce IL25, IL33 and thymic stromal lymphopoietin (TSLP), which recruits IL4 producing basophils into the lymph nodes. Thus epithelial

cells, basophils and the cytokines that they produce cooperate with DCs to induce the TH2 phenotype (figure 8).

TH17 cells express ROR $\gamma$ t, STAT3, ROR $\alpha$  transcription factors<sup>148</sup>, and secrete large amounts of IL17A, IL17F, IL22, GM-CSF and TNF $\alpha$ , cytokines that recruit and activate neutrophils to the site of infection<sup>151</sup>. TH17 cells can, therefore, protect us from pathogens but also play a pathogenic role in many autoimmune diseases<sup>152</sup>. TH17 development is dependent on MHCII/TCR, CD80 or CD86/CD28 interactions<sup>143</sup>. TH17 cells recognise mainly fungi and bacterial antigens presented by DCs. The recognition of fungi and bacterial antigens is mediated by DC receptor CLR, Dectin-1, which recognises  $\beta$ -glucans from fungal species such as *Candida albicans*<sup>153</sup>. This activates a signalling cascade that activates NF- $\kappa$ B and drives the production of pro-inflammatory cytokines such as IL1, IL6, IL23, all essential for TH17 development<sup>154</sup>. TGF $\beta$  is also essential for TH17 development, and IL17 and IL21 derived from  $\gamma\delta$ T cells can induce TH17 differentiation<sup>143</sup> (figure 8).

Tregs are recognised by their production of IL10 and TGF $\beta$  or the expression of FoxP3, STAT5, FOXO1, and FOXO3 transcription factors<sup>148</sup>. Their role is to regulate immune responses and prevent the development of autoimmunity and immunopathology<sup>143</sup>. Immature or partially mature DCs induce Treg development. Interactions of inhibitory molecules such as CTLA4/CD80 or CD86, and programmed cell death 1 with programmed cell death 1 ligand (PD1/PDL1) send inhibitory signals to T cells promoting anergy and immunosuppression<sup>155</sup>. Chronic bacteria such as *Bordetella pertussis* and parasites drive Treg development. CD11b/CD18 receptors recognise antigens on the DC surface and signal via p38 mitogen-activated protein (MAP) kinase and suppression of interferon regulatory factors IRF1 and IRF8, which

inhibit IL12. This signalling cascade results in IL10 and TGF $\beta$  secretion, essential for Treg development<sup>156</sup> (figure 8).

Tfh cells express BCL-6 and STAT3 transcription factors and produce IL-21. They have shown to play a role in the induction of germinal centre responses as well as providing help for B cell class switching<sup>148</sup>. Lastly, TH9 cells express the PU.1 transcription factor and produce IL9 and IL10. The role of TH9 cells has been documented in host defence against nematodes and promotion of allergic inflammation<sup>148</sup>.

### **1.2.2. Differential phenotypes of dendritic cells**

DC functions are diverse and depend on the type of antigen they process and the environment DCs are in, including the vicinity of other cells and cytokines that they produce. Besides, DCs can be classified based on their origin, location and function. The main subsets of DCs (pDCs, cDC1, cDC2), as well as MDSCs, have unique functions and properties with implications for an immune response that they induce. These will be briefly discussed here.

#### **1.2.2.1. Classical dendritic cells**

Classical dendritic cells, also known as conventional or myeloid, are derived from CDPs, which undergo differentiation in peripheral organs or tissues. They traffic to lymph nodes from peripheral tissue via afferent lymphatic vessels<sup>157</sup>. cDCs can be divided into two subsets: cDC1, which have high expression of BCMA-1, BDCA-3 and CD1c; and cDC2, which highly express CD141<sup>158</sup>. Both cDC1 and cDC2 subsets have similar antigen degradation, processing and presentation capabilities however they differ in TLR expression patterns and responsiveness<sup>132</sup>. cDC1 cells express all TLRs

except TLR9, and they secrete an array of cytokines including IL-1 $\beta$ , IL-12, IL-6, TNF $\alpha$ , CXCL8/IL-8, CCL3, CCL4, and CCL5 and CXCL-10 upon TLR3 stimulation<sup>132</sup>. Their primary function is to prime CD4 T cells, and they are known to induce TH1, TH2 and TH17, however, this ability is strongly dependent on the tissue in which they reside. Lymph node resident DCs can induce TH1 and TH2 differentiation, skin resident DCs promote TH2, whereas blood DCs are poor TH2 inducers<sup>132</sup>.

cDC2 cells express TLR3 and TLR10 and have low expression of TLR1-2, TLR6 and TLR8. They mainly respond to TLR3 ligands (poly I:C) and produce CXCL10/IP10, CCL5, IL12p70, IFN $\beta$  and IFN $\lambda$  cytokines (a type III interferon with anti-viral properties)<sup>132</sup>. cDC2 are also known to promote TH1 immune responses and play a role in cross-presentation of tumour and pathogen-derived antigens to induce effector CD8 T cell responses<sup>159</sup>.

#### **1.2.2.2. Plasmacytoid dendritic cells**

Plasmacytoid dendritic cells are a unique subset of dendritic cells that can be broadly characterised by the production of type I interferons and expression of the CD123 marker (figure 9). Their general role is mediation of antiviral immune responses however they have much broader roles in other areas of the immune system<sup>157</sup>. pDCs develop in the bone marrow, circulate in the blood and arrive in T cell areas of the lymph nodes via high endothelial venules (HEV). pDCs also migrate from secondary lymphoid organs or blood into peripheral tissues<sup>160</sup>.

A common DC progenitor (CDP) is characterised by a lack of lineage markers and expression of Fms-like tyrosine kinase 3 (FLT3 or CD135), macrophage colony-stimulating factor receptor (M-CSFR or CD115 or CSF1R) and the receptor tyrosine kinase KIT (CD117 or SCFR)<sup>157</sup>. CDP is found in the bone marrow and

can give rise to both pDCs and cDCs. The development of pDCs involves differentiation of CDP to pDC-like common DC precursor that is negative in CCR9 and low in MHCII expression<sup>161</sup>. These precursors later differentiate into pDCs, and all of these processes take place in the bone marrow<sup>157</sup>.

pDCs play a role in both innate and adaptive immune responses. They present antigens in the context of MHCI and MHCII and activate T cells with co-stimulatory molecules CD80, CD86 and CD40. They produce type I IFNs to maintain an antiviral state, activate NK cells, and promote effector CD8 and TH1 responses. They can release an array of cytokines and chemokines to drive recruitment of immune cells and have been shown to play a role in regulatory responses by driving Tregs as well as inducing cell death and T cell suppression<sup>157</sup>.

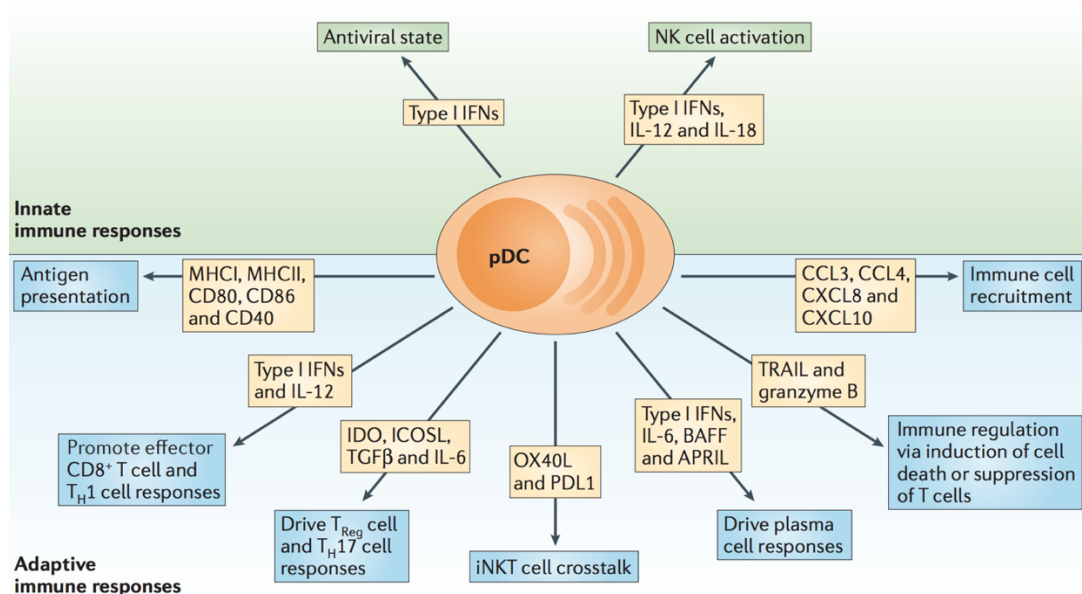


Figure 9. An overview of functions of plasmacytoid dendritic cells. Source: *The multifaceted biology of plasmacytoid dendritic cells*<sup>157</sup>

### 1.2.2.3. Monocyte-derived dendritic cells

Monocyte-derived dendritic cells are a unique DC subset that arises from blood circulating monocytes, which respond to local inflammation. Studies *in vitro* confirm differentiation of blood or bone marrow CD14 monocytes to DC-like cells after GM-CSF and IL4 stimulation<sup>162</sup>. These *in vitro* MDDCs have increased capacity to internalise, process and present antigens; they show increased stimulatory capacity in leukocyte reactions; and exposure to lipopolysaccharide (LPS) or BCG antigens induces HLADR, the maturation marker CD83 and T cell co-stimulatory markers CD80, CD86, and CD40<sup>163</sup>. *In vivo* mouse studies confirm that CD11b<sup>+</sup> F4/80<sup>-</sup> monocytes are recruited to the site of inflammation, and about 25% of them migrate to T cell areas of the draining lymph node and differentiate into DC-like cells, expressing upregulated MHCII, CD11c, CD80 and CD86<sup>164</sup>.

MDDCs become abundant in inflammation, and their increased levels were found in numerous fungal, protozoan and bacterial infections, including pulmonary Mtb<sup>165</sup>. They have been shown to play a role in both innate and adaptive parts of the immune response. MDDCs mediate iNOS dependent bacterial killing as well as iNOS mediated tissue toxicity in *Listeria monocytogenes*<sup>166</sup> and *Trypanosoma brucei*<sup>167</sup> infections respectively. They also phagocytose *Salmonella* bacteria during gastrointestinal infection<sup>168</sup>. As for the adaptive immune response, MDDCs have been shown to modulate T cell responses. Infection models confirm that MDDCs have an *ex vivo* (and some *in vivo*) capacity to stimulate CD4 T cell proliferation<sup>165</sup>. They have been shown to polarise T cells to their TH1<sup>169</sup>, TH2<sup>170</sup> or TH17<sup>171</sup> phenotypes and have been highlighted as cells that can induce TGFβ-dependent IgA production and class-switching<sup>172</sup>.

### 1.2.3. The role of dendritic cells in Mtb infection

The role of DCs in Mtb infection is complicated, and studies report contrasting results. Numerous studies report that DCs increase the cellular response against Mtb infection<sup>173</sup>. Both human<sup>174</sup> and mouse<sup>175</sup> DCs are able to phagocytose Mtb. At the onset of inflammation, DCs are highly represented<sup>176</sup>, and Mtb antigen presenting DCs mostly reside in airway epithelium, lung parenchyma, and visceral pleura<sup>176</sup>. Mucosal DCs are immature and specialise in antigen processing. TLRs recognise Mtb PAMPs resulting in DC maturation and trafficking to lymphoid organs where they prime T cells through MHCII expression and cytokine production such as IL12, essential for TH1 priming and effective Mtb killing<sup>173</sup>. In contrast, Mtb PAMPs recognised by DC-SIGN block NF $\kappa$ B activation resulting in a cascade of events: high IL10 levels, impaired DC maturation, masking of the Mtb pathogen in DCs, limited T cell activation, and subsequently impaired Mtb killing<sup>173,177</sup>. Mtb has evolved mechanisms that subvert host immune responses. A study has shown that mice Mtb infection delays the recruitment of DCs in the lungs impairing early immune responses and promoting Mtb replication<sup>178</sup>. Mtb infection has also shown to direct Th2 differentiation of CD4 T cells by inducing production of IL1 $\beta$  in dendritic cells<sup>179</sup>; it has also shown to promote the expansion of Tregs via the upregulation of PDL1 on dendritic cells<sup>180</sup>.

An *in vitro* infection of MDDCs with Mtb drives their maturation and upregulates expression of MHCI, MHCII, CD40, CD54, CD58, CD80, CD83; and increases their production of TNF $\alpha$ , IL1, and IL6<sup>181,182</sup>. MDDCs have also shown to be capable of an effective Mtb phagocytosis<sup>181</sup>. In contrast to MDDCs, Mtb infected macrophages show downregulation of MHCII and upregulation of CD40 and CD54. They were also unable to, unlike MDDCs, prime T cells to produce IFN $\gamma$ <sup>183</sup>. Mouse studies confirm

that DC depletion resulted in impaired CD4 responses and subsequent uncontrolled replication of Mtb in lungs and the spleen<sup>184</sup>. Human genetic deficiencies where DC functions are impaired have reported an uncontrolled BCG-osis and infection with *Mycobacterium kansasii*<sup>185</sup>.

#### **1.2.4. Dendritic cells: summary**

Dendritic cells vary in location, function and phenotype. They can elicit an array of immune responses to different stimuli. Thus, DC heterogeneity between populations could very well underlie variations in BCG immunogenicity. Going deeper, what are the molecular mechanisms that could be driving the underlying immune responses? Why do we observe sustained differences in immune responses? Could the newly emerging field of epigenetics explain some of these variations?

### **1.3. Epigenetic modifications as a molecular mechanism to shape immune processes.**

Epigenetics is a term used to describe heritable changes in DNA expression and activity that do not involve alterations to the DNA sequence<sup>186</sup>. The epigenetic modifications are accomplished using many mechanisms, including DNA methylation, histone modifications, and small interfering RNA (siRNA)<sup>186</sup>. Understanding epigenetics and mechanisms that drive epigenetic changes may help to explain why cells that carry identical DNA express different functions and differentiate into various phenotypes. Extrapolating the concept, through an understanding of epigenetics might explain diversities in immune responses following infection or vaccination<sup>187</sup>.



### 1.3.1. Chromatin structure

The distribution of genomic and eukaryotic DNA is regulated by proteins called histones, which modulate the formation of DNA/protein complexes, known as chromatin. The nucleosome, a basic unit of chromatin, consists of 146 DNA base pairs (bp), which are wrapped around an octamer of the four core histones (H2A, H2B, H3 and H4). These are tightly packed with amino-terminal tails extending from the globular region, making them accessible to histone modifying enzymes, allowing for epigenetic modifications<sup>188</sup>.

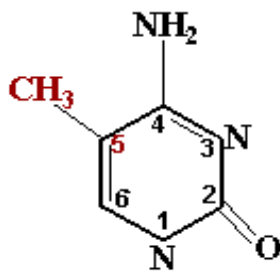
There are two types of chromatin: euchromatin and heterochromatin, both distinguishable by structure and function. Euchromatin is a less condensed, more open and accessible form of chromatin, ready for gene transcription and often associated with activated genes. Conversely, heterochromatin is a more packed and condensed form of chromatin, inaccessible for gene transcription and associated with gene silencing and inactivation<sup>189</sup>. Many epigenetic changes modulate the structure of the chromatin and its "readiness" for gene transcription. Thus, euchromatin is often associated with hypomethylated DNA and hyperacetylated histones, whereas hypermethylation of DNA and hypoacetylation of histones are characteristics of heterochromatin structure (table 1)<sup>187,190</sup>.

Table 1. An overview of structural and epigenetic differences between euchromatin and heterochromatin

	Chromatin Features			Epigenetic Markers		
Type of Chromatin	Structure	DNA Sequence	Activity	DNA Methylation	Histone Acetylation	Histone Methylation
<b>Euchromatin</b>	Less Condensed Open Accessible	Gene Rich	Expressed Active	Hypomethylation	Hyperacetylation of H3 H4	H3K4me2 H3K4me3 H3K9me1
<b>Heterochromatin</b>	Condensed Closed and Inaccessible	Repetitive Elements	Repressed Silent	Hypermethylation	Hypoacetylation of H3 H4	H3K27me2 H3K27me3 H3K9me2 H3K9me3

### 1.3.2.DNA Methylation

DNA methylation is the most characterised epigenetic modification, essential for gene regulation, development and tumorigenesis. In mammalian cells, it mostly occurs at the 5' position of the cytosine base within the CpG dinucleotide, creating 5-



*Figure 10. DNA methylation at 5' cytosine results in a formation of 5-methylcytosine.*

methylcytosine (5mC) (figure 10). The addition of 5' methyl group to cytosine is catalysed by DNA methyltransferases (DNMTs), including DNMT3a, DNMT3b (initiators of de novo methylation in stem cells and cancer cells) and DNMT1 (recognises and copies methylation patterns of CpGs, which were initiated by de novo DNMTs)<sup>186</sup>. Distribution and methylation of CpGs were found to regulate gene silencing, transcription and

chromosomal stability<sup>191</sup>. More specifically, CpG hypermethylation drives structural changes of chromatin, making hypermethylated genes inaccessible for transcription. Conversely, the absence of DNA methylation results in CpG dinucleotides being readily available to transcription factors, allowing gene transcription<sup>192</sup>. Abnormal methylation patterns can be observed in human tumours, which have a unique ability to regulate the expression of its oncogenes, thereby outperforming the host's cancer-fighting mechanisms<sup>187,191</sup>.

### 1.3.3.Histone Modifications

Core histones and more specifically their amino terminals undergo many epigenetic alterations, including most commonly acetylation and methylation, as well as phosphorylation, ubiquitination and sumoylation<sup>193</sup>. Histone acetyltransferase enzymes (HATs) and histone deacetylases (HDACs) regulate the acetylation and

deacetylation of histones respectively. Acetylation, removes positive charges between DNA and histones, thereby reducing affinity and shaping chromatin into euchromatin structure, allowing for DNA processes such as transcription and repair to take place. Conversely, the deacetylation of histones by HDACs introduces positive charges between DNA and histones, increases affinity and shapes chromatin into densely packed heterochromatin, inaccessible for transcription and other DNA processes<sup>186</sup>. In contrast to histone acetylation, which is positively associated with gene transcription, the role of histone methylation is more heterogeneous. Histone methylation can result in methylation of various lysine residues and can vary in its extent, which may be mono, duo or trimethylation. The literature suggests that H3K4me, H3K36me and H3K79me methylation patterns are associated with activated gene regions<sup>190</sup>, whereas H3K9me, H3K20me and H4K27me methylation patterns correlate with gene silencing and inactivation (table 1)<sup>193</sup>. The degree of methylation dictates gene expression. Trimethylation at H3K9me3 and H3K27me3 correlates with gene repression and silencing, whereas monomethylation of the same lysine residues (H3K9me1 and H3K27me1) is associated with gene transcription and activation (table 1)<sup>187,194</sup>.

#### **1.3.4. Non-coding RNA, transcriptional gene silencing and microRNAs**

Small RNAs were discovered as a potential template molecule to mediate epigenetic modifications. They play a role in a process known as transcription gene silencing (TGS)<sup>186</sup>. Small RNAs function within Argonaute (AGO) complex that targets nascent transcript scaffolds, mRNA, and long non-coding RNAs (lncRNAs), which all play a role in the recruitment of chromatin-modifying scaffolds. These scaffolds initiate epigenetic mediated changes in gene expression and maintain a persistent memory of epigenetic silencing by forming positive feedback loops within chromatin-based signals. lncRNAs from enhancer regions have been shown to activate gene

expression by bringing together enhancer and promoter regions, and activatory molecules to histones<sup>195</sup>.

In contrast, microRNAs (miRNAs) are non-coding RNAs that affect gene expression at the post-transcriptional level and target miRNAs directly. miRNAs mature and merge with the RNA-induced silencing complex (RISC) to inhibit mRNA mediated transcription. miRNAs have been shown to play a role in the regulation of multiple cellular and immune processes including the regulation of cytokine expression<sup>196</sup>.

### **1.3.5. The regulation of epigenetics by infection and environmental factors**

Because of the advances in mapping human DNA, it has become apparent that genes do not solely explain susceptibility to diseases and epigenetic mechanisms must be considered. There is growing evidence suggesting that environmental factors (nutritional supplements, low-dose radiation, reproductive factors, xenobiotic chemicals and others) present in the prenatal and postnatal period of life, modulate heritable changes in epigenetic markers and influence the susceptibility to a number of adult chronic diseases, including: cancer, diabetes, obesity, cardiovascular diseases; as well as behavioural disorders such as schizophrenia<sup>197</sup>. One of the most characterised examples of epigenetic modification following environmental exposure is the mouse Avy allele also known as agouti. There is substantial evidence that a degree of methylation of IAP, an element at the transcription site of the Avy allele, is associated with the phenotypic heterogeneity and disease susceptibility in mice, and many environmental factors modulate this methylation. A maternal diet rich in methyl-donor supplementation such as folic acid, vitamin B12, choline and betaine, hypermethylates the IAP element of the agouti gene resulting in offspring with brown fur. Conversely, a diet short of these supplements hypomethylates the IAP element

and shifts the fur colour distribution to yellow. Additionally, yellow fur mice were much more susceptible to diabetes, obesity and tumorigenesis whereas brown fur mice were protected from these chronic diseases, highlighting the importance of epigenetic mechanisms in susceptibility to chronic diseases<sup>197</sup>. Human studies conducted in The Gambia report that season at conception and associated maternal nutrition modulate DNA methylation of infants<sup>198</sup>.

Many studies are suggesting that an infection can alter the epigenetic landscape of the host and subsequently affect future immune responses. These studies have been extensively reviewed by Bierne et al.<sup>199</sup>. In short, the literature supports that infection can alter the host's epigenetic landscape by changing DNA methylation patterns, introducing histone modifications (acetylation, methylation, phosphorylation etc.), and inducing the expression of non-coding RNA (ncRNA). An excellent example of epigenetic modifications has recently been highlighted by Wen et al.<sup>200</sup>. It is well documented that a severe innate immune response such as septic shock is followed by deficiencies in subsequent immune responses in patients; however, the mechanism behind these deficiencies is unclear<sup>201</sup>. The research group has found that in mice, following peritonitis-induced sepsis, newly formed dendritic cells have significantly reduced expression of IL12, independently of immunoregulation by IL10. They found decreased levels of H3K4me3 and increased H3K27me2 histone modifications at IL12p35 and IL12p40 gene promoters, suggesting a heterochromatin conformation of chromatin and limited gene transcription<sup>200</sup>. In another example, an infection of macrophages with Mtb results in an upregulation of a chromatin-modifying complex Sin3a. Sin3a, along with HDAC modify chromatin into its densely packed form of heterochromatin, preventing transcription of genes such as IFN $\gamma$  inducible genes, including MHC class II<sup>202</sup>. The downregulation of MHC class II genes impairs the presentation of Mtb antigen, subsequently hindering host's immune responses and giving the pathogen survival advantage. Aside from modifying histones, bacteria

have been shown to have the ability to introduce aberrant DNA methylation patterns. Uropathogenic *Escherichia Coli* (UPEC) infection of human uroepithelial cells recruits DNMTs, including DNMT1, resulting in de novo methylation of CpG islands of the CDKN2A gene, encoding for cell cycle regulatory proteins. As a result, UPEC counteracts host cell apoptosis of uroepithelial cells and promotes their proliferation to maintain pathogen persistence<sup>187,203</sup>.

### **1.3.6. The epigenetic control of immune responses**

Epigenetic modifications can be modified by an infection, abnormal cell proliferation, vaccination and environmental factors. However, epigenetic modifications can also mediate multiple processes including cellular, metabolic, signalling and immune processes. Indeed, epigenetic modifications play an extensive role in the regulation of immune pathways<sup>204</sup>.

#### **1.3.6.1. The epigenetic regulation of innate immunity**

DNA methylation and histone modifications have been shown to play a role in the regulation of innate immunity. Increased methylation of CpG islands of *TLR2* gene leads to lower levels of TLR2 expression and is associated with increased risk of pulmonary TB<sup>205</sup>. Similarly, decreased methylation in the *TLR2* gene results in higher expression of TLR2 and an enhanced inflammatory response to bacterial challenges in cystic fibrosis patients<sup>206</sup>. Studies in Dutch adolescents have shown that BCG vaccination induced increased levels of H3K4me3 histone modifications of proinflammatory cytokines TNF $\alpha$  and IL6, as well as of the TLR4 receptor. These changes were associated with increased secretion of these cytokines and expression of TLR4 up to 3 months post BCG training<sup>87</sup>. Studies indicate that epigenetic mechanisms regulate macrophages and their functions. Engagement of TLRs in

macrophages induces a gene permissive histone acetylation of inflammatory genes<sup>207</sup> and a subsequent increase of proinflammatory cytokines in response to LPS. An increase in H3K4 methylation of the *TNFAIP3* gene (a facilitator of deubiquitination of NF $\kappa$ B and TRAF6) results in suppression of NF $\kappa$ B and mitogen-activated protein kinase signalling, resulting in decreased levels of IL6<sup>208</sup>.

#### **1.3.6.2. The epigenetic regulation of cell differentiation and adaptive immunity**

The differentiation of myeloid cells, their function and phenotype are regulated by epigenetic modifications. The polarisation of M2 macrophages has been shown to be mediated by epigenetic mechanisms. Satoh et al<sup>209</sup>. explained that a useful polarisation of macrophages requires a conversion of H3K27me3 to H3K27me1 via JMJD3, a demethylation protein that is induced by TLR stimulation in macrophages<sup>209</sup>. Another group has shown that in mice, a loss of histone deacetylase family 3 (HDAC) in macrophages makes them sensitive to IL4 and skews their differentiation to the M2 phenotype. This suggests a role for histone acetylation in responsiveness to IL4 and macrophage differentiation to their M2 state<sup>210</sup>.

The differentiation of monocyte-derived dendritic cells from monocytes is associated with a substantial loss of DNA methylation, mostly of enhancers and binding sites for transcription factors associated with the dendritic cell lineage. These findings were co-observed with dynamic expression changes of genes known to regulate DNA methylation: DNMT1, DNMT3, DNMT3B, and TET2<sup>211</sup>.

Naïve CD4 T cells differentiate into polarised TH phenotypes, and this process is mediated by changes in histone modifications and DNA methylation. The differentiation of T cells and the mediation of their function by epigenetic mechanisms



have been extensively reviewed <sup>212,213</sup>. High methylation of the *IL4* gene can characterise TH1 T cells whereas TH2 cells have high methylation of the *IFNG* gene<sup>214</sup>. Naïve T cells are marked by high levels of H3K9me2 (gene silencer) at the *IFNG*, *IL4*, *IL17A*, *TBX21*, *GATA3*, *RORC* and *FOXP3* genes. However, upon differentiation, modification of histones at these genes changes, allowing for gene transcription and translation. The H3K9me2 modification is removed from the *IFNG* and *TBX21* genes in TH1 cells; from the *IL4* and *GATA3* genes in TH2 cells; from the *IL17A* and *RORC* genes in TH17 cells; and from the *FOXP3* gene in Treg cells<sup>212</sup>. The differentiation and regulation of functions of CD8 T cells have also been shown to be regulated by epigenetic mechanisms<sup>215</sup>. Studies have found a loss of permissive H3K4me3 and H3K9ac modifications which are replaced by repressive H3K27me3 and DNA methylations at genes the expression of which are reduced in effector cells (*FOXO1*, *KLF2*, *LEF1*, *TCF7*, *IL2RA*, *CD27*, *TNF*, *CCR7*). Conversely, transcription factors (*EOMES*, *TBX21*, *PRDM1*) and effector genes (*GZMA*, *GZMB*, *PRF1*, *IFNG*, *KLRG1*) of which expression is increased in effector cells have a decrease in repressive and an increase in permissive epigenetic markers<sup>215</sup>.

Lastly, studies have investigated the role of epigenetics in the induction of vaccine-induced immune responses. A comparison of DNA methylation between low and high responders to Hepatitis B vaccine revealed numerous genes to be differentially methylated between two groups. A closer investigation revealed potential novel gene candidates: RNF39 was hypomethylated, and SULF2 was hypermethylated in indigent respondents to the Hepatitis B vaccine<sup>216</sup>. Another study investigated whether epigenetic modifications can mediate the immune response to the influenza vaccine. This showed that a decrease in DNA methylation of cellular differentiation, signalling and antigen presentation pathways was associated with an increased humoral response to influenza vaccine<sup>217</sup>.

The role of epigenetic modifications in immunity is vast and spans multiple arms of the immune system. Epigenetic modifications also play a role in pathogen-specific immune responses, and new evidence suggests a link between Mtb infection and epigenetics.

### **1.3.7. The role of epigenetics in Mtb infection**

The immune response to the Mtb is heterogeneous and often not optimal and sufficient to effectively kill the pathogen. Epigenetic modifications have been proposed as a mechanism to explain these variations. A growing number of studies show how the epigenome affects the immune response to Mtb and conversely, how Mtb can utilise epigenetic modifications to divert the host's immune response and sustain its own survival. The epigenome of the host can affect the immune response to Mtb. Gringhuis et al.<sup>218</sup> showed that following Mtb infection of dendritic cells, the methylation of NFκB is essential for its activation and increased transcription of IL10.

Mtb utilises epigenetic mechanisms to regulate host immune responses. It employs 19 kDa lipoprotein to antagonise TLR2 via deacetylation of histones of MHC2TA and class II transactivator gene (CIITA) promoters. The deacetylation of these genes results in a decreased presentation of Mtb antigens, T cell activation, and sustained survival of Mtb within cells<sup>219</sup>. Enhanced cellular survival protein (Eis) found in Mtb can inhibit ERK1/2 and JAK pathways in activated T cells. It does so by acetylation of DUSP16/MKP-7, both genes that are essential regulators of the ERK1/2 and JAK pathways. Thus Mtb can negatively inhibit T cell activation by acetylation of crucial ERK1/2 and JAK pathway genes<sup>220</sup>. Studies also demonstrate associations between TB disease or susceptibility and epigenetic modifications. Hypermethylation of the vitamin D receptor (VDR) gene results in increased susceptibility to TB, both in African and European populations<sup>221</sup>; the IL17 gene is hypermethylated in macrophages

isolated from active or latent TB when compared to healthy individuals<sup>222</sup>; and TB patients have hypermethylation of the TLR2 gene CpG islands<sup>223</sup>. Additionally, there is growing evidence of a role for miRNAs in response to Mtb infection. miRNA-21 targets IL12 mRNA and inhibits its translation<sup>224</sup>; miRNA-29 targets IFN $\gamma$  to suppress the immune response to Mtb<sup>225</sup>; and other miRNAs were able to target multiple inflammatory mRNAs subsequently attenuating the expression of pro-inflammatory cytokines and regulating the immune response to Mtb<sup>226</sup>.

In summary, studies demonstrate that Mtb uses epigenetic mechanisms to interfere with host immune responses. It does so by decreasing antigen presentation as well as inhibiting critical proinflammatory cytokines.

#### **1.3.8. Transcriptomic signatures in TB vaccine trials**

Epigenetic mechanisms modify the readiness of genes for mRNA transcription and subsequent protein translation. Therefore, one could expect, that mRNA and its signatures play a substantial role in the TB field. Because of technical advances, numerous studies have explored whether we can use transcriptomics to distinguish between healthy controls, active TB, LTBI, patients that are at risk of progressing to active TB, and patients that remain latently infected. Zak et al.<sup>227</sup> have identified a mRNA signature of 16 genes that prospectively identified people at risk of developing active TB in African adolescents<sup>227</sup>. Anderson et al.<sup>228</sup> have developed a 51-transcript signature that can distinguish TB from other diseases in African children<sup>228</sup>. Cliff et al.<sup>229</sup> have identified dynamic phases of blood gene expression patterns that change throughout TB treatment<sup>229</sup>. These and other studies highlight the growing role of transcriptomics in the TB field. They help to progress towards identifying reliable correlates of BCG protection or TB risk.

#### **1.4. Introduction summary**

The burden of TB remains high, and there is a growing threat of multi-drug resistant TB. Research consortia such as TBVAC2020 and international organisations like the European Union and United Nations highlight the need and facilitate research for the development of new TB vaccines. Despite a moderate number of TB vaccines in the pipeline BCG is still the only licensed vaccine for tuberculosis. The efficacy of BCG against pulmonary TB varies across geographical regions, and a comparison of immune responses between countries revealed distinct immune responses, potentially explaining the observed BCG efficacy disparities. The development of new TB vaccines and the improvement of BCG is impaired by a lack of reliable correlates of protection; limited understanding of the complex immunity that BCG induces across populations; and a lack of knowledge of molecular mechanisms that drive sustained BCG induced immune responses. Novel avenues of research must be explored to answer these questions. Dendritic cells have been highlighted previously to have a role in BCG mediated immunity; however, they have been poorly characterised across populations, and their contribution to BCG immunogenicity remains unclear. Molecular mechanisms have been largely neglected in vaccine efficacy trials, and epigenetic modifications have been indicated to play a role in vaccine-induced immune responses including following BCG as well as in Mtb immunity. This PhD will contribute to a better understanding of BCG induced immunity in the UK and Uganda and will investigate immune (dendritic cells) and molecular (epigenetic modifications) mechanisms that could explain underlying BCG immunogenicity profiles between these two countries.

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## **2. Aims, Objectives and hypotheses.**

BCG varies in efficacy and immunogenicity across geographical locations and reasons for these variations as well as the molecular mechanisms that drive them are still not well understood. This study will involve an examination of the immune responses of UK and Ugandan infants as well as an investigation into potential mechanisms, specifically, the involvement of dendritic cell phenotype and epigenetic profiles, which modulate these responses in a host. An in-depth understanding of immune and molecular mechanisms that determine vaccine efficacy is essential for the optimal design of new and improved vaccines, including BCG. The overarching hypothesis for this study is that DNA methylation patterns or dendritic cells phenotypes and composition are underlying mechanisms that can explain the spectrum of BCG immunogenicity and in this study, are associated with the immune response to the BCG vaccine. The study is split into four specific aims, each one addressed in a separate results chapter with the aim-specific hypothesis.

- 1) Aim 1 is to conduct a pilot study of DNA methylation profiles and their relationship to BCG-induced immune responses in BCG-vaccinated South African infants.
- 2) Aim 2 of this study is to compare immune responses to BCG vaccination in the UK and Uganda.
- 3) Aim 3 is to investigate the role of dendritic cell in Uganda in the context of BCG vaccination.
- 4) Aim 4 is to compare DNA methylation profiles between UK and Ugandan infants at 10 and 52 weeks after BCG vaccination.

### **3. Differential DNA methylation of potassium channel KCa3.1 and immune signalling pathways is associated with infant immune responses following BCG vaccination.**

In this chapter, I explore the relationship between the immune response in BCG vaccinated South African infants and their DNA methylation profile. I aim to answer whether there is differential methylation of genes that could predict characteristics of immune profiles to the BCG vaccine. I also conducted this study to establish protocols for the DNA methylation analysis of whole blood using the RRBS assay. I used this study as a platform to develop a data analysis pipeline for the DNA methylation data analysis.



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## RESEARCH PAPER COVER SHEET

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### SECTION A – Student Details

Student	Mateusz Hasso-Agopsowicz
Principal Supervisor	Dr Steven Smith
Thesis Title	A STUDY OF FACTORS UNDERLYING BCG IMMUNOGENICITY DIFFERENCES ACROSS COUNTRIES: THE INFLUENCE OF DNA METHYLATION PATTERNS AND ANTIGEN PRESENTING CELLS

**If the Research Paper has previously been published please complete Section B, if not please move to Section C**

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Where was the work published?	
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Where is the work intended to be published?	Scientific Reports
Please list the paper's authors in the intended authorship order:	Mateusz Hasso-Agopsowicz, Thomas J Scriba, Willem Hanekom, Hazel M Dockrell, Steven G Smith
Stage of publication	Responded to reviewers comments, waiting for approval

### SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I stained samples for flow cytometry analysis. Analysed the flow cytometry data and chose samples for the DNA methylation analysis. I extracted the DNA, prepared the DNA methylation library and sequenced samples. I prepared FASTA files and established a data analysis pipeline. I analysed the DNA methylation data and consulted with experts on the best approach. I prepared the graphs and wrote the manuscript.
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Student Signature: \_\_\_\_\_

Date: 23/07/2018

Supervisor Signature: \_\_\_\_\_

Date: \_\_\_\_\_

**Differential DNA methylation of potassium channel KCa3.1 and immune signalling pathways is associated with infant immune responses following BCG vaccination.**

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## Summary

*Bacillus Calmette–Guérin* (BCG) is the only licensed vaccine for tuberculosis (TB) and induces highly variable protection against pulmonary disease in different countries. We hypothesised that DNA methylation is one of the molecular mechanisms driving variability in BCG-induced immune responses. DNA methylation in peripheral blood mononuclear cells (PBMC) from BCG vaccinated infants was measured and comparisons made between low and high BCG-specific cytokines responders. We found 318 genes and 67 pathways with distinct patterns of DNA methylation, including immune pathways, e.g. for T cell activation, which are known to affect immune responses directly. We also highlight signalling pathways that could indirectly affect the BCG-induced immune response: potassium and calcium channel, muscarinic acetylcholine receptor, G Protein-coupled receptor (GPCR), glutamate signalling and WNT pathway. This study suggests that in addition to immune pathways, cellular processes could influence vaccine-induced immune responses. Our results highlight mechanisms that require consideration when designing new TB vaccines.



## Introduction

*Mycobacterium bovis* Bacillus Calmette–Guérin (BCG) is the only licensed vaccine against tuberculosis (TB). BCG protects against severe childhood cases of miliary TB and tuberculous meningitis<sup>1</sup>. However, the protection afforded by the vaccine against pulmonary TB varies across geographical regions with studies reporting vaccine efficacy ranging from 0% in India<sup>2</sup> to 80% in the United Kingdom (UK)<sup>3</sup>. Reasons for these variations remain unknown. Possible explanations include comorbidities<sup>4</sup>, pre-exposure to environmental mycobacteria<sup>5</sup>, and genetics<sup>6</sup>. Previous studies in Malawi<sup>7</sup>, The Gambia<sup>8</sup>, Indonesia<sup>9</sup> and the UK<sup>8,10</sup> have shown disparate immune responses across these populations, with a dominant production of IFN $\gamma$  in the UK and higher production of T helper (Th) 2 cytokines in Malawi and The Gambia<sup>10</sup>. Multiple efforts are being undertaken to identify correlates of protection following BCG vaccination<sup>11,12</sup>. The importance of IFN $\gamma$  has been highlighted in numerous studies<sup>8,13–16</sup>, however, the Th1 boosting candidate TB vaccine MVA85A (Modified Vaccinia virus Ankara expressing Ag85A from *M. tuberculosis*) failed to improve on protection afforded by BCG in vaccinated infants<sup>17</sup>; a large South African study did not find that BCG specific Th1 response correlated with risk of TB disease<sup>18</sup>; however further analysis of the MVA85A clinical trial showed that the frequencies of cells producing BCG-specific IFN $\gamma$  were associated with a reduced risk of developing disease<sup>16</sup>.

Another unknown factor in the immune response to the BCG vaccine is the molecular mechanism that drives these immune responses. Recent studies highlight the role of transcriptomics<sup>19–21</sup> as a means of identifying the fundamental mechanisms and the importance of measuring RNA profiles in vaccine trials. Epigenetics, a mechanism known to play a role in the regulation of gene expression, is another mechanism of growing importance. Specifically, DNA methylation of CpG dinucleotides in mammals is known to regulate gene expression and subsequent protein production<sup>22,23</sup>. *In-vitro*

methylation of herpes thymidine kinase (Tk) genes resulted in *in-vivo* downregulation of gene expression <sup>24</sup> and methylation of the O<sup>6</sup>-methylguanine-DNA-methyltransferase (MGMT) gene was negatively correlated with its protein concentration in humans <sup>25</sup>. In the context of immune responses, two recent studies have examined the role of epigenetic regulation, gene expression and protein production in responses to Hepatitis B <sup>26</sup> and Influenza <sup>27</sup> vaccines. Several differentially methylated genes were found between low and high immune responders to Hep B vaccine, and DNA methylation was correlated with gene expression and protein levels for multiple genes following influenza vaccination. Lastly, recent evidence shows that DNA methylation of macrophages is associated with anti-mycobacterial activity in BCG vaccinated participants <sup>28</sup>.

We have examined whether the DNA methylation profile of peripheral blood mononuclear cells (PBMC) in BCG vaccinated infants is associated with the magnitude of BCG specific immune responses. We found novel pathways and genes that were differentially methylated between high and low BCG responders amongst South African infants who received BCG vaccination at birth. This knowledge will allow us to understand molecular mechanisms that drive vaccine-induced immune responses, paving the way to design better and more effective TB vaccines.

## Results

### *Sample collection and processing*

We used 60 archived frozen PBMC samples from a previous study<sup>18</sup>. 36.7% were female, 83.3% Cape Mixed Ancestry, 13.3% Black African and 3.3% Asian. All participants received the Japanese BCG vaccine at birth, and their blood was collected at ten weeks post vaccination.

### *An increase in cytokine production after BCG stimulation is not correlated with cell phenotype.*

To investigate the magnitude of immune responses to the BCG vaccine, we measured the cytokine production after stimulation with BCG (SSI strain,  $1.2 \times 10^6$  organisms/ml) or Staphylococcus Enterotoxin B (SEB) using an intracellular cytokine staining (ICS) assay. We looked at cytokine responses in the PBMC population (see gating strategy in figure 1A). There was a consistent increase in the production of all cytokines after BCG stimulation and IFN $\gamma$ , TNF $\alpha$ , IL2 but not IL8 and IL4/5/13 after SEB stimulation (figure 1B). We examined whether the observed magnitude of immune responses was due to changes in T cell frequency and conducted a Spearman correlation analysis of frequencies of IFN $\gamma$ -expressing cells following BCG stimulation with other immune parameters. The frequencies of BCG-specific IFN $\gamma$  cells (hereafter called IFN $\gamma$  BCG) were correlated with IL4/5/13, IL2 and IL8 secreted following stimulation with BCG but not with any T cell population measured (table S1). This indicates that in this assay, the BCG induced IFN $\gamma$  production is not correlated with T cell and lymphocyte composition. All samples were stratified based on their IFN $\gamma$  BCG production and 15 highest (IFN $\gamma$  Low) and 15

lowest (IFN $\gamma$  High) IFN $\gamma$  BCG producers were selected for the analysis of DNA methylation (Figure 1C)

*Differential methylation of 318 genes between low and high BCG cytokine respondents*

A total of 30 samples were selected for the DNA methylation analysis (figure 1C). These were stratified based on their BCG specific immune responses forming low vs high groups for five cytokines: IFN $\gamma$ ; IL4/5/13; IL2; TNF $\alpha$ ; and IL8. For each cytokine, DNA methylation of low responders was compared to high responders using logistic regression with a minimum difference in methylation of 5%. The analysis showed several probes to be differentially methylated between low and high groups of: IFN $\gamma$  (70), IL4/5/13 (108), IL2 (50), TNF $\alpha$  (146), and IL8 (122) (figure 2). A gene feature report highlighted 318 genes (table 1) that span the differentially methylated probes. Several novel genes of which levels of methylation can indirectly affect the magnitude of BCG immune responses were identified. Notably, the ZFP57 gene was found to be differentially methylated in all cytokine low and high groups comparisons. ZFP57 is a transcriptional regulator of gene imprinting, and it acts by controlling DNA methylation during the earliest stages of multicellular development<sup>29,30</sup>. Another gene candidate KCa3.1 is a protein that forms the voltage-independent potassium channel and was found to be differentially methylated across four cytokine low and high groups (IFN $\gamma$ , IL2, IL4/5/13 and IL8). Kca3.1 regulates calcium influx and can influence TH1 and TH2 development<sup>31</sup>. We also found ERICH-1 to be differentially methylated across cytokine low and high groups. ERICH-1 encodes the glutamate-rich 1 protein and glutamate is known to regulate Ca<sup>2+</sup> and K<sup>+</sup> efflux thereby affecting T cell activation<sup>32</sup>.

*Hierarchical cluster analysis reveals clusters of differentially methylated genes.*

We conducted a t-distributed stochastic neighbour embedding (tSNE) analysis of gene methylation for the 30 examined samples, however, this did not show a clear separation of IFN $\gamma$  low and IFN $\gamma$  high nor the other cytokine low and high groups (data not shown). This indicates subtle differences in DNA methylation between cytokine low and high groups, suggesting that there might also be other mechanisms that drive immune responses to the BCG vaccine. We then proceeded with the hierarchical cluster analysis of the differentially methylated probes when stratified by cytokine respondents. We saw visible differences in cluster formation for all the cytokine groups measured (Figure 3).

*Differentially methylated genes are part of immune and cellular processes pathways.*

We examined biological functions of the differentially methylated genes for all the cytokines using the Panther Pathway Analysis (PPA). Differentially methylated genes are included in 67 pathways that are involved in multiple biological processes including immune functions (2.8%), metabolic (20.8%) and cellular (25.3%) processes and other processes (table 2). Several pathways of the immune system could explain the polarisation of cytokine responses. Notably, T cell activation, interferon gamma signalling, interleukin and JAK/STAT signalling pathways were all involved in the regulation of cytokine responses. B cell activation, TGF-beta and chemokine and cytokine-mediated inflammation were also highlighted as pathways containing genes that were differentially methylated. A large proportion of highlighted pathways regulate cellular and metabolic processes. We found differentially methylated genes that are part of the G-protein signalling pathways, which are known to regulate T cell migration and activation <sup>33</sup>. We also found six differentially methylated genes in the Wnt pathway, which is known to regulate IL12 signalling in

antigen presenting cells (APCs) <sup>34</sup>. Lastly, we highlight the muscarinic acetylcholine receptor signalling pathway (mAChRs), which can mediate cytokine production <sup>35</sup>. By regulating the immune signalling and cellular processes, these pathways can directly and indirectly affect the magnitude of the BCG induced immune response that is observed in our samples.

## Discussion

BCG vaccination induces variable immunity across populations <sup>36,37</sup> translating to inconsistent protection against pulmonary tuberculosis <sup>1</sup>. This study aimed to answer whether epigenetic modifications, specifically DNA methylation, could be one of the molecular mechanisms that drive the observed disparate immune responses.

We first measured the magnitude of immune responses induced after the BCG vaccination. We chose to include all cells in the PBMC population as the DNA used for the methylation analysis was extracted from matching vials of PBMC. Similar to the previous study <sup>18</sup>, we saw an increase in all the measured cytokines following BCG stimulation *in vitro*. The magnitude of immune responses for IFN $\gamma$  ranged from 0% to 2.61% measured as the frequency of cytokine-producing PBMCs. As expected, BCG and SEB stimulation decreased the expression of the CD3 protein and had no effect on the expression of CD4 and CD8 proteins (data not shown) and SEB, an inflammatory superantigen <sup>38</sup>, failed to increase the production of TH2 cytokines and IL8. CD4+ and CD8+ T cells are known to be the primary producers of IFN $\gamma$  <sup>39</sup>, however, we saw that none of the T cell phenotypes measured correlated with levels of BCG induced IFN $\gamma$  (table S1). This indicates that the magnitude of immune responses is independent of cellular T cell composition and is likely driven by molecular mechanisms within individual cells. Importantly, the magnitude of cytokine responses for BCG and SEB stimulations did not correlate (not shown), confirming that observed immune responses are antigen specific. The frequencies of BCG-induced IFN $\gamma$  had a strong positive correlation with IL2 and IL4/5/13 and a weak negative correlation with IL8. IL2 is known to activate T cells, which are one of the main IFN $\gamma$  producers <sup>40</sup>, and the BCG vaccine was shown to induce both TH1 and TH2 cytokines <sup>7,9</sup> previously. We cannot exclude that changes in cell numbers of Natural Killer (NK) cells and monocytes between groups could explain observed

differences in cytokine levels. This could be addressed by analysing DNA methylation patterns in purified cell populations, which due to a limited sample size was beyond the scope of the project.

Having measured the immune responses and selected samples for further analysis, we measured DNA methylation and stratified samples by high and low cytokine responses. We found probes that were differentially methylated between high and low cytokine responders and identified the 318 corresponding genes. Interestingly, genes that were differentially methylated in many of the cytokine groups were involved in potassium, calcium and neurotransmitter signalling.

KCNN4 or Kca3.1, Potassium Calcium-Activated Channel Subfamily N Member 4, was found to be differentially methylated in IFN $\gamma$ , IL2, IL4/5/13, and IL8 high and low groups. The Kca3.1 channel is a voltage-independent potassium channel, which is activated by an increase in intracellular calcium, resulting in membrane hyperpolarisation and continuous calcium influx<sup>31</sup>. Calcium regulates multiple cellular processes. A prolonged contact between a CD4<sup>+</sup> T cell and an APCs increases intracellular Ca<sup>2+</sup> levels. The primary sources of Ca<sup>2+</sup> in T cells are store-operated calcium channels (SOCE) and calcium-release-activated calcium (CRAC)<sup>41–44</sup>. A SOCE and CRAC dependent release of calcium inside CD4<sup>+</sup> T cells changes gene expression, and thus cytokine production, drives the differentiation of naïve T cells to TH1 or TH2 cells, and the development of immature T cells<sup>41</sup>. A strong Ca<sup>2+</sup> signal favours TH1 differentiation<sup>45</sup> whereas weak Ca<sup>2+</sup> release skews cells to a TH2 phenotype<sup>46–48</sup>. Kca3.1 can thus affect T cell activation by maintaining a negative membrane potential and continuing Ca<sup>2+</sup> release via CRAC channels<sup>31</sup>.

ERICH1, which encodes the glutamate-rich 1 protein, was found to be differentially methylated in 4 out of 5 low and high cytokine groups. Glutamate is an excitatory



neurotransmitter of the nervous system, which is critical for brain development and function and plays a signalling role in peripheral organs, including T lymphocytes<sup>32,49</sup>. It can be produced by several cells of the immune system including T cells, dendritic cells and others. Glutamate mediates intracellular  $\text{Ca}^{2+}$  fluxes and outward  $\text{K}^+$  flows thereby affecting adhesion, migration, proliferation, survival, activation and metabolism of T cells<sup>32,49,50</sup>. Differential methylation of ERICH1 could, therefore, lead to varied intracellular glutamate levels affecting the immune response to the BCG vaccine.

To look more broadly at differentially methylated genes, we investigated the array of pathways which contained differentially methylated genes. As expected, we found several immune pathways: T cell activation, JAK/STAT signalling, Interleukin signalling, Interferon-gamma signalling, inflammation mediated by chemokine and cytokine signalling, integrin signalling, TGF-beta signalling, B cell activation. All these pathways could contribute to the underlying differences observed in the cytokine response to the BCG vaccine. Interestingly, we also found a large proportion of pathways that were involved in metabolic and cellular processes. Several neurotransmitter mediating pathways were highlighted.

Muscarinic acetylcholine receptor signalling pathway signals via muscarinic acetylcholine receptors (mAChRs), which is made up of five types ( $\text{M}_1$ - $\text{M}_5$ ) of class A G protein-coupled receptors (GPCRs)<sup>51</sup>. Activation of T cells with PHA and PMA upregulates the expression of mAChRs<sup>52,53</sup>; a stimulation via CD11a upregulates  $\text{M}_5$  gene expression<sup>52</sup> suggesting an increase in cholinergic transmission following T cell activation; a stimulation of mAChRs in PBMC upregulated the IL2 production<sup>35</sup>; and  $\text{M}_1$ - $\text{M}_5$  knockout mice produced lower levels of OVA-Specific IgG1, TNF $\alpha$  and IL-6<sup>54</sup>. Seven genes in the GPCRs signalling pathway were differentially methylated across low and high cytokine groups. G proteins act as molecular switches to control

downstream effector molecules including chemokines and chemokine receptors<sup>33</sup>. In immune cells, G proteins impact signal transduction and affect survival, proliferation, differentiation and cell migration, and recent evidence suggests non-canonical GPCR signalling in immune cells<sup>33,55</sup>.

The WNT pathway, of which six genes were differentially methylated, is known to influence the immune system indirectly. There are 19 WNT genes in the human genome, and they all encode lipid-modified secreted glycoproteins. There is numerous evidence that show how the WNT proteins affect the immune system: WNT1 and WNT4 deficient mice have impaired T and B cell development<sup>56,57</sup>; WNT proteins are crucial for the initial proliferation of thymocytes before  $\beta$ -selection<sup>58</sup>; the overexpression of WNT signalling increases the survival of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Tregs)<sup>59</sup>; and WNT signalling in APCs results in increased IL12 production and subsequent TH1 differentiation<sup>34</sup>. Variation in methylation of WNT genes could, therefore, lead to changes in WNT proteins, ultimately leading to mediating the immune response to the BCG vaccine.

A report by Verma et al.<sup>28</sup> has recently investigated the effect of BCG vaccination on the DNA methylation of PBMCs *in vivo*. They found an enrichment of the DNA methylation in genes of immune pathways, including T cell activation, after the BCG vaccine vs before vaccination. Their study shows that several non-immune pathways such as regulation of biological processes are enriched however these processes were not discussed in the report. It is essential to highlight notable differences between the current study and that of Verma et al. We investigated the preceding methylation patterns induced by BCG vaccination and how they regulate the immune response to BCG stimulation in infants whereas the Verma et al. study examined how BCG vaccination modulates the DNA methylation of PBMCs in adults.

Hierarchical cluster analysis of DNA methylation revealed visible differences in cluster formation between high and low IFN $\gamma$  groups as well as other cytokines but tSNE analysis failed to separate infants based on their DNA methylation patterns. Importantly, tSNE analysis incorporates all methylated probes whereas hierarchical clustering methodology focuses on probes that are differentially methylated, allowing for clear separation and formation of clusters. These findings suggest that DNA methylation is probably not the primary mechanism to regulate the immune response to the BCG vaccine and other epigenetic mechanisms might play a role. Histone modifications and microRNA are other types of epigenetic modifications that could explain differences in observed immune responses. Histone modifications are known to control the transcriptional profile of memory lymphocytes thereby shaping their function<sup>60</sup>. They also regulate mechanisms of adaptive features of trained immunity<sup>61</sup>. microRNAs have shown to play a role in the modulation of inflammatory responses<sup>62</sup>.

We acknowledge that the observed differences in DNA methylation patterns may not be specific to the BCG vaccine responses and may, in fact, represent regulation of general vaccine-induced immune responses. However, it is important to highlight that in a context of this study, cytokine levels were measured after BCG stimulation, hence they are BCG specific. Thus, the differences in cytokine levels and the subsequent stratification to low and high cytokine responding groups is BCG specific. The DNA methylation differences observed between these groups are associated with BCG specific differences in cytokine responses, however, we cannot exclude that they may also be associated with immune responses to other vaccines.

Taken together, the data reported here identified genes and pathways that were differentially methylated in PBMC from infants with high or low cytokine responses to BCG vaccination. As expected we observed differential methylation of multiple immune pathways that could directly influence the disparate immune responses to

the BCG vaccine. Unexpectedly, we identified several genes and pathways that could indirectly affect the BCG specific cytokine production. These findings suggest that in addition to immune pathways, vaccine-induced immune responses could be modulated by molecular mechanisms and mediators that regulate cellular processes such as the glutamate, the flux of potassium and calcium through membrane channels, the GPCRs and mAChRs signalling and the WNT pathway. Studies should focus on investigating the role of methylation as well as other epigenetic marks in the regulation of the genes and pathways in question as well as measuring differences in gene expression when stratified by high and low cytokine responses. This knowledge will allow us to understand the molecular mechanisms that drive vaccine-induced immune responses, paving a way to design better and more effective TB vaccines.

## Methods

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

### *Study participants*

We retrieved blood samples collected from a subset of 10 week old infants who were enrolled into a large study of BCG vaccination at the South African Vaccine Initiative (SATVI) field site in the Worcester area, near Cape Town, South Africa (Hawkrige et al., BMJ 2008; <sup>18</sup>. All participants were vaccinated with BCG at birth and samples were collected ten weeks after birth. Participants were excluded from the study if any of the exclusion criteria were met: infant not immunised with BCG within 24 hours; mother infected with human immunodeficiency virus (HIV); chronic and acute disease in the infant at the time of enrolment; clinical anaemia in the infant; significant perinatal complications in the infant; and contact with any person with TB disease or anyone who was coughing. Participants were followed for two years to observe the development of TB disease. Only healthy infants who did not develop TB disease were selected for this study. Parents gave consent for their infants to participate in the study. The study was conducted according to the U.S. Department of Health and Human Services and Good Clinical Practice guidelines, and included protocol approval by the University of Cape Town Research Ethics Committee and written informed consent from the parent or legal guardian. The study also received ethical approval from the Ethics Committee of the London School of Hygiene and Tropical Medicine (LSHTM, #8720).

### *Cell separation, processing and stimulation*

The methods below have been previously published in other studies<sup>18,63</sup>. Heparinized blood samples were collected from infants. Peripheral blood mononuclear cells were isolated using the density gradient centrifugation. The remaining 1 ml of blood was incubated with BCG (SSI,  $1.2 \times 10^6$  organisms/ml), medium alone, and staphylococcal enterotoxin B (10 µg/ml; Sigma-Aldrich) (SEB). The co-stimulatory antibodies, anti-CD28 and anti-CD49d antibodies (1 µg/ml each; BD Biosciences, San Jose, CA) were added to all conditions. Samples were incubated for 7 hours at 37°C. Later, Brefeldin-A was added and samples were incubated for another 5 hours. The role of Brefeldin-A is to capture cytokines inside the cell. The time point of Brefeldin-A incubation was decided in the previous study to best reflect dynamics of cytokine production. Red blood cells were lysed and white cells fixed using FACS Lysing Solution (BD Biosciences). Cells were collected, fixed and cryopreserved as described elsewhere<sup>63</sup>. Thus, for each participant, non-stimulated PBMC sample and three stimulated and fixed whole blood samples were available.

### *Intracellular cytokine staining*

Previously stimulated samples were thawed and washed in FACS buffer (PBS, 5% FBS v/v, 0.05% sodium azide w/v) and stained for 30 minutes at 4°C in the dark, with a cocktail of surface marker antibodies: CD3-BV650 (clone SK7), CD4-BV605 (clone S3.5), CCR7-PECF594 (clone 150503), CD45RO-APC-H7 (clone UCHL1), (all BD Bioscience) and CD8-BV570 (clone RPA-T8; BioLegend). Samples were then permeabilised using the Cytoperm/Cytofix (BD Pharmingen) solution for 20 mins. After permeabilisation, cells were stained for 30 minutes at room temperature in the dark with a cocktail of intracellular cytokine antibodies: TNFα-PE-Cy7 (clone Mab11), IFNγ-V450 (clone B27), IL-2-FITC (clone MQ1-17H12), (BD Bioscience), IL-4-PE

(clone 3010.211; FastImmune), IL-5-PE (clone TRFK5), IL-13-PE (JES10-5A2) and IL-8-APC (clone E8A1), (BioLegend). IL4, 5 and 13 were collected in one PE channel. After staining, samples were washed and stored at 4°C in the dark to be acquired within 24 hours using BD LSRII Flow Cytometer and obtaining a minimum of 200,00 events.

#### *Sample selection and DNA isolation*

Samples were stratified based on the results of the ICS assay and the BCG specific IFN $\gamma$  response. Corresponding PBMC samples of the 15 lowest (hereafter called IFN $\gamma$  Low) and 15 highest IFN $\gamma$  (hereafter called IFN $\gamma$  High) respondents were selected for the DNA methylation analysis. The selected 30 samples were later stratified based on their IL2, IL8, IL4/5/13 and TNF $\alpha$  responses effectively forming five low and high cytokine groups. DNA was extracted using the Dneasy Blood & Tissue Kit (Qiagen). The quality of the DNA was checked with NanoDrop (Thermo Fisher Scientific) and the concentration measured with Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific).

#### *Measurement of the DNA methylation*

The DNA methylation was examined using a reduced representation bisulphite sequencing (RRBS) method and with the Premium Reduced Representation Bisulphite Sequencing Kit (Diagenode) according to manufacturer's instructions.

#### *DNA quality control and MiSeq sequencing*

DNA concentration was measured using the Qubit dsDNA HS Assay Kit and size examined with the High Sensitivity DNA Analysis Kit (Genomics Agilent) using

Bioanalyzer 2100 (Genomics Agilent). DNA was denatured with 0.2M NaOH, and its concentration was adjusted to 16 pM. Samples were sequenced with the MiSeq Reagent Kit v3 (Illumina) at 51 cycles per run and single-end reads. A 12.5 pM PhiX v3 library (Illumina) was added for positive control.

#### *Data processing*

FASTA files were generated as the DNA sequencing output. Sequences were trimmed using the TrimGalore (v0.4.3) and Cutadapt software discarding all reads below 30 Phred quality score. The quality of samples was measured using the FastQC software (v0.11.5). A bisulphite treated human genome reference sequence was prepared using the GrCh38p7 assembly and Bismark Genome Preparation (v0.16.3) and Bowtie2 (v2.2.9) software. Samples were mapped to the reference genome using Bismark (v0.16.3) and Bowtie2. The DNA methylation was extracted using Bismark Methylation Extractor (v0.16.3).

#### *Data analysis: DNA methylation*

The methylation of DNA sequences was analysed using SeqMonk (v1.38.2) and Rstudio (1.0.44) software. Contig methylation probes were generated with a 10-fold depth cut off, ignoring duplicate reads and merging probes closer than 500bp. Probes were quantified using read count quantitation and filtered to include probes measured at least ten times. Chromosomes X, Y and any mitochondrial DNA were removed, and probes were quantitated using bisulphite methylation pipeline embedded in the SeqMonk software. Based on the previous ICS responses of whole blood samples, DNA samples were then separated into two groups: IFN $\gamma$  low & IFN $\gamma$  high and logistic regression with a P-value cut-off of 0.05 and multiple testing correction was run. The logistic regression test was then performed on other cytokine groups: IL4/5/13 low &



IL4/5/13 high; IL2 low & IL2 high; TNF $\alpha$  low & TNF $\alpha$  high; and IL8 low and IL8 high. Genes that were differentially methylated were visualised using SeqMonk, Panther Pathway Analysis and Cytospace (v3.5.1).

#### *Data analysis: correlation of immune responses*

We used StataSE (v15.0) software to measure whether the magnitude of the BCG induced immune response is cell population dependent. Frequencies of BCG specific cytokines (TNF $\alpha$ , IFN $\gamma$ , IL2, IL8 and IL4/5/13) and cell phenotypes (CD3+CD4+CCR7+, CD3+CD4+CD45RO+, CD3+, CD3+CD4+, CD3+CD8+, % of Lymphocytes) were correlated with the BCG specific IFN $\gamma$  response using the Spearman correlation.

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#### **Author Contributions**

Conceptualization M.H.A, S.S. and H.M.D.; Methodology M.H.A. and S.S.; Investigation and Formal Analysis M.H.A.; Resources T.S., W.H.; Writing – Original

Draft M.H.A.; Writing – Review & Editing S.S., T.S. and H.M.D.; Visualization M.H.A.,  
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#### **Declaration of Interests**

None

Figures

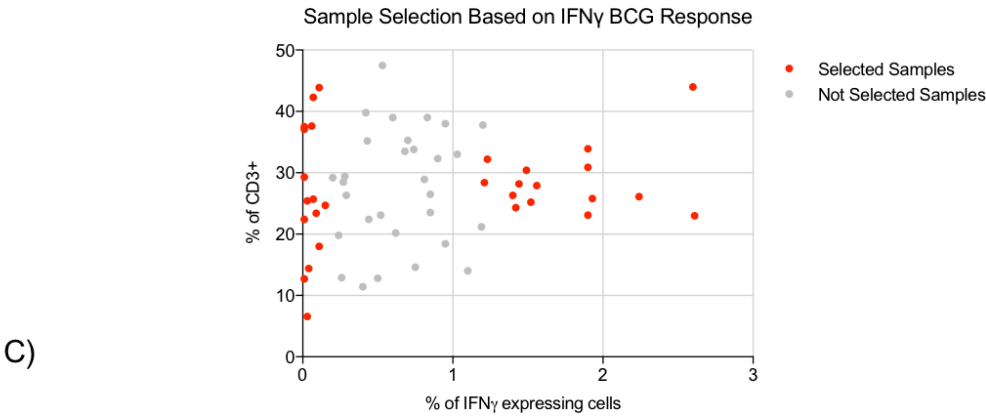
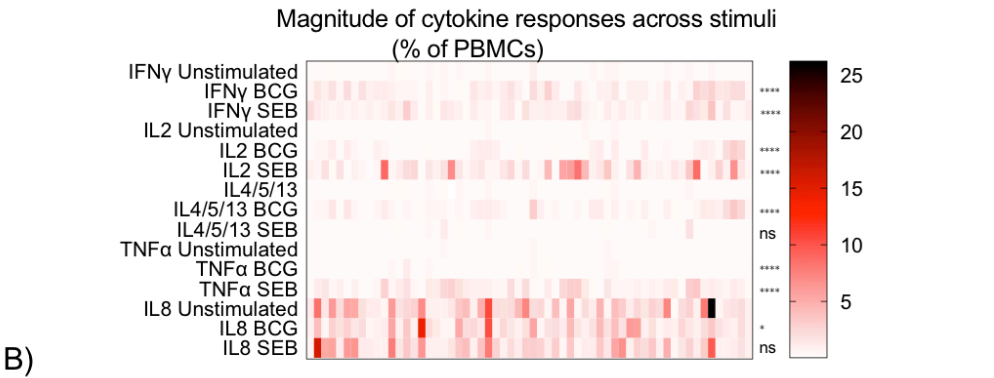
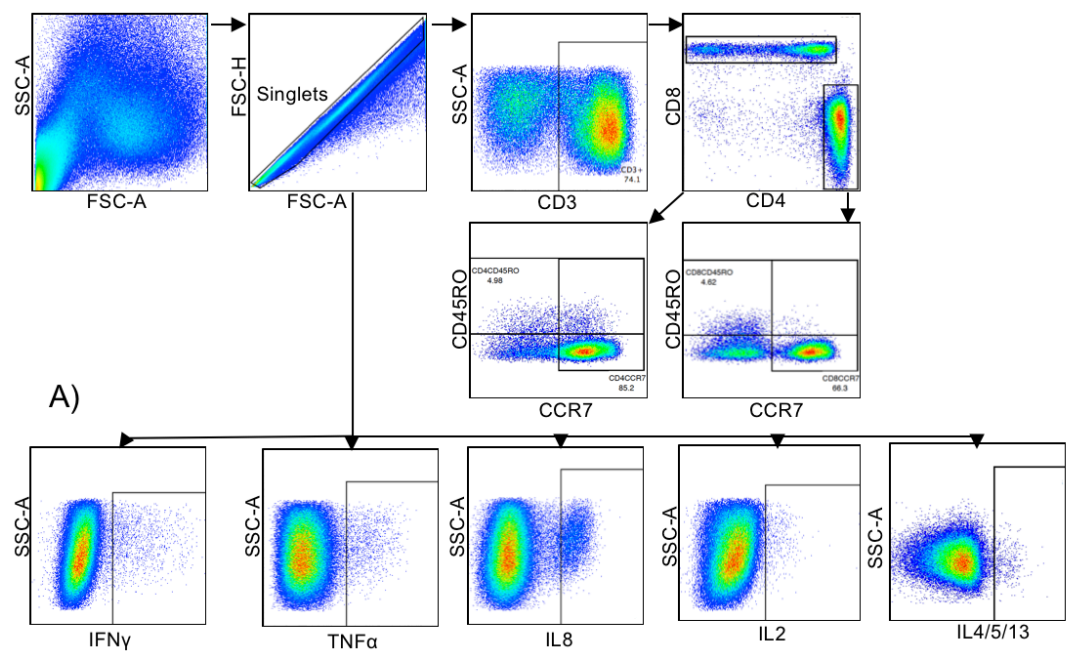


Figure 1. The immune response to the BCG vaccine was used to select samples for analysis.

A) The FACS gating strategy used to measure all immune responses. Singlet cells and CD3+ populations were chosen to measure intracellular cytokine responses as well as phenotypic markers such as CD3+, CD3+CD4+, CD3+CD8+, CD3+CD4+CCR7+, CD3+CD4+CD45RO+.

B) The magnitude of immune responses of PBMCs after 12 hours stimulation with BCG and SEB (% of PBMCs). Each column represents one donor sample. Stimulated samples were compared to non-stimulated with the Wilcoxon paired t-test. N=60; ns=not significant; \*= p< 0.05; \*\*\*\*= p<0.0001

C) A magnitude of IFN $\gamma$  BCG responses when stratified by origin (CD3+ population). Fifteen lowest (non-responders) and fifteen highest (responders) IFN $\gamma$  BCG producers (red) were selected for the DNA methylation analysis. Samples coloured in grey were not selected. N=60

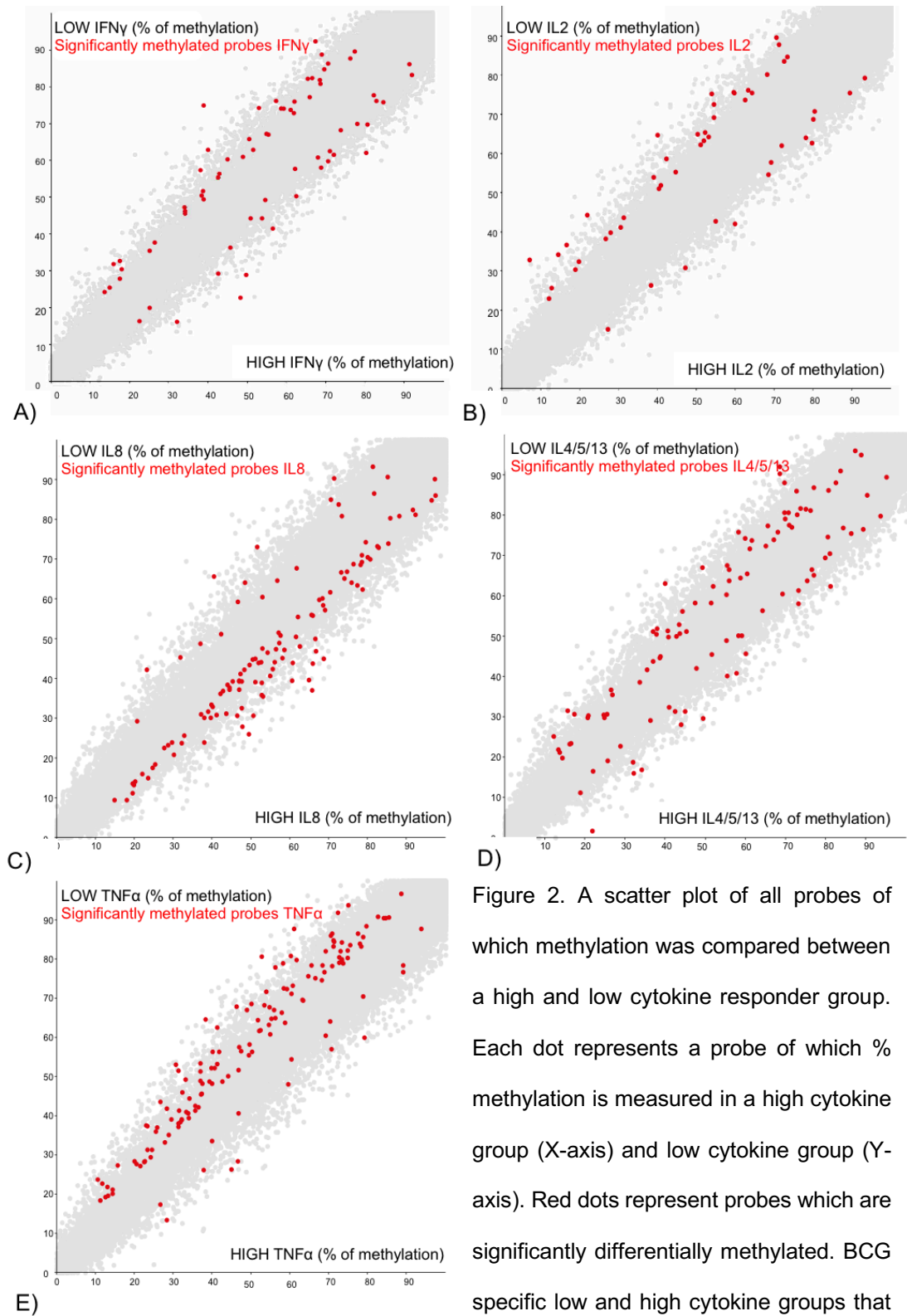


Figure 2. A scatter plot of all probes of which methylation was compared between a high and low cytokine responder group. Each dot represents a probe of which % methylation is measured in a high cytokine group (X-axis) and low cytokine group (Y-axis). Red dots represent probes which are significantly differentially methylated. BCG specific low and high cytokine groups that were compared included: IFN $\gamma$  (A), IL2 (B), IL8 (C), IL4/5/13 (D), and TNF $\alpha$  (E).

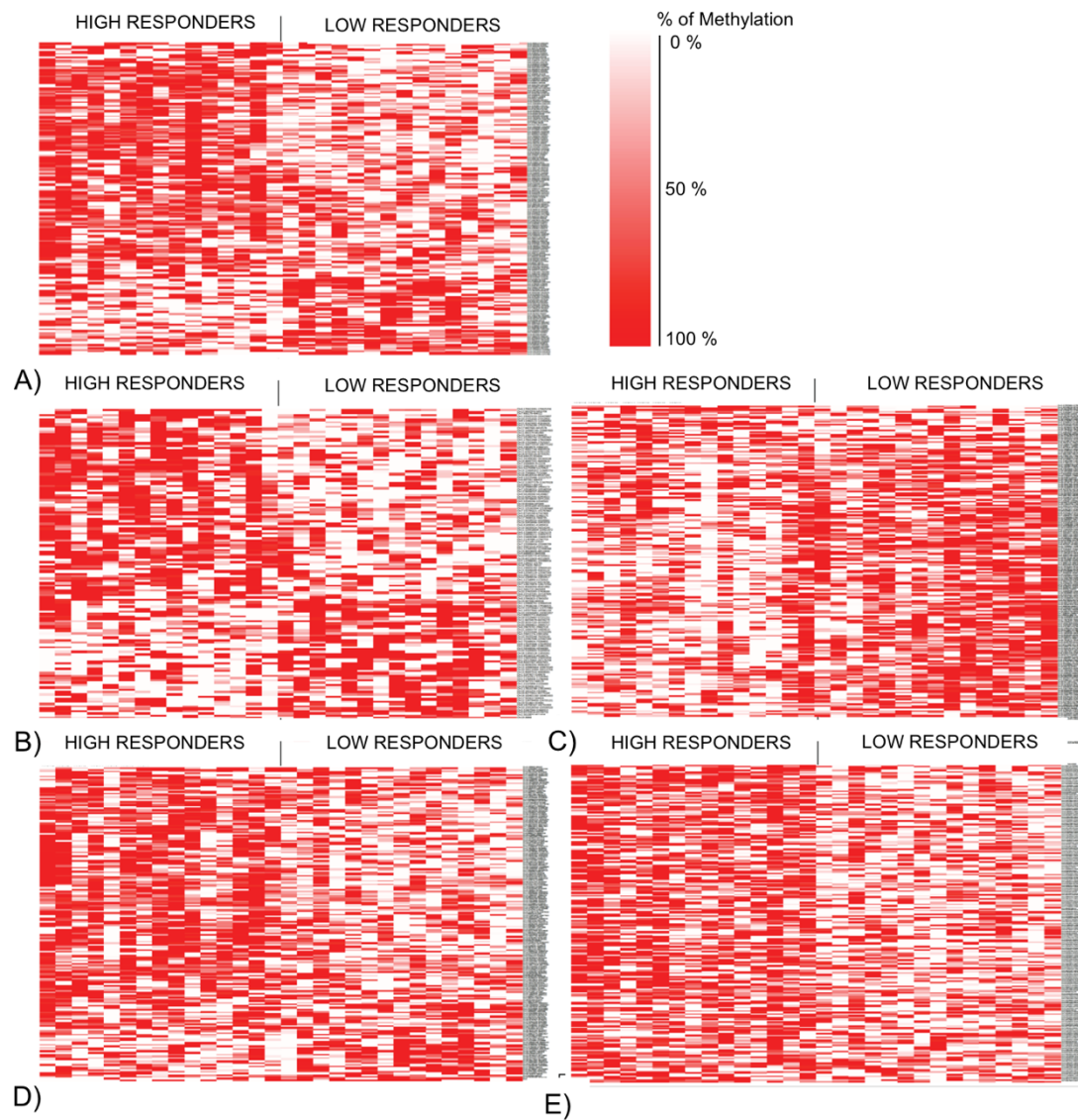


Figure 3. A hierarchical per probe normalised clustering analysis of the DNA methylation profile between high and low IFN $\gamma$  (A), IL2 (B), IL8 (C), IL4/5/13 (D) and TNF $\alpha$  (E) responder groups. First fifteen columns represent methylation of probes in cytokine high responder group and last fifteen columns represent methylation of probes in the low responder group. Each horizontal line represents a differentially methylated probe. The scale represents the percentage of methylation of each probe. The white colour represents low DNA methylation whereas the red colour represents high DNA methylation. n=30.

## Tables

Table 1: A summary of all genes that were differentially methylated across all low and high cytokine groups. Each row shows single or multiple genes that were differentially methylated across one or multiple cytokine groups. n=30

Name of Cytokine Pairs	Total Number of Differentially Methylated Genes	Names of Genes
IFN $\gamma$ IL2 IL4/5/13 IL8 TNF $\alpha$	1	ZFP57
IFN $\gamma$ IL2 IL4/5/13 IL8	2	PTPRN2 KCNN4
IFN $\gamma$ IL2 IL4/5/13 TNF $\alpha$	4	ERICH1 GPR124 RP5-1029F21.3 COLEC11
IL2 IL4/5/13 IL8 TNF $\alpha$	2	CTC-281F24.1 MIR4520-1
IFN $\gamma$ IL2 IL4/5/13	5	RP11-609L3.1 ZMIZ1 AURKC NEURL1B SEPT7P3
IFN $\gamma$ IL2 TNF $\alpha$	1	DNAH1
IFN $\gamma$ IL4/5/13 IL8	1	AZU1
IFN $\gamma$ IL4/5/13 TNF $\alpha$	3	LMTK3 LY6K ANO8
IL2 IL8 TNF $\alpha$	1	CRYGEP
IL2 IL4/5/13 TNF $\alpha$	1	DTX1
IL4/5/13 IL8 TNF $\alpha$	2	EHBP1L1 SIPA1

IFN $\gamma$ IL2	2	PRSS36 MVB12B
IFN $\gamma$ IL8	1	GCSAML
IFN $\gamma$ IL4/5/13	17	TTN DEAF1 CARS2 RP11-108K14.12 HELZ2 RGS3 COL1A1 MTHFR GRK1 DUSP15 CYP2E1 FIP1L1 LPIN1 TTN-AS1 C1orf167 FBXO41 CMTM7
IFN $\gamma$ TNF $\alpha$	1	LINC00539
IL2 IL8	1	HCN2
IL2 IL4/5/13	3	PDIA3P2 CATSPER2 KCNAB1
IL2 TNF $\alpha$	2	PROSER3 MAN1C1
IL8 TNF $\alpha$	3	CUX1 PLEC SLC43A2
IL4/5/13 TNF $\alpha$	9	PKNOX2 HNRNPH1 AC010907.2 RP11-687M24.8 MAST4 LHX6 ADARB2 AC096649.3 NLGN2
IFN $\gamma$	13	MED15P5 NTM CTD-3080P12.3 PIAS4 SPG7 TIMP2 CAMSAP3 SOGA3 CD9 MALRD1 PXDN NTNG2 RP11-599J14.2
IL2	15	RP4-724E13.2 TGFB3 RP11-452L6.8 COX6A2 CARD11 NINJ2 SNRPN MAP1LC3BP1 RP11-218M22.1 C20orf96 ANK1 FANCC MEGF6 RP4-559A3.7 LEFTY1
IL8	88	RN7SL646P FAM222B ZFP41 PBX4 LINC01169 KCNQ1OT1 TRPV2 EDARADD UMODL1-AS1 DTNB bP-21264C1.2 RP11-216L13.18 KIAA1161 CDH23 ZNF473 LINC01266 COL23A1 AC011850.2 FLJ26850 RP11-394I13.3 LINC00467 SOX10 MSL3P1 AC018688.1 HIST1H3E FAM217B 5_8S_rRNA RNMTL1 RAI1 RELT LRRC24 NFATC1 UNC93B1 ZNF790-AS1 EMC9 ITPR1 POU5F1 EGOT UMODL1 MIR4508 AC005481.5 VOPP1 AC005538.3 ATP8B3 POLR2F MIR6820 SDK1 MIR138-2 FRRS1 MIR6813 WDR60 COL5A1 CTD-2126E3.3 THSD4 OPCML TMPRSS12 RABL6 RP11-133L14.5 PROSER2-AS1 PIK3R2 PRSS21 RYR1 CLEC9A CTD-2269E23.4 MAN2B1 ARHGEF10 GLI4 RP11-497G19.1 ATG16L2 LZTS3 WDR20 SH3GL1 EXD3 AMOTL2 LRRC14 MROH1 CTD-2192J16.22 TARID CFD TNFRSF11A MMP17 RGS19 KCNQ1 C8orf82 RNF165 LOXL1 LINC01312 SYCP2



IL4/5/13	41	TPM4P1 GNAL AC131097.4 RP11-244F12.1 RP11-867G23.12 NBPF13P RNVU1-8 RIMBP2 LINC01237 ZNF718 RP11-680G24.4 TAF1C TBCE PHACTR3 RP11-662M24.2 RP11-452D2.2 RP11-545A16.3 HLA-DRB9 PRDM15 KCNN3 PRDM16 DRAXIN ARID3A SNRNP27 COL18A1 RP11-1260E13.1 RPH3AL RP3-522J7.6 AP001347.6 LRRC37A6P PRDM7 MYBPC2 PNPLA6 AC147651.1 PDXDC1 SRC NYAP2 ADAMTS12 ANKRD20A18P C5orf17 RIN1
TNF $\alpha$	98	GABRB3 MKRN3 PRKAR1B PAX8-AS1 FAM189A1 RNA5-8S5 ACOT2 WT1 ACVR1B LINC00839 AOX3P PRDM8 LDLRAD2 CHCHD3 FBXO16 PEBP4 MARK4 CTU1 HLA-DPA1 ATP5J2 RPL13A MEST RASAL2 PRAM1 PRKCQ GNAI2 RP11-127L20.3 RP11-726G23.10 BRSK1 BCAN CROCC GABRA5 LINC00887 PTCD1 SDF4 FNDC3B RPL13P12 CYGB NCOR2 CLIP2 GALNT9 BCR AOX2P OPRL1 RP11-377G16.2 FLT3LG CTD-3148I10.9 CPT1A RP11-54O7.17 RPS6KA4 RP11-284F21.7 PIGQ RP11-492E3.2 PRCD AC093609.1 HLA-DPB1 SEMA6B CH507-513H4.1 UHRF1 CTD-3148I10.15 SEMA3B ZNF331 GEMIN7 MPPED1 ERICH1-AS1 RP5-906A24.1 PPAPDC3 MGRN1 ZNF395 FLT4 ITPRIP RP11-426C22.5 KAZN MST1L PSPN ITFG2 ARL5C RP11-137H2.4 FBXW4P1 PAX8 ALKBH7 C17orf98 RBM19 NOS3 CCNYL2 RP11-273G15.2 HTRA4 RP11-231D20.2 RP11-65I12.1 SCARF1 ITGAX RP11-230C9.1 PPP1R37 RP11-56P9.10 OSBPL5 ATP5J2-PTCD1 CBFA2T3 PLEKHA2

Table 2: An overview of biological processes and selected pathways of the differentially methylated genes when stratified by low and high cytokine groups. Each row shows a biological process or a pathway and a number of differentially methylated genes associated with it.

Biological Processes Of Differentially Methylated Genes		
Name of the Biological Process	Number of Genes Involved	% of Total Genes
Cellular Process	80	25.3
Metabolic Process	66	20.8
Response To Stimulus	34	10.8
Biological Regulation	30	9.4
Developmental Process	30	9.4
Multicellular Organismal Process	25	7.8
Localization	18	5.6
Cellular Component Organization Or Biogenesis	13	4.2
Immune System Process	9	2.8
Biological Adhesion	6	1.9
Locomotion	4	1.1
Reproduction	3	0.8
Highlighted Pathways of Differentially Methylated Genes		
Pathway Name	Number of Genes Involved	% of Total Genes
Heterotrimeric G-Protein Signaling Pathway-Gi Alpha And Gs Alpha Mediated Pathway	7	4.2
Integrin Signaling Pathway	6	3.6
Heterotrimeric G-Protein Signaling Pathway-Gq Alpha And Go Alpha Mediated Pathway	6	3.6
Wnt Signalling Pathway	6	3.6
T Cell Activation	5	3.0

Inflammation Mediated By Chemokine And Cytokine Signaling Pathway	3	1.8
Muscarinic Acetylcholine Receptor 1 And 3 Signaling Pathway	3	1.8

Table S1: The Spearman correlation analysis of IFN $\gamma$  BCG levels with immune parameters.

There is a strong correlation between IFN $\gamma$  BCG and IL4/5/13 BCG, IL2 BCG and a weak correlation with IL8 BCG. No other parameters were correlated with IFN $\gamma$  BCG. n=60.

Immune Parameter	Correlation "R"	P value
IFN $\gamma$ BCG	1.00	0.0000
IL4/5/13 BCG	0.53	0.0000
IL2 BCG	0.53	0.0000
IL8 BCG	-0.28	0.0313
CD3+CD4+CCR7+	-0.24	0.0614
CD3+CD4+CD45RO+	0.22	0.0959
TNF $\alpha$ BCG	0.21	0.1042
CD3+	0.11	0.3999
% of Lymphocytes	0.10	0.4340
CD3+CD4+	0.09	0.4867
CD3+CD8+	0.05	0.7093

Table S2: An overview of all pathways of the differentially methylated genes when stratified by low and high cytokine response. Each row shows a biological process or a pathway and a number of differentially methylated genes associated with it.

Pathway	Genes Involved	% of Total
Axon guidance mediated by netrin (P00009)	2	1.20%
Beta3 adrenergic receptor signalling pathway (P04379)	1	0.60%
Axon guidance mediated by Slit/Robo (P00008)	1	0.60%
Metabotropic glutamate receptor group III pathway (P00039)	2	1.20%
Beta2 adrenergic receptor signalling pathway (P04378)	2	1.20%
JAK/STAT signalling pathway (P00038)	1	0.60%
Beta1 adrenergic receptor signalling pathway (P04377)	2	1.20%
Apoptosis signalling pathway (P00006)	3	1.80%
5HT4 type receptor-mediated signalling pathway (P04376)	1	0.60%
Angiogenesis (P00005)	7	4.20%
Interleukin signalling pathway (P00036)	1	0.60%
Alzheimer disease-presenting pathway (P00004)	1	0.60%
Interferon-gamma signalling pathway (P00035)	1	0.60%
5HT2 type receptor-mediated signalling pathway (P04374)	2	1.20%
Alzheimer disease-amyloid secretase pathway (P00003)	2	1.20%
Integrin signalling pathway (P00034)	6	3.60%
5HT1 type receptor-mediated signalling pathway (P04373)	1	0.60%
Alpha-adrenergic receptor signalling pathway (P00002)	2	1.20%
Insulin/IGF pathway-protein kinase B signalling cascade (P00033)	1	0.60%
Insulin/IGF pathway-mitogen activated protein kinase/MAP kinase cascade (P00032)	1	0.60%
Inflammation mediated by chemokine and cytokine signalling pathway (P00031)	3	1.80%
Hypoxia response via HIF activation (P00030)	1	0.60%
GABA-B receptor II signalling (P05731)	1	0.60%
Huntington disease (P00029)	2	1.20%
Heterotrimeric G-protein signalling pathway-rod outer segment phototransduction (P00028)	1	0.60%
p53 pathway (P00059)	1	0.60%
p53 pathway feedback loops 2 (P04398)	1	0.60%
Heterotrimeric G-protein signalling pathway-Gq alpha and Go alpha-mediated pathway (P00027)	6	3.60%

Heterotrimeric G-protein signalling pathway-Gi alpha and Gs alpha-mediated pathway (P00026)	7	4.20%
Wnt signalling pathway (P00057)	6	3.60%
Hedgehog signalling pathway (P00025)	1	0.60%
VEGF signalling pathway (P00056)	5	3.00%
Allantoin degradation (P02725)	1	0.60%
Transcription regulation by bZIP transcription factor (P00055)	3	1.80%
Thyrotropin-releasing hormone receptor signalling pathway (P04394)	2	1.20%
General transcription regulation (P00023)	2	1.20%
Ras Pathway (P04393)	1	0.60%
General transcription by RNA polymerase I (P00022)	1	0.60%
T cell activation (P00053)	5	3.00%
FGF signalling pathway (P00021)	3	1.80%
TGF-beta signalling pathway (P00052)	3	1.80%
Oxytocin receptor mediated signalling pathway (P04391)	2	1.20%
Endothelin signalling pathway (P00019)	7	4.20%
EGF receptor signalling pathway (P00018)	4	2.40%
p38 MAPK pathway (P05918)	1	0.60%
Parkinson disease (P00049)	1	0.60%
PI3 kinase pathway (P00048)	3	1.80%
Opioid proopiomelanocortin pathway (P05917)	1	0.60%
PDGF signalling pathway (P00047)	4	2.40%
Opioid prodynorphin pathway (P05916)	1	0.60%
Histamine H2 receptor mediated signalling pathway (P04386)	1	0.60%
Oxidative stress response (P00046)	2	1.20%
Opioid proenkephalin pathway (P05915)	1	0.60%
Histamine H1 receptor mediated signalling pathway (P04385)	3	1.80%
Notch signalling pathway (P00045)	1	0.60%
Cadherin signalling pathway (P00012)	2	1.20%
Enkephalin release (P05913)	2	1.20%
Muscarinic acetylcholine receptor 2 and 4 signalling pathway (P00043)	2	1.20%
Dopamine receptor mediated signalling pathway (P05912)	1	0.60%
Muscarinic acetylcholine receptor 1 and 3 signalling pathway (P00042)	3	1.80%

B cell activation (P00010)	3	1.80%
Angiotensin II-stimulated signalling through G proteins and beta-arrestin (P05911)	1	0.60%
Metabotropic glutamate receptor group I pathway (P00041)	2	1.20%
Corticotropin releasing factor receptor signalling pathway (P04380)	1	0.60%
Metabotropic glutamate receptor group II pathway (P00040)	2	1.20%
CCKR signalling map (P06959)	6	3.60%
Gonadotropin-releasing hormone receptor pathway (P06664)	12	7.30%

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**4. Terminally differentiated T cells and inflammatory cytokines define the immunogenicity profile of Ugandan compared to UK infants following BCG vaccination.**

In this chapter I compare the immune response to the BCG vaccine in UK and Ugandan infants. I aim to answer whether Ugandan infants have a different BCG-induced immune profiles to their UK peers. I also investigate T cell phenotypes and explain how they could influence the observed differences in BCG immunogenicity.



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## RESEARCH PAPER COVER SHEET

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### SECTION A – Student Details

Student	Mateusz Hasso-Agopsowicz
Principal Supervisor	Dr Steven Smith
Thesis Title	A STUDY OF FACTORS UNDERLYING BCG IMMUNOGENICITY DIFFERENCES ACROSS COUNTRIES: THE INFLUENCE OF DNA METHYLATION PATTERNS AND ANTIGEN PRESENTING CELLS

**If the Research Paper has previously been published please complete Section B, if not please move to Section C**

### SECTION B – Paper already published

Where was the work published?	
When was the work published?	
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion	
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### SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	Frontiers in Immunology
Please list the paper's authors in the intended authorship order:	Mateusz Hasso-Agopsowicz, Grace Nabakooza, Marjorie Nakibuule, John Vianney Tushabe, JiSook Lee, Patrice Mawa, Dorothy Aibo, Joel Serubanja, Emily L Webb, Hazel Dockrell, Alison Elliott, Stephen Cose, Steven G Smith
Stage of publication	In preparation for submission

### SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I processed all samples recruited from UK infants. I received .fcs and luminex files for samples collected from Ugandan infants. I established a data analysis pipeline and analysed all flow cytometry and luminex samples. I established a common patients characteristics database and analysed patients characteristics across samples. I analysed flow cytometry data using tSNE and SPADE algorithms. I prepared graphs and wrote the manuscript.
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Student Signature: \_\_\_\_\_ Date: 23/07/2018

Supervisor Signature: \_\_\_\_\_ Date: \_\_\_\_\_

**Terminally differentiated T cells and inflammatory cytokines define the immunogenicity profile of Ugandan compared to UK infants following BCG vaccination.**

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## Abstract

**Introduction:** *Bacillus Calmette–Guérin* (BCG) is the only licensed vaccine for tuberculosis (TB), and its efficacy against pulmonary TB is lower in countries closer to the equator. Previous studies have investigated the immune signature of BCG vaccinated infants in UK, Malawian and Gambian infants. They found a predominant T helper 1-like (TH1) immune response in the UK and TH2 responses in Malawi and The Gambia. However, the cellular phenotypes in these populations have not been studied.

**Methods:** We investigated cytokines responses in BCG vaccinated, UK and Ugandan infants using a six-day whole blood stimulation assay. We also investigated phenotypes of T cells and their cytokine production across these populations. Lastly, we employed novel flow cytometry data analysis techniques to find rare cell populations and describe their dynamics in the UK and Uganda.

**Results:** We report that Ugandan infants produce higher levels of IL1 $\beta$  and TNF $\alpha$  cytokines compared to UK infants. This might be mediated by a higher proportion of terminally differentiated T cells, and a lower proportion of naïve T cells at 52 weeks in Uganda. We report no differences in TH1 cytokines when measured using the whole blood assay, and moderately higher frequencies of IFN $\gamma$  or IL2 producing CD4 or CD8 T cells at 52 weeks in the UK.

**Implications:** These results reveal that Ugandan infants have a different immunogenicity profile following BCG vaccination when compared with previous findings in Malawian and Gambian infants. We highlight the role of these immune

differences in the mediation of BCG immunogenicity and their potential impact on efficacy.

## Introduction

Despite continuous research efforts, the burden of tuberculosis (TB) remains high with 10.4 million people falling ill with TB in 2016 alone<sup>1</sup>. The disease disproportionately affects low-income countries, especially in Africa, where 82% of TB related deaths occurred<sup>1</sup>. *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG) is the only licensed vaccine for TB. Efficacy studies show that BCG protects well against severe childhood cases of TB meningitis and tuberculous miliary<sup>2</sup>. However, the protection against pulmonary TB varies across the globe and studies reported efficacy ranging from 0% in India<sup>3</sup> to 80% in the United Kingdom<sup>4</sup> (UK). Numerous reasons have been proposed to explain these variations. They include co-infections with malaria or helminthic parasites<sup>5</sup>; pre-exposure to environmental mycobacteria, including non-tuberculous mycobacteria<sup>6</sup>; and genetics<sup>7</sup>. Interferon  $\gamma$  (IFN $\gamma$ ) and T helper 1 (TH1) immune responses have been highlighted as paramount in an effective immune response against TB<sup>8</sup>, however, a South African study found no evidence for an association between TH1 responses following BCG vaccination and reduced risk of pulmonary TB<sup>9</sup>. A recent vaccine candidate MVA85A (Modified Vaccinia virus Ankara expressing Ag85A from *M. tuberculosis*) that boosts TH1 responses failed to demonstrate efficacy in BCG vaccinated infants<sup>10</sup>; however, in contrast to the study mentioned above<sup>8</sup>, the frequencies of BCG-specific IFN $\gamma$ -producing cells correlated with a reduced risk of developing TB<sup>11</sup>. The levels of IFN $\gamma$ , TH1, TH2, TH17 and other cytokines were investigated in studies in Malawi<sup>12</sup>, The Gambia<sup>13</sup>, Indonesia<sup>14</sup> and the UK<sup>12,13</sup>. A comparative analysis of immune responses following BCG vaccination of infants in Malawi and the UK revealed differences in 27 out of 42 cytokines, chemokines and growth factors measured in PPD-stimulated,

diluted whole blood. UK infants had more prominent type 1 responses with a high IFN $\gamma$  production, whereas Malawian infants had a type-2 and regulatory immune profile with higher IL13 and IL10 production<sup>12</sup>. The differences in immune responses could help to explain observed variations in BCG efficacy between these countries.

To fully understand the mechanisms underpinning BCG efficacy, we need to characterise further the underlying BCG-specific immune responses in countries where efficacy varies. In Malawi and The Gambia, we measured whole blood cytokine concentrations but were unable to investigate cell populations that produce them. In this study, we investigated BCG-induced immune responses in the UK and Uganda, a country where BCG efficacy is predicted to be low due to its equatorial latitude. We conducted a comparative analysis of diluted whole blood cytokine concentrations following antigen stimulation, T cell phenotypes and frequencies of cytokine-producing T-cells between BCG vaccinated infants residing in these two countries. We hypothesise that, as in Malawi, Ugandan infants would have a disparate profile of immune responses with a dominating type-2 and regulatory phenotype compared to stronger type-1 immune responses in UK infants.

## Methods

### *Ethical approval and informed consent*

Only infants whose mothers gave written, informed consent for participation were included in the study. This project was approved by the NRES Committee London – Camden & Islington (#15/LO/0048), the Research Ethics Committee of the London School of Hygiene and Tropical Medicine (LSHTM) (#8720), the Uganda National Council for Science and Technology (UNCST) (#HS1526), and the Uganda Virus Research Institute (UVRI) Research and Ethics Committee (#GC/127/14/02/432).

### *Study design and sample collection in Uganda*

This was a nested study within a large cohort study aimed at examining the effect of maternal LTBI on infants' BCG immune responses. Mothers and their infants were included in this study if they delivered in Entebbe General Hospital; resided in Entebbe Municipality and Katabi sub-county, Wakiso district, Uganda; were HIV negative; and had a singleton pregnancy with a healthy delivery of a neonate above 2500g. Infant cord blood was taken at delivery and screened for LTBI one week after birth. T-SPOT.TB assay (Oxford Immunotec) and a tuberculin skin test (TST; 2 tuberculin units, Statens Serum Institut) were used to detect LTBI. Mothers were considered LTBI positive when both tests were positive, and LTBI negative when both tests were negative. Mothers with discordant assay results were excluded from the study. If TB disease was suspected, mothers were referred for treatment. All infants were BCG vaccinated at birth with BCG SSI strain. Up to 5ml of heparinised peripheral venous blood and up to 10ml of cord blood was obtained from each infant. In the larger study, infants gave blood at 0, 1, 4, 6, 10, 14, 24 and 52 weeks but due to multiple blood draws, infants were randomised to give blood at either 1 and 6

weeks, or 4 and 10 weeks, and all gave blood at 0 and 52 weeks. For this study, and to allow comparison with UK responses, only samples at 10 and 52 weeks were used. Overall, 313 Ugandan samples were used in this analysis (108 samples at 10 weeks and 205 samples at 52 weeks, out of which 82 samples were paired). Due to limited blood volume at sample collection, not all infant samples were available for all analyses.

#### *Study design and sample collection in the United Kingdom*

Infants were included in the study if their mothers had a singleton pregnancy in North Middlesex Hospital, London, United Kingdom; had an uncomplicated delivery of a neonate above 2500g; and were LTBI negative as determined by T-SPOT.TB assay (Oxford Immunotec). All infants were BCG vaccinated at birth or within the first week of life with BCG SSI strain. Up to 5ml of heparinised peripheral venous blood was collected from each infant at 10 and 52 weeks of age. We collected samples from 28 infants at 10 weeks of age and collected follow-up samples from 12 of these infants at 52 weeks of age. Due to limited blood volume at sample collection, not all infant samples were available for all analyses.

#### *EBV and CMV viral DNA detection from whole blood using droplet digital PCR (ddPCR) assay*

DNA was extracted from 200µl whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) in 100µl elution buffer and according to manufacturer's guidelines. The ddPCR reaction mix included probe mixture (final concentration 250 nM) for EBV and CMV (table S1), 10 µl ddPCR™ Supermix for Probes (Bio-Rad Laboratories, Hercules, USA), 10 µl of template DNA, and 1.1 µl of 20 x primers (final concentration 900nM). 20 µl of the mixture was partitioned in oil-in-water with 70 µl of

droplet generator oil using a QX-100 droplet generator (Bio-Rad). The generated droplets (around 40  $\mu$ l) were then heated for 5 sec at 170 °C using a PX1™ PCR Plate Sealer (Bio-rad). PCR amplification was performed with the following thermal cycling: 95 °C for 10 min, 40 cycles consisting of 94 °C for 30 sec (denaturation) and 60 °C for 1 min (extension), followed by 98°C for 10 min and holding at 12 °C. Droplets were analyzed using a QX100 droplet reader (Bio-rad) and the target DNA concentration was calculated using QuantaSoft software (Bio-Rad). All samples were tested in duplicate.

#### *Immune cell phenotyping and flow cytometry*

Standard operating procedures (SOPs) were shared between the UK and Uganda, and an assay harmonisation exercise involving members of both teams took place in Uganda. Reagents from the same manufacturers were used. Heparinised whole blood was diluted 1:3 with RPMI 1640 (Life Technology, #21875-091) supplemented with a mixture of penicillin and streptomycin (Sigma, P4458) (final concentration of penicillin 5 units/ml, final concentration of streptomycin 50 $\mu$ g/ml) and L-glutamine (Sigma, #59202C) (final concentration 2mM). Diluted blood was aliquoted, and samples were stimulated with PPD (Tuberculin PPD-SSI batch RT 50) (final concentration 10 $\mu$ g/ml) and PHA (Sigma, #L8902) (final concentration 10 $\mu$ g/ml). An unstimulated sample was also included. GolgiPlug with brefeldin A (BD, #512301KZ), anti-CD28 (BD, #555726) and anti-CD49d (BD, #340976) were added to all samples at 1 $\mu$ l each. Samples were incubated with loose lids at 37°C in 5% CO<sub>2</sub> for 24 hours. After incubation, red blood cells were lysed with 1x BDPharmLyse (BD, #555899) for 10 minutes in the dark. Samples were centrifuged at 400g for 5 minutes and washed in 2ml of PBS. After another centrifugation (400g, 5 minutes), samples were stained with a viability dye (LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit, L34957) for 20 minutes in the dark at 4°C; washed with FACS buffer (1XPBS, 5% FBS, 0.05%

Sodium Azide) and stained with FcR blocking antibody (14-9161-73, eBiosciences) for 10 minutes in the dark at 4°C. Samples were washed with FACS buffer and stained with a mixture of surface stain antibodies: CD3 BV650 (BD, #563999, clone SK7), CD4 BV605 (Life Technology, #562658, clone S3.5), CD8 BV570 (Biolegend, #301038, clone RPA-T8), CCR7 PECF594 (BD Horizon, #562382, clone 120503), CD45RO APC-H7 (BD Pharmingen, #561137, clone UCHL1) for 30 mins at 4°C in the dark. After incubation, samples were washed with FACS buffer and permeabilised using 200µl of Cytoperm/Cytofix (BD Pharmingen, #554722) for 20 minutes at room temperature in the dark. After permeabilisation, samples were washed with 1x BDPerm wash buffer (BD, #554723). Samples were stained with a cocktail of intracellular antibodies: IFN $\gamma$  V450 (BD, #560371, clone B27), TNF $\alpha$  Pe-Cy7 (eBioscience, #557647, clone Mab11), IL2 FITC (BD Pharmingen, #554565, clone MQ1-17H12), IL4 PE (BD FastImmune, #340451, clone 3010.211), IL5 PE (Biolegend, #504304, clone TRFK5), IL13 PE (Biolegend, #501903, clone JES10-5A2), and IL8 APC (Biolegend, #511410, clone E8N1) for 30 minutes at room temperature in the dark. After staining, samples were washed with perm wash buffer and stored at 4°C in the dark in PBS with 1% paraformaldehyde (PFA) to be acquired within 24 hours using a BD LSRII Flow Cytometer and acquiring a minimum of 100,000 events.

#### *Whole blood stimulation and Luminex assay*

Heparinised whole blood was diluted 1:4 in RPMI 1640 (Life Technology, #21875-091) supplemented with a mixture of penicillin and streptomycin (Sigma, P4458) (1:100, v:v) and L-glutamine (Sigma, #59202C) (1:100, v:v). 200 µl of diluted whole blood was stimulated in duplicate with PPD (Tuberculin PPD-SSI batch RT 50) (final concentration 10µg/ml) and PHA (Sigma, #L8902) (final concentration 10µg/ml). An unstimulated sample was also included. Samples were incubated at 37°C in 5% CO<sub>2</sub>

for six days. After incubation, supernatants were collected, and a viral incubation buffer (1:188 TNBP, Sigma Aldrich T4908; 1:100 Tween 80, Sigma Aldrich P-1754; RPMI 1640, Life Technology #21875-091 supplemented with a mixture of penicillin and streptomycin, Sigma, P4458, final concentration of penicillin 5 units/ml, final concentration of streptomycin 50µg/ml, and L-glutamine, Sigma, #59202C, final concentration 2mM) was added to each sample for 30 minutes. Samples were stored at -80°C until analysis. Reference samples were included at both sites to ensure data compatibility. Cytokine concentrations were measured using Bio-Plex Pro™ Human Cytokine 17-plex (GM-CSF, IFN $\gamma$ , IL12p40, IL13, IL17A, IL1 $\alpha$ , IL1 $\beta$ , IL1RA, IL2, IL5, IL8, IP10, MCP1, MIP1 $\alpha$ , MIP1 $\beta$ , TNF $\alpha$ ) Assay (Bio-Rad, #m5000031yv) according to manufacturer's instructions. Samples were acquired using Bio-Rad Luminex reader.

#### *Data analysis Luminex and Flow cytometry assays*

For the Luminex assay, extrapolated and out of range values were assigned 1.6 pg/ml at the low end of the scale (half the bottom standard concentration) and 11000 pg/ml at the upper end of the scale. Background values measured in unstimulated samples were subtracted from concentrations of stimulated samples. If concentration values were less than 1.6 pg/ml, 1.6 pg/ml was assigned.

In the flow cytometry assay, cells were gated for T cell phenotype. Live, singlet, CD3+ lymphocytes were included. Within that population T cell phenotypic markers (CD4, CD8, CCR7, CD45RO) and intracellular cytokines (IFN $\gamma$ , IL2, TNF $\alpha$ , IL4/5/13, and IL8) were measured. We investigated phenotypes of CD4+, CD4 Naïve (CD4+CCR7+CD45RO-), CD4 central memory (CM, CD4+CCR7+CD45RO+), CD4 effector memory (EM, CD4+CCR7-CD45RO), CD4 terminally differentiated (TD,



CD4+CCR7-CD45RO-), total CD8+, CD8 Naïve (CD8+CCR7+CD45RO-), CD8 CM (CD8+CCR7+CD45RO+), CD8 EM (CD8+CCR7-CD45R) and CD8 TD (CD8+CCR7-CD45RO-). The full gating strategy is described in figure S1. For cytokine responses, values measured in unstimulated samples were subtracted from those measured in stimulated ones, and negative values were assigned 0. Only unstimulated samples were selected to measure changes in phenotypes. In Uganda, 94/111 samples were stained for IL8 as IL8 staining was introduced later into the assay.

#### *Data analysis viSNE and SPADE algorithms*

The “downsample” plugin available in FlowJo was used to select 1000 most representative cells per sample of the lymphocyte gate. Downsampled events were concatenated and the t Stochastic Neighbour Embedding (tSNE) algorithm was used to reduce data dimensionality with the following parameters: perplexity 40; iterations 1000, Eta learning rate 200 and Theta 0.5. Samples were loaded to Cytobank ([www.cytobank.org](http://www.cytobank.org)), where Spanning-tree Progression Analysis of Density-normalized Events (SPADE) was used to identify distinct cell clusters from previously calculated tSNE parameters. The viSNE algorithm was used to analyse phenotypes of T cells.

The viSNE algorithm distributed cells based on the expression of markers (CD4, CD8, CD45RO, CCR7, IFN $\gamma$ , IL2, TNF $\alpha$ , IL4/5/13, and IL8) and the SPADE algorithm identified 50 distinct T cell clusters. Clusters are defined as groups of cells with similar phenotypes. Cluster size is defined as the number of cells within a cluster. Cluster size can change in response to a stimulant, sample characteristics, time etc. These clusters were overlaid onto a viSNE plot, and cell ontology was assigned based on marker expression within each cluster. Samples were chosen randomly for viSNE analysis. The number of samples for viSNE plots must be equal across conditions.

As the maximum available number of samples was 12 at 52 weeks in the UK, we had to select 12 samples at each time point in each country. Due to technical limitations (UK and Ugandan samples were acquired at different sites) viSNE and SPADE analyses were calculated independently for each country, and thus viSNE plots and ontology differ.

### *Statistical analysis*

To test whether sample characteristics differed across countries the Mann-Whitney test was used for continuous variables (mother's median age and birth weight) and the Chi-square test for categorical variables (the proportion of tuberculosis infected mothers, the proportion of CMV infected infants, the proportion of EBV infected infants). Cytokine concentrations and T cell phenotypes measured by Luminex or flow cytometry were compared between countries using linear regression adjusting for mother's age; and between time points using the Mann-Whitney test. Some samples were paired and to avoid type 1 error they were checked for correlation of cytokine concentrations. There was no evidence of the Spearman correlation between paired samples (not shown). To compare cytokine concentrations across countries, median responses were compared. If median concentrations were equal, mean responses were compared.

## Results

*Higher levels of proinflammatory cytokine secretion in Ugandan infant blood after PPD stimulation.*

Overall 313 Ugandan and 40 UK samples were included in this analysis. Mothers of Ugandan infants had a lower median age (34 years in the UK vs 25 years in Uganda,  $p<0.001$ ); a larger proportion of mothers were tuberculosis-infected (146/313 in Uganda vs 0/40 in the UK,  $p<0.001$ ); a larger proportion of infants were CMV infected (44/168 in Uganda vs 0/40 in the UK,  $p<0.001$ ); and a larger proportion of infants were EBV infected (47/168 in Uganda vs 1/40 in the UK,  $p=0.001$ ) (table 1). Using the Luminex assay, we measured concentrations of 17 cytokines in 10 and 52 weeks diluted whole blood UK and Ugandan infant samples stimulated with PPD.

After background subtraction, we found that Ugandan infants produced up to seven-fold higher PPD specific concentrations of IL1 $\beta$  at 10 weeks (8.6 pg/ml in the UK, 60.06 pg/ml in Uganda,  $p<0.001$ ), and up to five-fold higher concentrations at 52 weeks (5.1 pg/ml in the UK, 24.1 pg/ml in Uganda,  $p=0.012$ ). The concentrations of IL1 $\beta$  remained elevated in Uganda at both time points when CMV or EBV infected, or infants born to LTBI mothers were excluded from the analysis (figure 1, table 2). In Uganda, we also observed two-fold higher concentrations of tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) at 10 (59.5 pg/ml in the UK, 127.6 pg/ml in Uganda,  $p=0.036$ ) and 52 (37.6 pg/ml in the UK, 69.6 pg/ml in Uganda,  $p=0.010$ ) weeks and these concentrations remained elevated when CMV and EBV infected infants were taken out of the analysis (figure 1, table 2). When infants born to LTBI mothers were taken out of the analysis, TNF $\alpha$  remained higher in Ugandan infants at 52 (37.6 pg/ml in the UK, 66.9 pg/ml in Uganda,  $p=0.042$ ) but not 10 (59.5 pg/ml in the UK, 111.1 pg/ml in Uganda,  $p=0.139$ )

weeks. Interestingly, both  $\text{TNF}\alpha$  and  $\text{IL1}\beta$  levels were elevated in Uganda in unstimulated and phytohaemagglutinin (PHA) stimulated samples at 10 and 52 weeks (table S2, table S3). Conversely, we report that at 10 weeks UK infants produced higher concentrations of IL10 (8.4 pg/ml in the UK, 1.6 pg/ml in Uganda,  $p=0.007$ ) and IL2 (32.2 pg/ml in the UK, 7.0 pg/ml in Uganda,  $p=0.017$ ). When CMV or EBV infected infants were taken out of the analysis, the levels of IL10 were similar in the UK and Uganda (8.4 pg/ml in the UK, 10.0 pg/ml in Uganda without CMV infants) suggesting that CMV and EBV infections decrease PPD specific IL10 levels. Similarly, the analysis without CMV or EBV infected infants indicated higher interferon  $\gamma$  ( $\text{IFN}\gamma$ ) levels in Uganda at 10 (349.8 pg/ml in the UK, 855.4 pg/ml in Uganda,  $p=0.030$  without CMV infants; 349.8 pg/ml in the UK, 1002.6 pg/ml in Uganda,  $p=0.025$  without EBV infants) but not at 52 (266.7 pg/ml in the UK, 261.4 pg/ml in Uganda,  $p=0.554$  without CMV infants; 266.7 pg/ml in the UK, 252.5 pg/ml in Uganda,  $p=0.594$  without EBV infants) weeks of age. We report no differences in TH1 cytokine concentrations IL12p40 (6.7 pg/ml in the UK, 3.8 pg/ml in Uganda,  $p=0.977$  at 10 weeks; 5.2 pg/ml in the UK, 2.9 pg/ml in Uganda,  $p=0.567$  at 52 weeks); for TH17 cytokine IL17A (21.2 pg/ml in the UK, 18.5 pg/ml in Uganda,  $p=0.907$  at 10 weeks; 15.0 pg/ml in the UK, 16.2 pg/ml in Uganda,  $p=0.843$  at 52 weeks); TH2 cytokines IL13 (330.0 pg/ml in the UK, 165.4 pg/ml in Uganda,  $p=0.508$  at 10 weeks; 140.1 pg/ml in the UK, 120.8 pg/ml in Uganda,  $p=0.811$  at 52 weeks) and IL5 (38.0 pg/ml in the UK, 38.4 pg/ml in Uganda,  $p=0.292$  at 10 weeks; 40.0 pg/ml in the UK, 29.5 pg/ml in Uganda,  $p=0.711$  at 52 weeks) and other cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF),  $\text{IL1}\alpha$ ,  $\text{IL1RA}$ , IL8, interferon gamma-induced protein 10 (IP10), monocyte chemoattractant protein 1 (MCP1), macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ ), and MIP-1 $\beta$  (figure 1, table 2).

*Ugandan infants have lower frequencies of total CD8, Effector Memory (EM) CD8 and Naïve CD8 T cells at 52 weeks*

Having measured cytokine responses after PPD stimulation, we decided to further investigate the immune response following BCG vaccination and measured T cell phenotypes in the UK and Ugandan infants. For this analysis samples from 111 Ugandan and 35 UK infants were available. The Ugandan infants had younger mothers (median age 34 in Uganda and 25 in the UK  $p<0.001$ ), had a larger proportion of LTBI mothers (56/111 in Uganda vs 0/35,  $p<0.001$ ), a larger proportion of infants were CMV infected (17/88 in Uganda vs 0/35 in the UK,  $p=0.005$ ), and a larger proportion of infants were EBV infected (26/88 in Uganda vs 1/35 in the UK,  $p=0.001$ ) (table 3). In Uganda, there was a decrease in the frequency of CD4 ( $p=0.035$ ), naïve CD4 ( $p<0.001$ ), CM CD4 ( $p<0.001$ ), and an increase in CD4 TD ( $p<0.001$ ) T cells over time (figure 2A). We also observed a decrease in naïve CD8 ( $p<0.001$ ), CM CD8 ( $p<0.001$ ), and an increase in EM CD8 ( $p=0.028$ ) and TD CD8 ( $p<0.001$ ) T cells over time (figure 2B). In the UK, we report a decrease in CD4 ( $p=0.031$ ), and an increase in EM CD4 ( $p=0.022$ ), CD8 ( $p<0.001$ ), and EM CD8 (0.010) T cells over time (figure 2A). When T cell phenotypes were compared between countries, we found that UK infants had higher frequencies of naïve CD4 ( $p<0.001$ ), CM CD4 ( $p<0.001$ ), CD8 ( $p<0.001$ ), naïve CD8 ( $p<0.001$ ), CM CD8 ( $p<0.001$ ), and EM CD4 ( $p<0.001$ ) T cells at 52 weeks of life. We also found that Ugandan infants had higher frequencies of EM CD4 ( $p=0.017$ ) at 10 weeks of life, higher TD CD4 ( $p<0.001$ ), and TD CD8 ( $p<0.001$ ) T cells at 52 weeks of life (figure 2A, B).

We also investigated CD4:CD8 T cell ratios and found that there was no difference in the CD4:CD8 ratio between UK and Uganda at 10 weeks ( $p=0.477$ ), but at 52 weeks,

Ugandan infants had a higher CD4/CD8 ratio ( $p=0.001$ ) (figure 3A). We have also found that the CD4:CD8 ratio has decreased in the UK over time ( $p<0.001$ ), but remained unchanged in Uganda ( $p=0.460$ ) (figure 3A). The observed differences in CD4:CD8 ratios are mainly driven by the increased frequency of CD8 T cells over time. Notably, the median frequency of CD8 T cells is 26.8% of all CD3+ T cells in the UK and 15.4% in Uganda at 52 weeks (figure 3B). In summary, we observed an increase in CD8 populations in the UK but not in Uganda. We also report that Ugandan infants have a higher proportion of terminally differentiated T cells.

*Frequencies of IL2, IL4/5/13 and IL8 CD8 producing T cells are higher in the UK than Uganda*

Having measured T cell phenotypes, we also investigated cytokines that they produce. We report that frequencies of PPD specific IFN $\gamma$  or IL2 producing CD4 (0.017% in the UK, 0.006 in Uganda,  $p<0.001$  for IFN $\gamma$ ; 0.141% in the UK, 0.006 in Uganda,  $p<0.001$  for IL2) or CD8 (mean frequency 0.023% in the UK, 0.006 in Uganda,  $p=0.010$  for IFN $\gamma$ ; 0.003% in the UK, 0.000% in Uganda,  $p=0.005$  for IL2) T cells are higher in the UK than Uganda at 52 weeks of age (figure 4A, 4B, table 4). We also observed that frequencies IL8 CD8 producing T cells are higher in the UK at 52 weeks (0.500% in the UK, 0.011 in Uganda,  $p=0.001$ ). In contrast, in Uganda, frequencies of TNF $\alpha$  producing CD4 T cells were higher at 10 weeks (0.016% in the UK, 0.050 in Uganda,  $p=0.033$ ), and frequencies of IL4/5/13 producing CD8 T cells were increased at 52 weeks (0.001% in the UK, 0.013 in Uganda,  $p=0.024$ ) when compared to the UK. Interestingly, the frequencies of unstimulated TNF $\alpha$  producing CD4 or CD8 T cells were elevated in Uganda at 10 and 52 weeks (table S4), and frequencies of PHA stimulated TNF $\alpha$  producing CD4 or CD8 T cells were increased in Uganda at 10 weeks of life (table S5). When infants that were CMV or EBV infected,

or born to LTBI mothers were taken out of the analysis we found that frequencies of IFN $\gamma$ , IL2 and IL8 producing T cells remained higher in the UK, however, we did not observe higher frequencies of TNF $\alpha$  producing T cells in Uganda. This suggests that CMV or EBV infection, or being born to an LTBI mother, increases the frequency of PPD specific TNF $\alpha$  producing T cells at 10 weeks of age in Ugandan infants, however, this finding is not supported by the Luminex data discussed earlier. Taken together, we report increased frequencies in IFN $\gamma$  or IL2 producing CD4 or CD8 T cells at 52 weeks in the UK when compared to Uganda.

*viSNE analysis reveals that CD8 clusters increase in size with age*

To study rare populations of T cells and how they change over time and in response to PPD stimulation, we adopted viSNE and SPADE techniques to reduce the dimension and cluster T cells in the UK and Uganda. The acquisition of samples in different laboratories introduced technical limitations due to which, we were unable to calculate one viSNE plot for both countries. Thus, direct comparisons between countries cannot be made, however, observed trends over time and PPD stimulation remain informative. For this analysis, we used 24 samples from the UK (12 at 10 weeks, 12 at 52 weeks) and 24 samples from Uganda (12 at 10 weeks, 12 at 52 weeks). Ugandan infants had younger mothers (median mother age in Uganda 24 years, median mother age in the UK 35.5 years,  $p < 0.001$ ) and a higher proportion of tuberculosis infected mothers (8/24 in Uganda and 0/24 in the UK,  $p = 0.002$ ) (table 5).

In both countries, PPD had a limited effect on T cell cluster size (figure 5A, 5B, 5C-D left two columns) and age had a much higher impact on the dynamics of T cell populations. In the UK, non-cytokine producing CD4 (clusters 24, 26, 29, 36, 37, 4, 44 and 48) and the naïve CD4 (clusters 28, 45 and 47) T cell populations decrease

in size at 52 weeks of age. The diminished non-cytokine producing CD4 T cells are replenished by cytokine producing CD4 populations (clusters 13, 5, 34, 14), and cytokine-producing CD8 T cells (clusters 33 and 42) (figure 5A, 5B right three columns). In Uganda, changes with age are less pronounced. Similarly as in the UK, the non-cytokine producing CD4 clusters decrease with age (clusters 25-27), giving way to a number of unidentified clusters (30, 33, 34, 36, 37, 40-44, 49), cytokine (clusters 8, 18) and non-cytokine (cluster 29) producing CD8 populations, as well as EM CD4 (cluster 10) and EM CD8 (cluster 13) T cells (figure 5A, 5C right three columns). In contrast to the UK, we also observed a decrease in cytokine-producing CD8 populations (clusters 3 and 19). Taken together, we report that in both countries, non-cytokine producing CD4 T cells decrease with age and these cells are replenished by cytokine-producing CD8 T cells.

Having analysed sizes of T cell clusters, we examined the MFI expression of T cell markers and assigned ontology to 45/50 (90%) T cell populations in the UK and 27/50 (54%) in Uganda (figure 6A and 6B respectively). We found limited evidence of changes in marker expression after PPD stimulation in both countries (figure 6C, 6D). We also examined the effect of time on MFI and found that in the UK, there is an overall decrease in the IFN $\gamma$  producing clusters in unstimulated and PPD stimulated cells with time (figure 7A). In Uganda, we observed an increase in IL8 producing clusters in unstimulated and PPD stimulated cells (figure 7B). Overall, we found that age had an aberrant effect on T cell clusters, and no clear pattern of marker expression emerged.



## Discussion

BCG efficacy varies across geographical regions and between low and high-income countries<sup>2</sup>. This study aimed to investigate the immunogenicity of BCG in UK and Ugandan infants.

We first examined the PPD-stimulated, diluted whole blood cytokine responses measured by the Luminex assay. Both at 10 and 52 weeks, we observed an increase in  $\text{TNF}\alpha$  and  $\text{IL1}\beta$  concentrations in Uganda, and this increase was independent of CMV, EBV or maternal LTBI. We have also examined unstimulated and PHA responses and confirmed that Ugandan unstimulated and PHA responses of  $\text{TNF}\alpha$  and  $\text{IL1}\beta$  were higher at both time points. One study, conducted in Malawian and UK adolescents, confirms increased levels of  $\text{TNF}\alpha$  and  $\text{IL1}\beta$  before and after BCG vaccination in Malawi<sup>15</sup>. However, our findings contrast with previous observations between UK, Malawian and Gambian infants, who had similar levels of  $\text{IL1}\beta$  and  $\text{TNF}\alpha$ <sup>13,16</sup>. Importantly, in our study, all infants were vaccinated within the first week of life with BCG SSI, and samples were collected 10 and 52 weeks after BCG immunisation. However, in the study comparing UK and Malawian responses, UK infants were vaccinated around the fifth week of life and Malawian infants around the seventh week of life, and their samples were collected three months after BCG immunisation. The Gambian infants were vaccinated in the first week of life but received a different vaccine strain, BCG Russia, which is known to elicit distinct immune responses<sup>17</sup>. Previous studies have reported higher inflammatory signals in individuals of African descent. A study in African American women highlights that they are more likely to carry allelic variants that up-regulate proinflammatory cytokines, including  $\text{IL1}$ <sup>18</sup>. Another study has investigated the inflammatory profile of macrophages and found that individuals of African ancestry have a markedly higher

expression of pro-inflammatory genes and better control of bacterial growth<sup>19</sup>. One of the explanations proposed by Nédélec et. al<sup>19</sup>, could be that as European ancestors have moved out of Africa, they were exposed to fewer of bacterial pathogens<sup>20</sup>, whereas African ancestors and their exposure to bacterial pathogens remained high due to a hotter climate. As a consequence, our immune systems have adapted accordingly, and individuals of European ancestry produce lower amounts of proinflammatory cytokines than their African colleagues<sup>21</sup>. On a molecular level, these differences in levels of proinflammatory cytokines could be driven by changes in frequencies of non-T cell populations (monocytes, macrophages, natural killer (NK) cells, neutrophils, mast cells, or eosinophils), epigenetic modifications, higher expression of Toll-like receptors (TLRs), induced gene expression and protein transcription and translation, or other mechanisms.

Surprisingly, we found no differences in TH1 or TH2 cytokines between UK and Uganda. This is in contrast to the Malawian study, where higher IFN $\gamma$  and IL12p40 were observed in the UK, and higher IL13 in Malawi. This could be explained by the study differences outlined before (time of vaccination, time of sample collection), but could also be a reflection of the characteristics of study cohorts. In the Malawian and Gambian studies, both cohorts were rural, whereas in our study, the Ugandan cohort resides in Entebbe, a prosperous city with an urban population. This has significant implications as urban populations have a reported reduced exposure to environmental mycobacteria<sup>22</sup>, one of the mechanisms proposed to influence the immunogenicity and efficacy of the BCG vaccine, also known as BCG masking<sup>23</sup>.

To dissect the source of cytokines, we investigated T cell function and phenotype in both countries. We found that in both countries, the frequency of CD4 T cells decreased over time, and in the UK the frequency of CD8 T cells increased. This is in agreement with a previously published study in US infants, where the frequency of

CD4 T cells decreased, and CD8 T cells increased during the first year of life<sup>24</sup>. Changes in T cell frequencies are not well understood. Viral infections such as CMV and EBV, as well as vaccines, the majority of which are delivered in the first year of life, can change the frequency of CD4 and CD8 T cells. Due to the increase in CD8 T cell frequency in the UK at 52 weeks, we have observed an increased CD4:CD8 ratio in Uganda. A healthy CD4:CD8 ratio is around 2 and above, and an inverted ratio (less than 1) has been associated with increased morbidity and mortality across age groups<sup>25</sup>. Interestingly, we report that the CD4 and CD8 naïve populations, as well as the TD CD4 and the TD CD8 populations, are higher in frequency in Uganda than the UK at 52 weeks of life. At birth and before antigen exposure, a majority of T cells are naïve. The exposure to antigens, usually within the first year of life differentiates them from central memory T cells, which traffic to lymphoid tissues; and effector memory T cells, which migrate to peripheral tissues. Repetitive antigen exposure and proliferation further differentiate memory T cells into their terminally differentiated state<sup>26</sup>. We hypothesise that differences in naïve and TD T cells observed in the UK and Uganda could be explained by higher and repetitive antigen exposure in Uganda, differentiating naïve T cells to memory and eventually to a terminal state, all within the first year of life. In the UK, where pathogen exposure is lower, this T cell differentiation could happen later in life.

To complement our understanding of the BCG induced immune responses, we examined frequencies of cytokine-producing cells in the UK and Uganda. We reported moderately higher frequencies of IFN $\gamma$  or IL2-producing CD4 and CD8 T cells in the UK at 52 weeks. IFN $\gamma$  is an important cytokine that stimulates macrophage-mediated killing of *Mycobacterium tuberculosis* (*Mtb*)<sup>27</sup>. An increase in IFN $\gamma$  producing polyfunctional T cells was observed after BCG vaccination<sup>28</sup>; IFN $\gamma$  has been studied extensively as a potential correlate of protection<sup>29</sup>; and higher levels of

IFN $\gamma$  were measured in whole blood stimulation assays in the UK as compared to Malawi or The Gambia<sup>13</sup>. We have also observed higher frequencies of IL4/5/13 producing CD8 T cells at 52 weeks in Uganda. TH2 cytokine-producing CD8 T cells have been observed before *in vitro*, however, their role is unclear<sup>30</sup>. Higher production of TH2 cytokines could impair TH1 immune responses, and induce alternative activation of macrophages, both factors that could impair effective *Mtb* killing resulting ultimately in a lower BCG efficacy. However, the observed differences and median frequencies are small, and the effect could diminish when, like in the Luminex assay, other non-T cell populations and their cytokines are measured. This could explain why frequencies of cytokines producing T cells do not agree with Luminex results and *vice versa*, i.e. ICS measurement is restricted to T cells, whereas the Luminex assay is not.

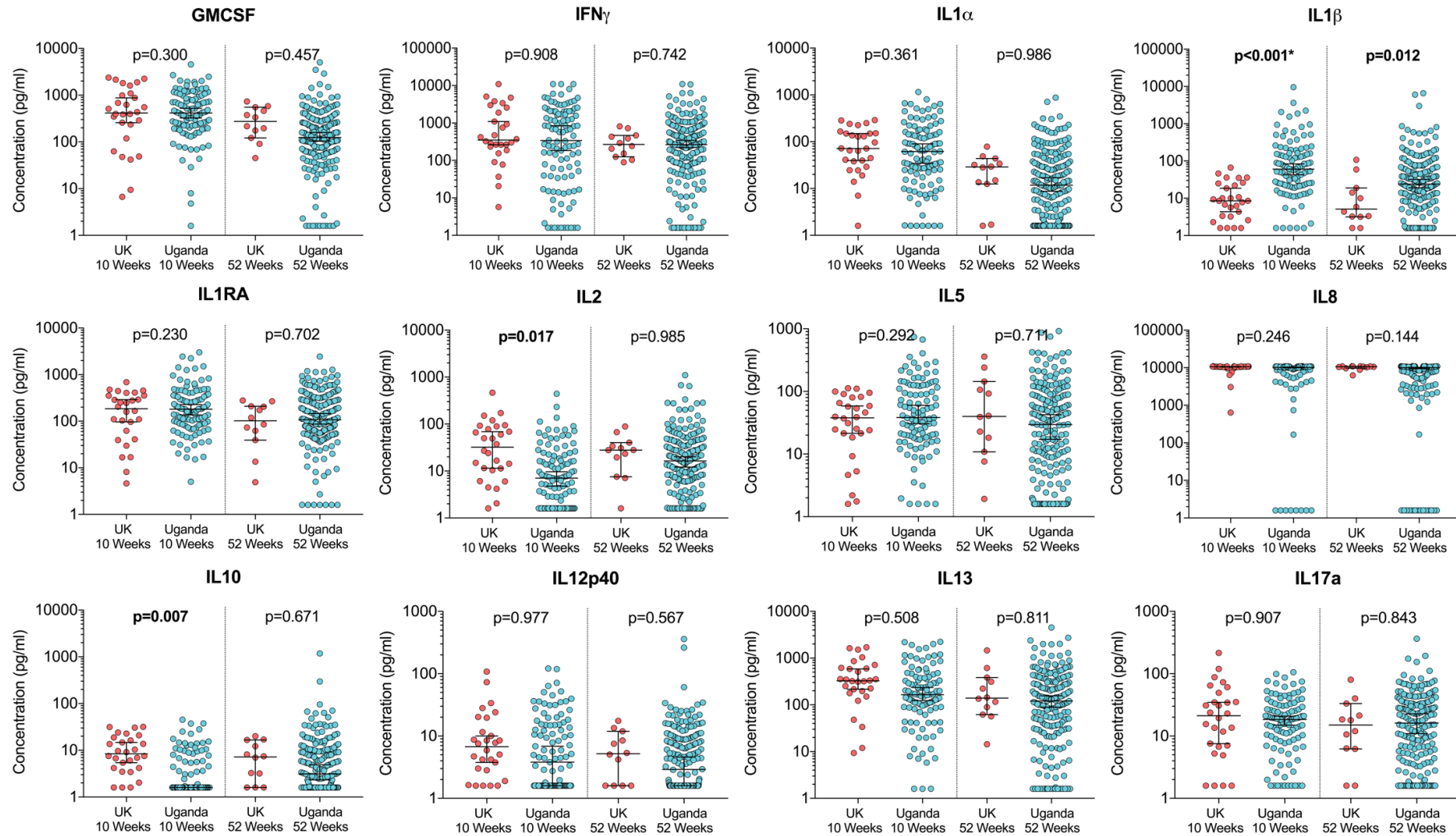
Lastly, we employed tSNE and SPADE algorithms to visualise rare populations of T cells and examine their dynamics. We found that over time there was a decrease in non-cytokine producing T cell populations and an increase in cytokine-producing T cell populations in both countries. We suggest that this could be explained by a development of the immune system over time. Exposure to pathogenic antigens and vaccines drives T cell differentiation from their naïve state (with limited cytokine production) to a memory and TD state (with increased cytokine production and proliferation)<sup>26</sup>.

We have identified some limitations to our study. We measured immune parameters in peripheral blood. Cells that are present in peripheral blood are not representative of the whole cell population and peripheral blood T cells represent only ~2.5% of total T cells in the body<sup>26</sup>. Secondly, more Ugandan infants were infected with CMV, EBV, or born to LTBI mothers. We adjusted for these coinfections in the Luminex analysis, but not in the flow cytometry analysis, mainly due to a smaller number of samples.

However, the limited effect of CMV, EBV and maternal LTBI on cytokine levels measured by Luminex gives us confidence in the flow cytometry analysis.

This paper describes a comprehensive comparison of the immune response following BCG vaccination of UK and Ugandan infants. We report that the immune response of Ugandan infants has an inflammatory profile, which could be mediated by lower levels of naïve T cell populations, and higher levels of terminally differentiated T cells in Uganda at 52 weeks. We emphasise that IFN $\gamma$ , IL2 and TH2 cytokines appear to be important, as we observed different frequencies in cytokine-producing T cells. We highlight the role of these immune differences in the mediation of BCG immunogenicity and their implied impact on efficacy. Vaccinologists should continue to design vaccines that focus on the expansion of TH1, TH17 or other immune compartments, while having in mind and continuing to explore the observed differences in inflammatory profiles as well as frequencies of terminally differentiated T cells. Future studies that dissect BCG immunogenicity should be conducted, and include multiple countries in both urban and rural settings, as well as investigate other cell types such as NK cells.

## Figures



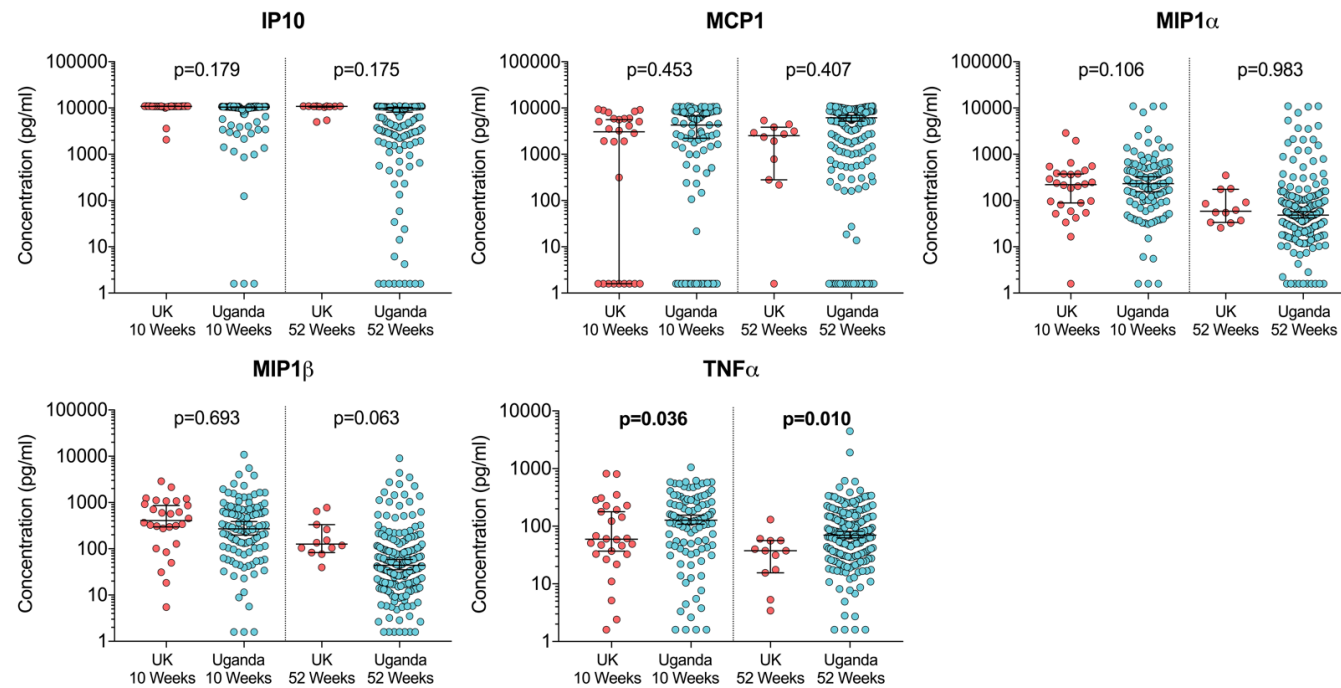
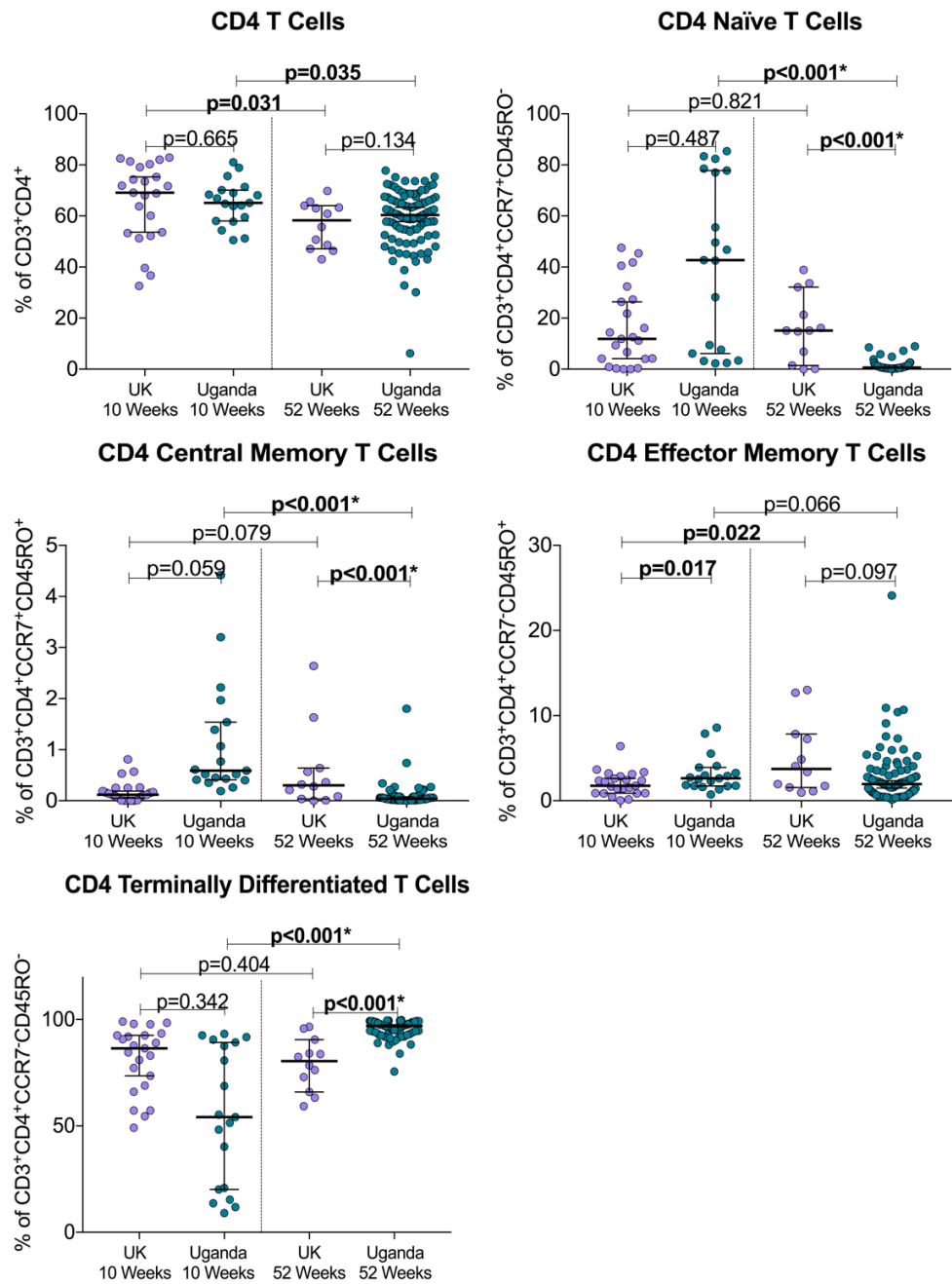


Figure 1. PPD specific cytokine levels corrected for background and acquired using a 17-plex Luminex assay after 6-day whole blood stimulation. Cytokines were examined in Uganda at 10 (n=108) and 52 weeks (n=205); in the UK at 10 (n=28) and 52 weeks (n=12). The linear regression was used to test for significance adjusting for mother's age between the UK and Uganda over a single time point. P value  $\leq 0.05$  was considered significant, is highlighted in bold and printed above each compared pair of countries. The asterisk indicates that the p-value remains significant after the Bonferroni multiple testing adjustment. The horizontal line depicts the median value with 95% confidence intervals (CI).



A



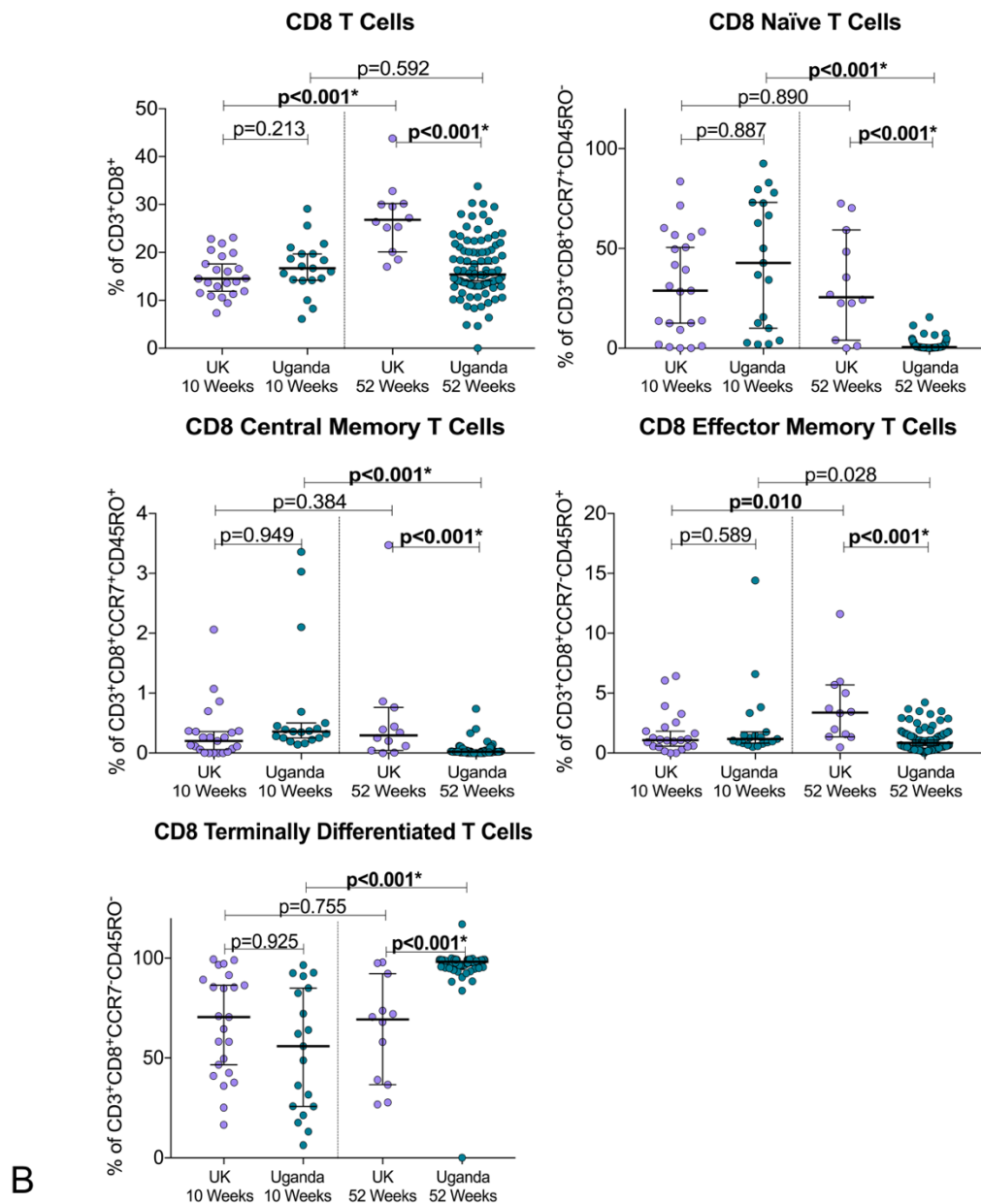


Figure 2. CD4 (A) and CD8 (B) T cell phenotypes of unstimulated samples examined with flow cytometry. Phenotypes were examined in Uganda at 10 (n=19) and 52 weeks (n=92); in the UK at 10 (n=23) and 52 weeks (n=12). The Linear regression was used to test for significance adjusting for mother's age between the UK and Uganda over a single time point. The Mann-Whitney test was used to test for significance between 10 and 52 weeks over a single country. P value  $\leq 0.05$  was considered significant, highlighted in bold and printed above each comparison. The asterisk indicates that the p value remains significant after the Bonferroni multiple testing adjustment. The horizontal line depicts the median value with 95% confidence intervals (CI).

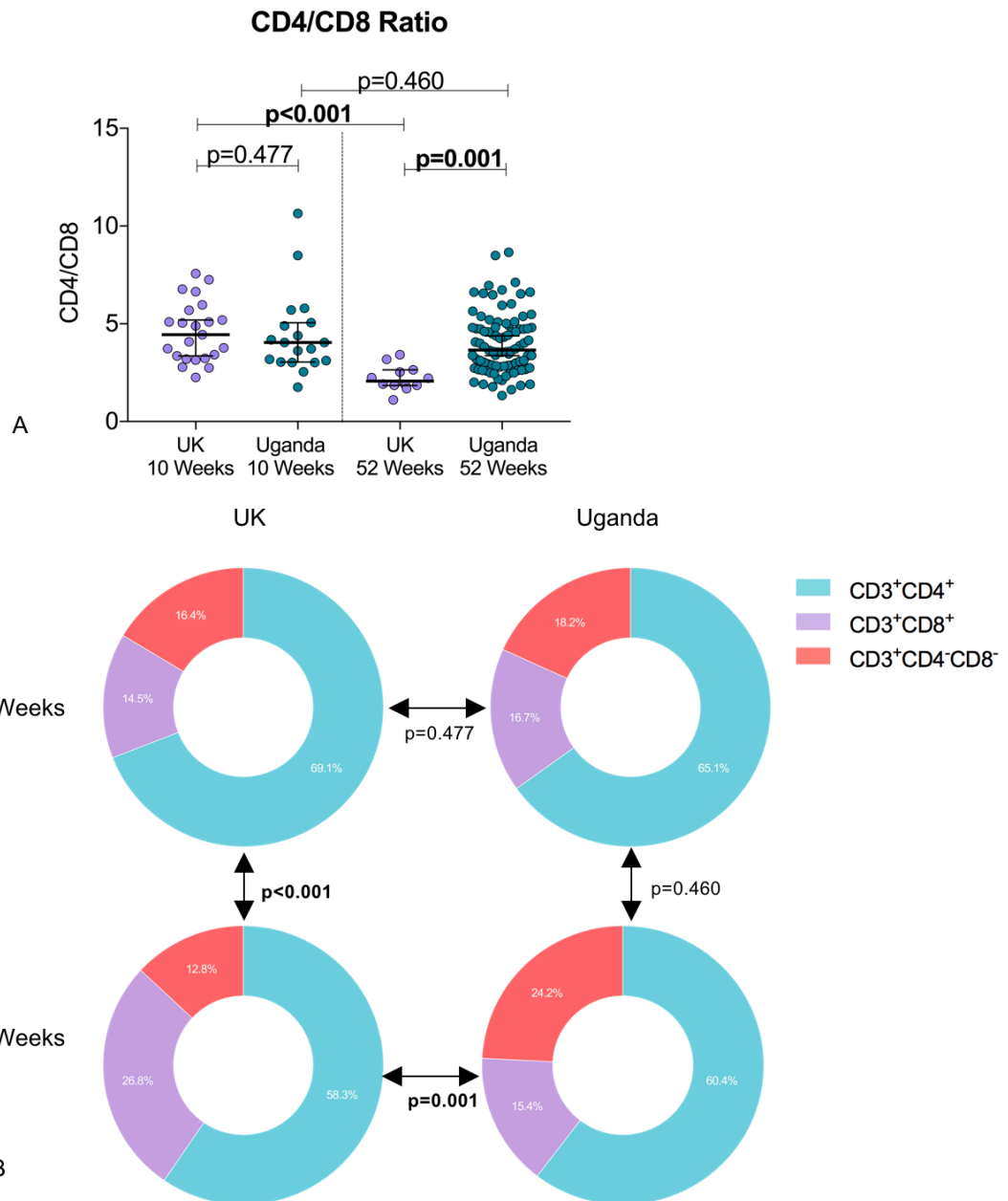
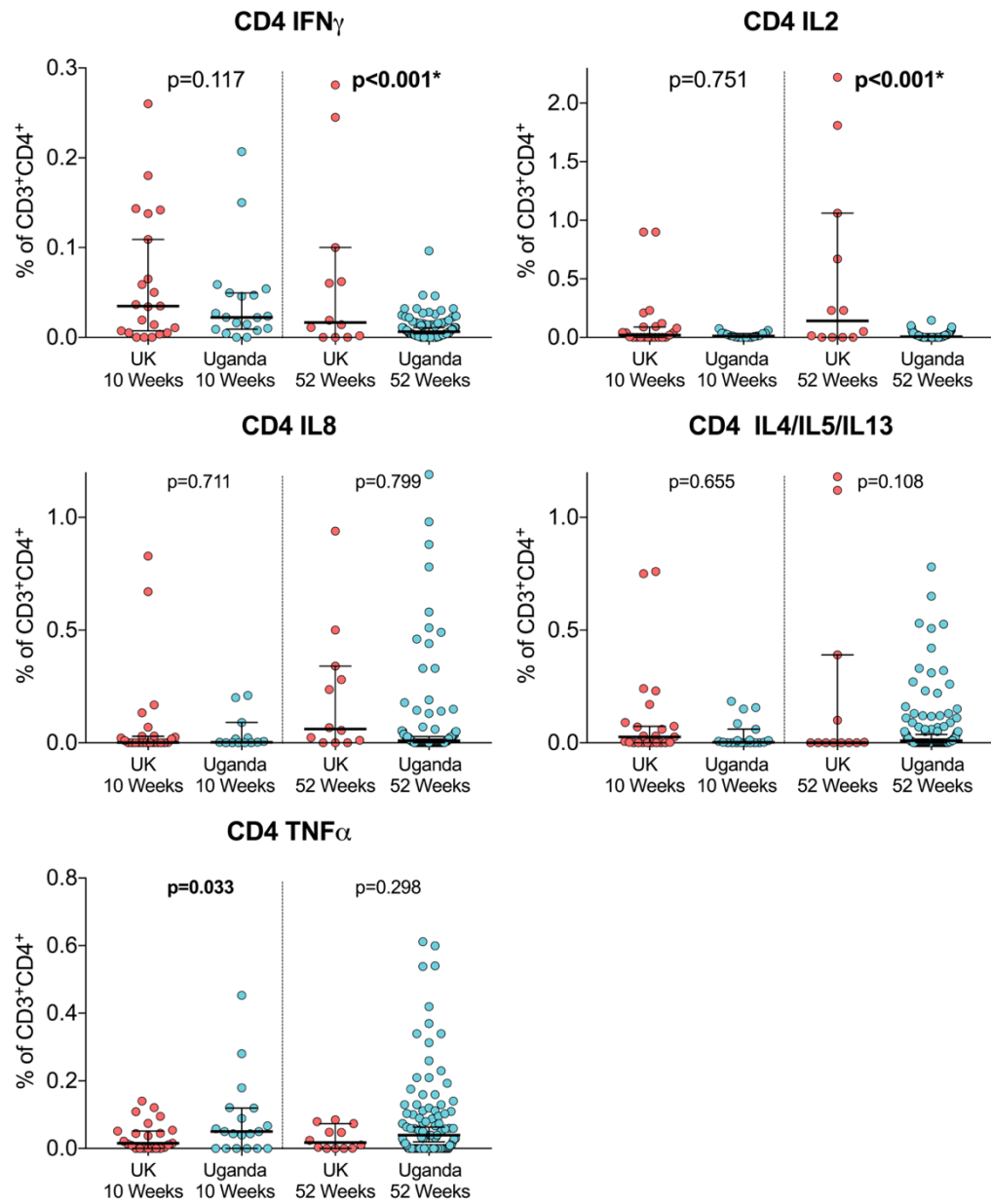


Figure 3. The median frequencies of CD3<sup>+</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup> and CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup> T cell phenotypes of unstimulated samples were examined in Uganda at 10 (n=19) and 52 weeks (n=92); in the UK at 10 (n=23) and 52 weeks (n=12), and measured with flow cytometry. A comparison of CD4/CD8 ratios over time and country (A). A breakdown of median frequencies of each T cell population over time and country (B). The Mann-Whitney test was used to test for significance of CD4/CD8 ratio between 10 and 52 weeks over a single country and the linear regression adjusting for mother's age was used to test between the UK and Uganda over a single time point. P value  $\leq 0.05$  was considered significant, is highlighted in bold and printed in between each compared pair of countries or time points. The horizontal line depicts the median value with 95% confidence intervals (CI).



A

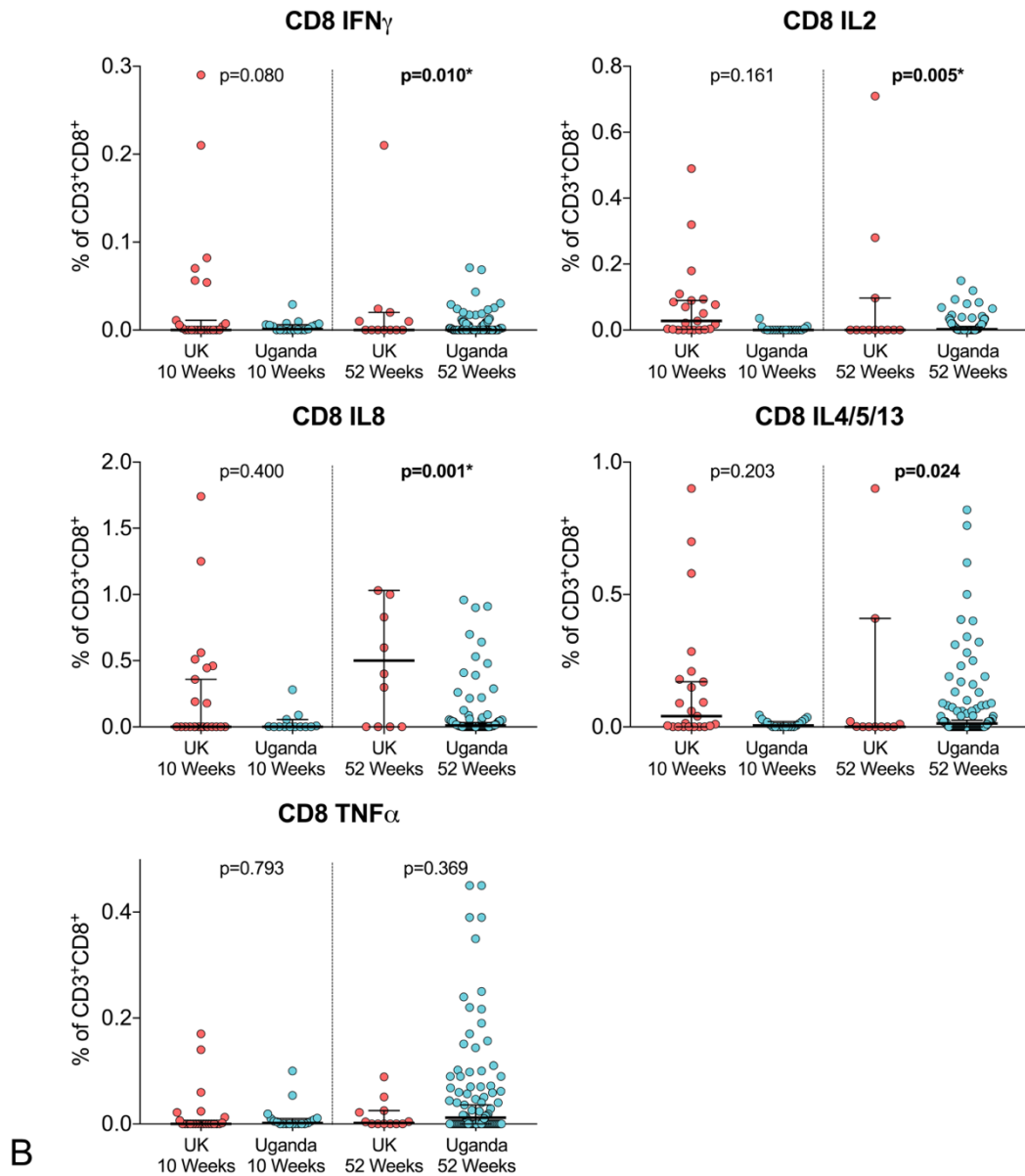
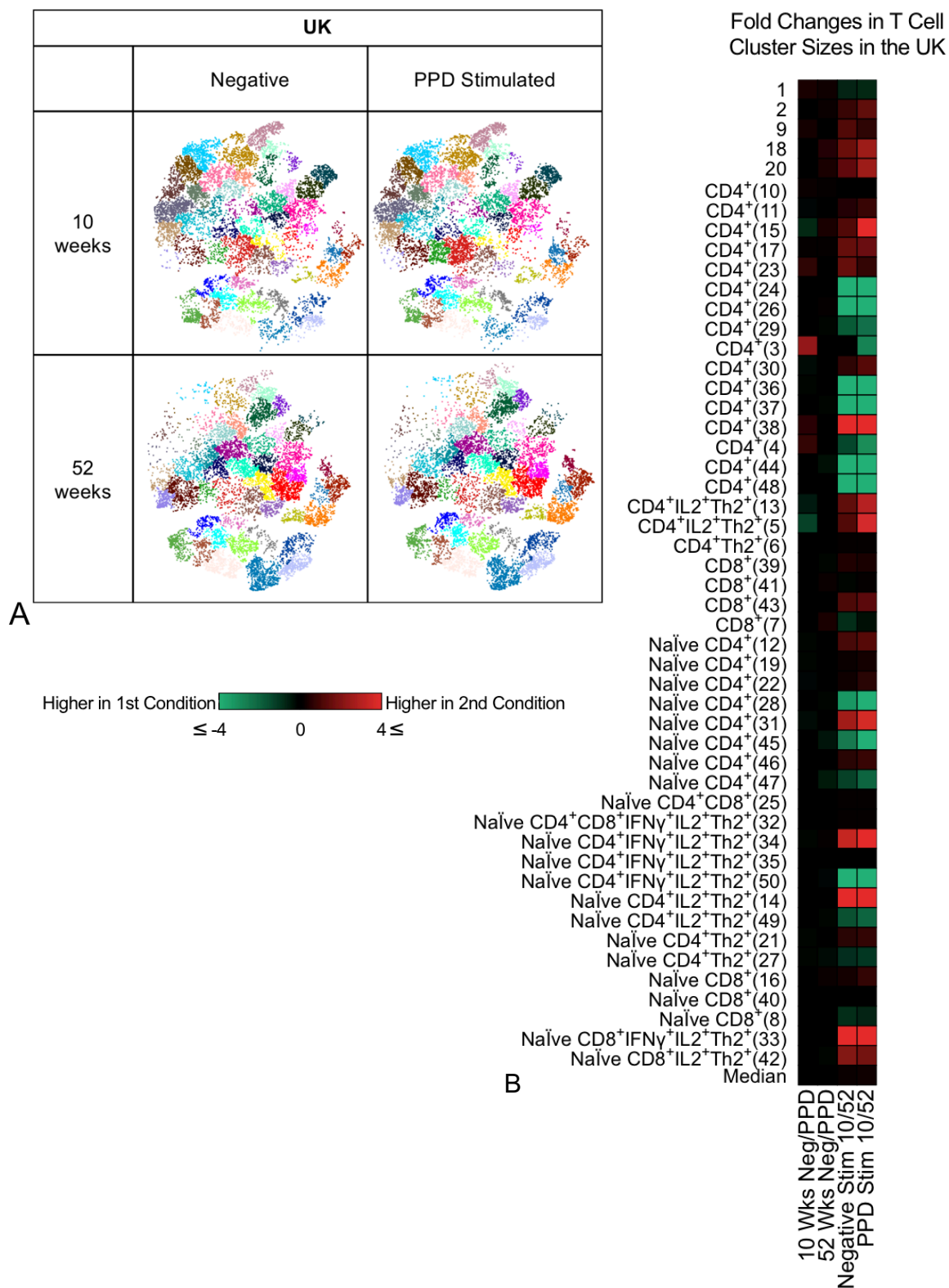


Figure 4. PPD specific cytokine responses corrected for background and measured by flow cytometry in CD4 (A) and CD8 (B) cells. Cytokines were examined in Uganda at 10 (n=19) and 52 weeks (n=92); in the UK at 10 (n=23) and 52 weeks (n=12). The linear regression was used to test for significance adjusting for mother's age between the UK and Uganda over a single time point. P value  $\leq 0.05$  was considered significant, is highlighted in bold and printed above each compared pair of countries. The asterisk indicates that the p-value remains significant after the Bonferroni multiple testing adjustment. The horizontal line depicts the median value with 95% confidence intervals (CI).



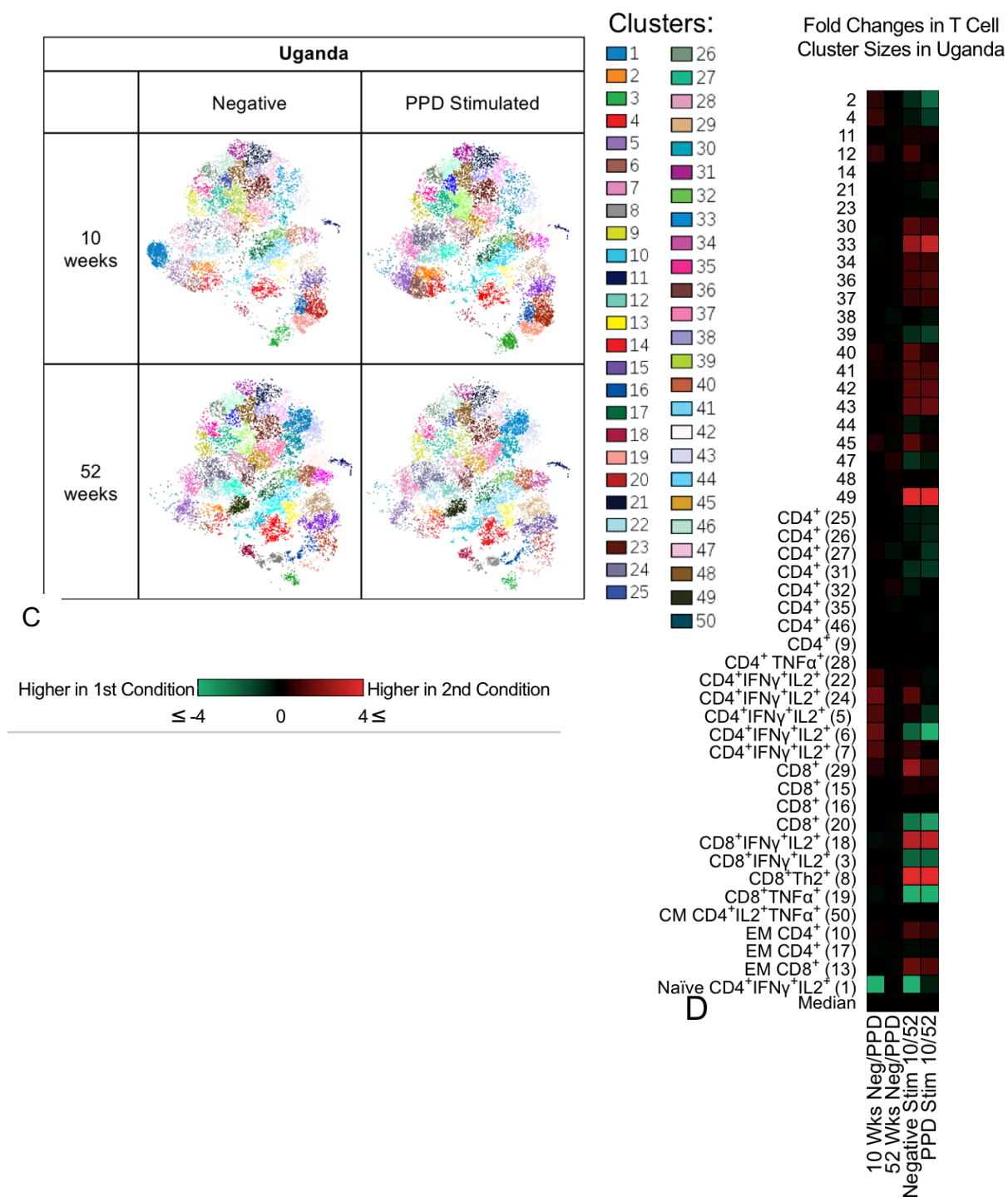
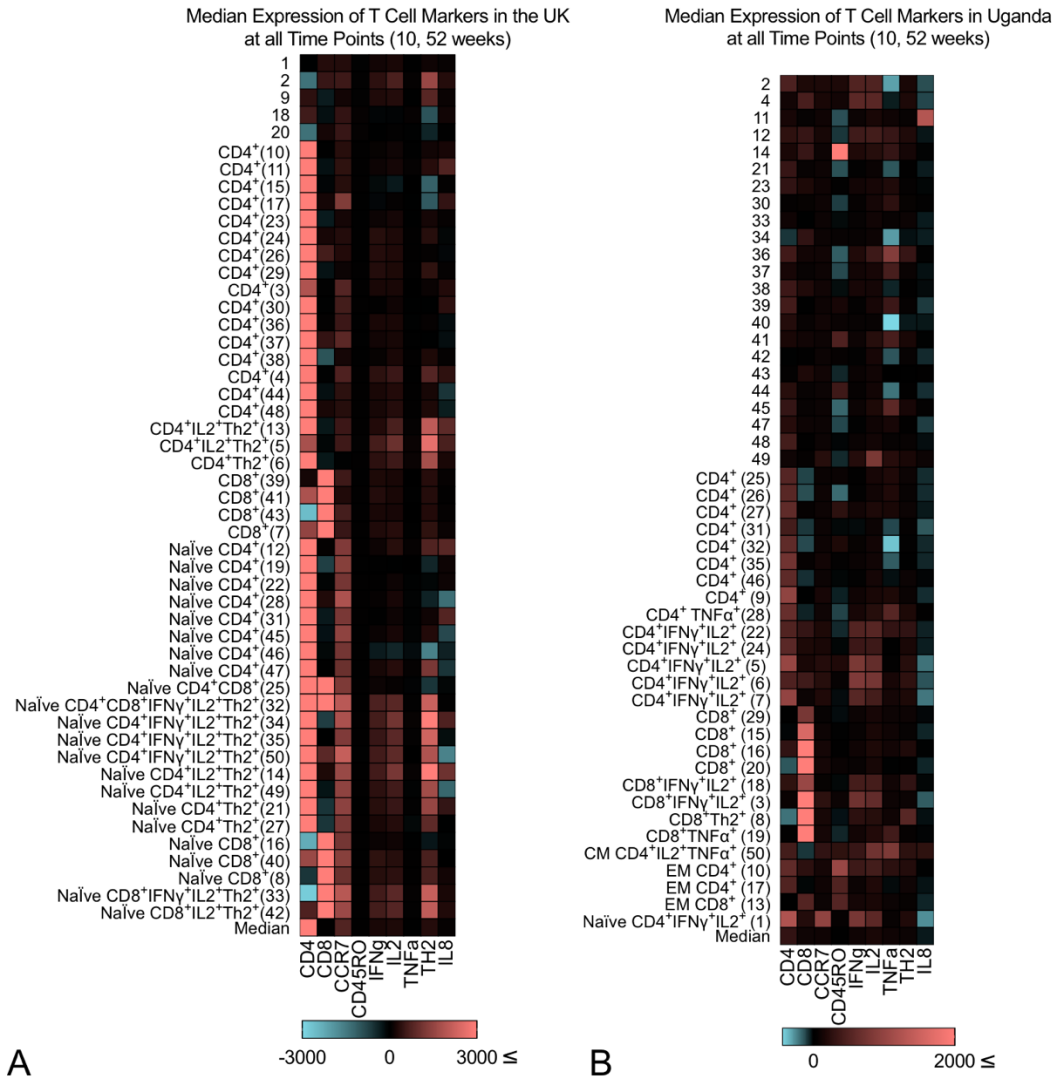


Figure 5. viSNE analysis that represents changes in cluster sizes of T cell populations over time and PPD stimulation in the UK (n=24) (A) and Uganda (n=24) (C). Different clusters are represented by the associated colour palette. Fold change of cluster size over time points and PPD stimulation in the UK (B) and Uganda (D). The green colour indicates that cluster size was larger in the first condition whereas the red colour indicates that cluster size was larger in the second condition. Numbers in brackets indicate cluster numbers. Abbreviations used: wks (weeks), Th2 (IL4/5/13).





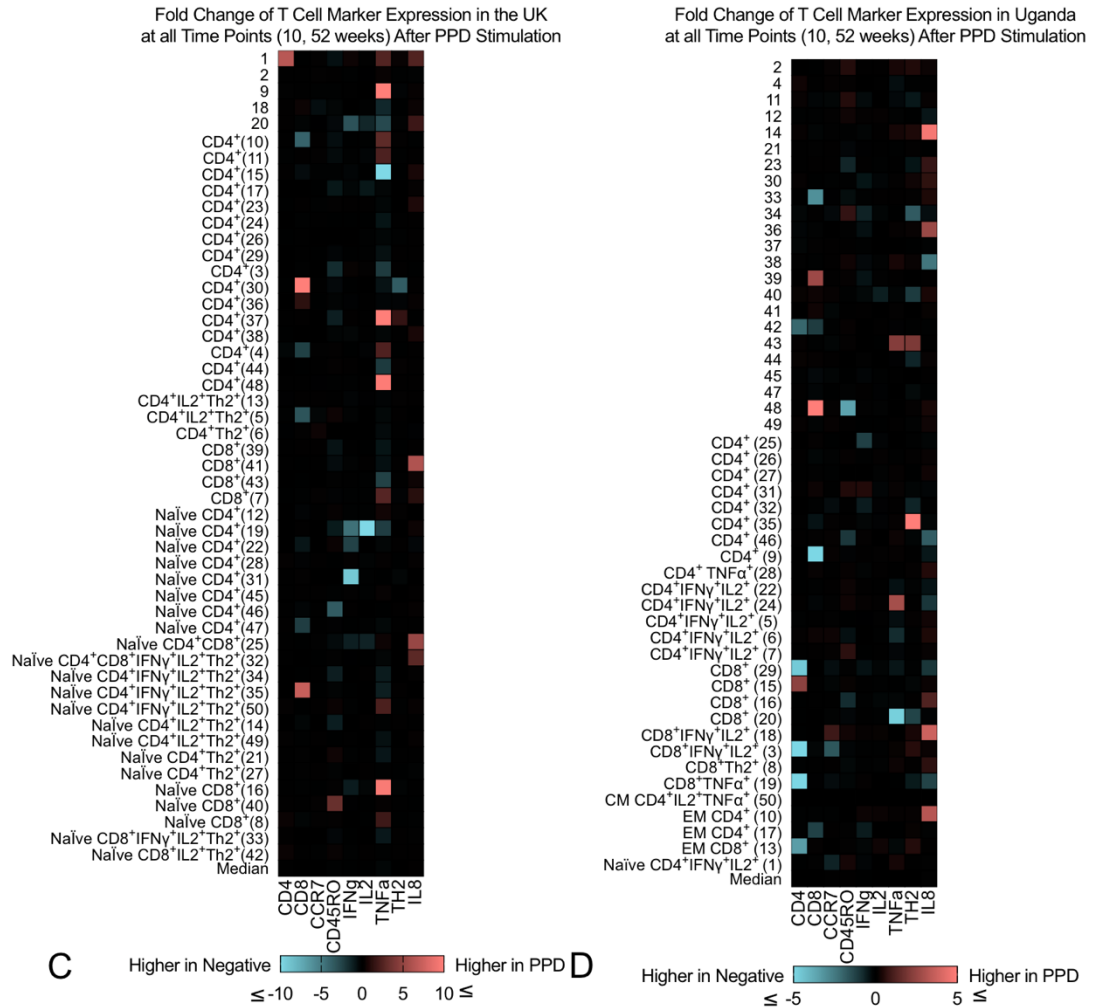


Figure 6. viSNE analysis that represents median fluorescence intensity (MFI) across clusters in the UK (A) and Uganda (B). Fold change of the MFI across clusters after PPD stimulation at all time points in the UK (C) and Uganda (D). The pink colour indicates higher MFI whereas the black and the blue lower MFI(A,B). The pink colour indicates higher MFI after PPD stimulation for a given cluster whereas the blue colour indicates lower MFI after PPD stimulation for a given cluster (C,D). Numbers in brackets indicate cluster numbers. Abbreviations used: Th2 (IL4/5/13), EM (effector memory), CM (central memory).

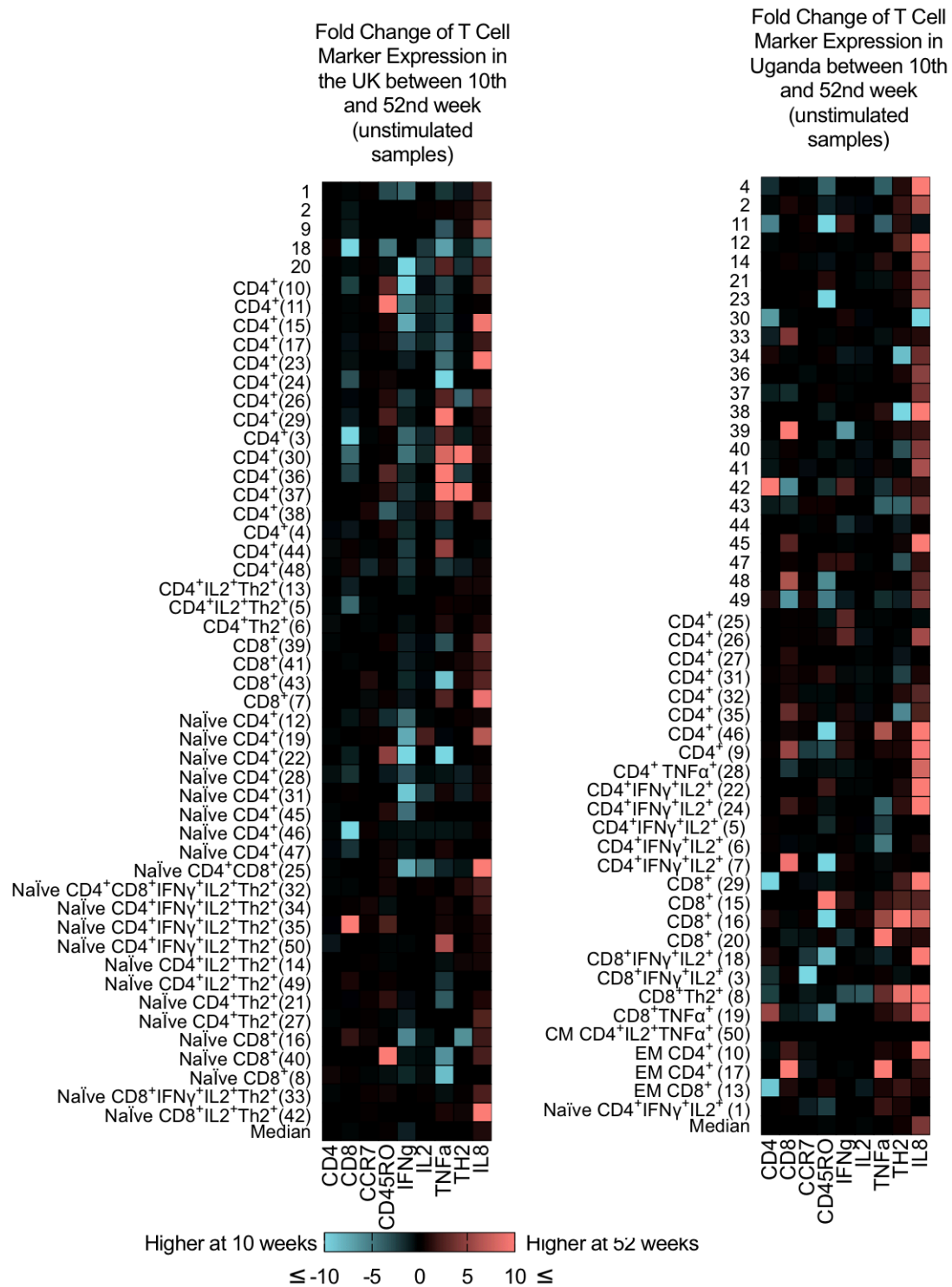


Figure 7. viSNE analysis fold change of the MFI across clusters between 10<sup>th</sup> and 52<sup>nd</sup> week in the UK (A) and Uganda (B) in unstimulated samples. The pink colour indicates higher MFI at 52<sup>nd</sup> week of life for a given cluster whereas the blue colour indicates lower MFI at 52<sup>nd</sup> week of life for a given cluster. Numbers in brackets indicate cluster numbers. Abbreviations used: Th2 (IL4/5/13), EM (effector memory), CM (central memory).

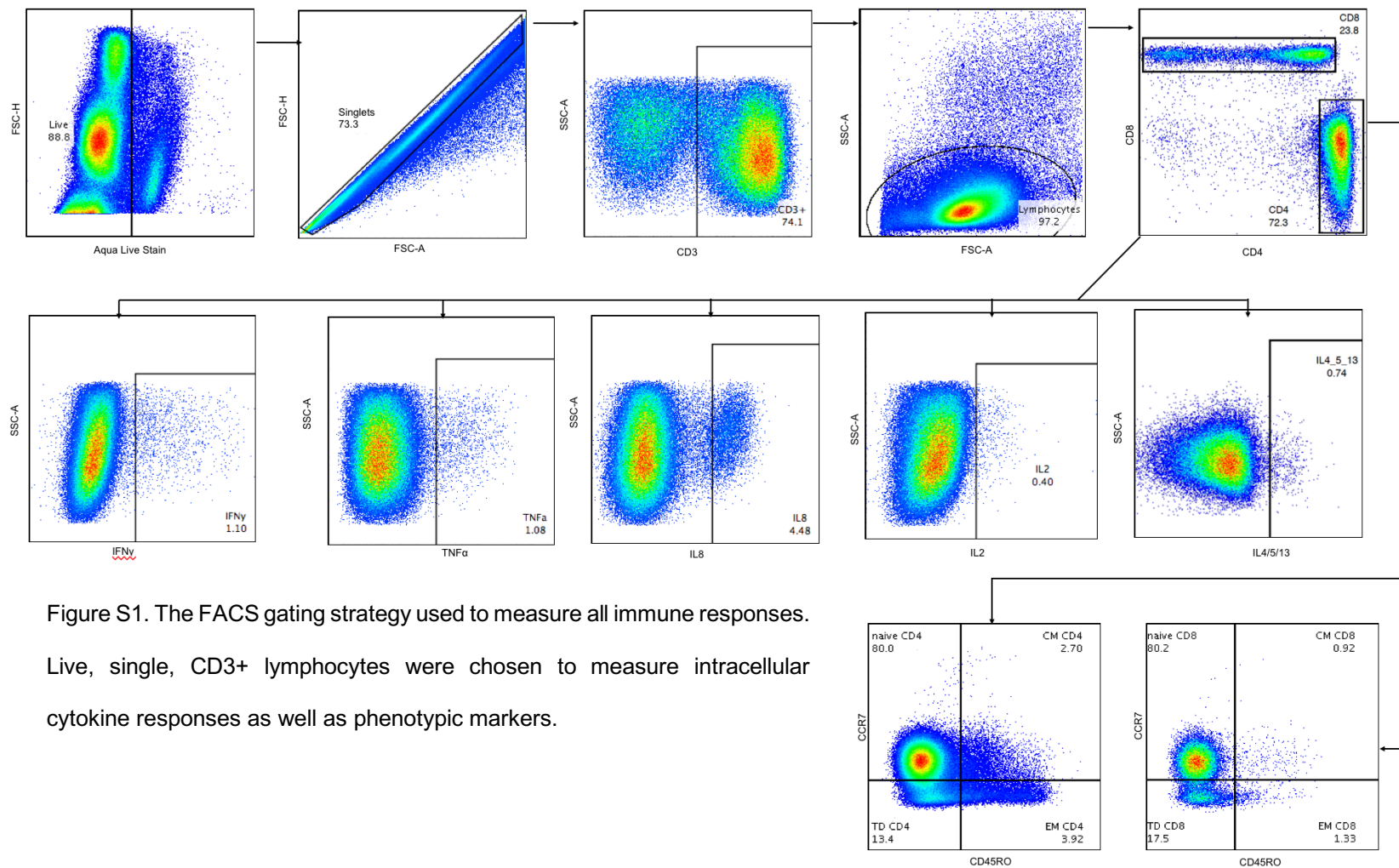


Figure S1. The FACS gating strategy used to measure all immune responses. Live, single, CD3+ lymphocytes were chosen to measure intracellular cytokine responses as well as phenotypic markers.

## Tables

Characteristics	Uganda	UK	P value
Mother's Age (Median Years)	25.0	34.0	<b>&lt;0.001</b>
Birth Weight (kg)	3.2	3.3	0.310
Proportion of Boys	170/313	20/40	0.606
Proportion of Tuberculosis Infected Mothers	146/313 (46.6%)	0/40 (0.0%)	<b>&lt;0.001</b>
Proportion of CMV Infected Infants	44/168 (26.2%)	0/40 (0.0%)	<b>&lt;0.001</b>
Proportion of EBV Infected Infants	47/168 (28.0%)	1/40 (2.5%)	<b>0.001</b>

Table 1: Characteristics of all samples included in the Luminex analysis in Uganda (n=313) and the UK (n=40). P value  $\leq 0.05$  was considered significant and highlighted in bold. The Mann-Whitney test was used for continuous variables (mother's median age and birth weight) and the Chi-square test for categorical variables (the proportion of tuberculosis-infected mothers, the proportion of CMV infected infants, the proportion of EBV infected infants). Due to a limited sample volume, not all infants were tested for CMV and EBV in Uganda.

Visit	Cytokine	Median UK	Median Uganda	Median Uganda LTBI <sup>-a</sup>	Median Uganda CMV <sup>-b</sup>	Median Uganda EBV <sup>-c</sup>	Fold Difference <sup>d</sup>	P <sup>e</sup>	P LTBI <sup>f</sup>	P CMV <sup>-g</sup>	P EBV <sup>-h</sup>
10	GMCSF	416.1	414.7	432.1	372.9	347.4	1.0	0.300	0.605	0.104	0.093
	IFN $\gamma$	349.8	339.1	346.8	855.4	1002.6	1.0	0.908	0.566	<b>0.030</b>	<b>0.025</b>
	IL10	8.4	1.6	1.6	10.0	9.4	<b>5.2</b>	<b>0.007</b>	<b>0.023</b>	0.410	0.506
	IL12p40	6.7	3.8	2.0	9.7	14.8	1.8	0.977	0.666	0.169	0.152
	IL13	330.0	165.4	153.0	376.3	323.8	2.0	0.508	0.366	0.179	0.191
	IL17A	21.2	18.5	15.1	14.0	17.9	1.1	0.907	0.677	0.909	0.912
	IL1 $\alpha$	71.8	61.7	62.5	56.4	35.0	1.2	0.361	0.053	0.542	0.739
	IL1 $\beta$	8.6	60.6	67.2	54.3	38.4	<b>7.1</b>	<b>&lt;0.001*</b>	<b>&lt;0.001*</b>	<b>0.004</b>	<b>0.010</b>
	IL1RA	185.8	181.1	183.5	184.0	159.3	1.0	0.230	0.105	0.203	0.311
	IL2	32.2	7.0	7.0	5.7	6.7	<b>4.6</b>	<b>0.017</b>	0.194	0.242	0.246
	IL5	38.0	38.4	35.9	24.7	21.0	1.0	0.292	0.846	0.151	0.191
	IL8	10607.2	10158.8	10161.9	10527.7	10510.7	1.0	0.246	0.143	0.064	<b>0.047</b>
	IP10	10865.7	10408.7	10411.8	10747.3	10658.0	1.0	0.179	0.249	0.337	0.258
	MCP1	3050.0	4294.1	2788.1	5493.6	4893.8	1.4	0.453	0.920	0.138	0.288
	MIP1 $\alpha$	220.4	231.4	169.5	204.0	141.3	1.0	0.106	0.124	0.203	0.356
52	MIP1 $\beta$	409.2	269.7	258.8	213.8	139.2	1.5	0.693	0.864	0.978	0.790
	TNF $\alpha$	59.5	127.6	111.1	184.0	1485.0	<b>2.1</b>	<b>0.036</b>	0.139	<b>0.025</b>	<b>0.030</b>
	GMCSF	275.3	124.1	117.0	153.9	123.4	2.2	0.457	0.216	0.886	0.847
	IFN $\gamma$	266.7	264.7	225.0	261.4	252.5	1.0	0.742	0.833	0.554	0.594
	IL10	7.2	3.1	2.8	3.7	5.3	2.3	0.671	0.722	0.582	0.803
	IL12p40	5.2	2.9	2.0	2.9	2.1	1.8	0.567	0.265	0.608	0.719
	IL13	140.1	120.8	112.0	142.2	99.3	1.2	0.811	0.967	0.784	0.805
	IL17A	15.0	16.2	15.7	17.1	17.6	1.1	0.843	0.652	0.961	0.531
	IL1 $\alpha$	28.8	11.8	12.4	9.1	9.7	2.4	0.986	0.830	0.946	0.936
	IL1 $\beta$	5.1	24.1	25.9	18.8	21.2	<b>4.7</b>	<b>0.012</b>	<b>0.036</b>	<b>0.012</b>	<b>0.006</b>
	IL1RA	102.0	107.5	93.0	102.9	94.6	1.1	0.702	0.508	0.491	0.228
	IL2	27.9	16.5	13.3	19.0	19.0	1.7	0.985	0.756	0.824	0.867
	IL5	40.0	29.5	31.8	31.4	24.0	1.4	0.711	0.692	0.738	0.978
	IL8	10609.2	10003.2	9943.4	10245.2	10137.3	1.1	0.144	0.194	0.154	0.396
	IP10	10881.7	9846.4	8413.4	10209.3	10103.2	1.1	0.175	0.233	0.274	0.235
	MCP1	2535.5	6132.5	5491.0	6183.4	6183.4	2.4	0.407	0.881	0.231	0.259
	MIP1 $\alpha$	58.7	48.6	44.7	50.8	46.8	1.2	0.983	0.708	0.621	0.536
	MIP1 $\beta$	126.8	43.9	38.0	51.5	37.8	2.9	0.063	0.084	0.173	0.145
	TNF $\alpha$	37.6	69.9	66.9	67.1	65.7	<b>1.9</b>	<b>0.010</b>	<b>0.042</b>	<b>0.009</b>	<b>&lt;0.001*</b>

Higher in the UK

Higher in Uganda

\* P value Remains Significant After Bonferroni Correction

Table 2. A summary of median background-corrected concentrations (pg/ml) and p values of cytokines between all infants, CMV uninfected, EBV uninfected, or infants born to non-LTBI mothers in the UK and Uganda at 10 and 52 weeks of life. Cytokines were examined in Uganda at 10 (n=108) and 52 weeks (n=205); in the UK at 10 (n=28) and 52 weeks (n=12). None of the UK infants had CMV, or were born to LTBI mothers, and one infant had EBV (not shown). Cytokine levels were acquired using a 17-plex Luminex assay after a 6-day whole blood stimulation assay. The linear regression was used to test for significance adjusting for mother's age between the UK and Uganda over a single time point. P value  $\leq 0.05$  was considered significant and highlighted in bold. The asterisk indicates that the p-value remains significant after the Bonferroni multiple testing adjustment. The blue colour means that the median cytokine concentration was higher in the UK whereas the red colour indicates that the median cytokine concentration was higher in Uganda. If medians were equal, then the mean concentration was compared. Abbreviations used: United Kingdom (UK), Uganda (UG), LTBI (latent TB infection), CMV (cytomegalovirus), EBV (Epstein-Barr virus).

<sup>a</sup> Median concentrations of cytokines in Uganda in infants born to LTBI negative mothers only

<sup>b</sup> Median concentrations of cytokines in Uganda in infants uninfected with CMV

<sup>c</sup> Median concentrations of cytokines in Uganda in infants uninfected with EBV

<sup>d</sup> Fold difference between all UK and all Ugandan infants

<sup>e</sup> P values for a comparison between all UK and all Ugandan infants

<sup>f</sup> P values for a comparison between UK and Ugandan infants born to LTBI negative mothers only

<sup>g</sup> P values for a comparison between UK and Ugandan infants uninfected with CMV

<sup>h</sup> P values for a comparison between UK and Ugandan infants uninfected with EBV

Characteristics	Uganda	UK	P value
Mother's Age (Median Years)	25.0	34.0	<b>&lt;0.001</b>
Birth Weight (kg)	3.2	3.3	0.327
Proportion of Boys	57/111	19/35	0.762
Proportion of Tuberculosis Infected Mothers	56/111	0/35	<b>&lt;0.001</b>
Proportion of CMV Infected Infants	17/88	0/35	<b>0.005</b>
Proportion of EBV Infected Infants	26/88	1/35	<b>0.001</b>

Table 3: Characteristics of all samples included in the flow cytometry analysis in Uganda (n=111) and the UK (n=35). P value  $\leq 0.05$  was considered significant and highlighted in bold. The Mann-Whitney test was used for continuous variables (mother's median age and birth weight) and the Chi-square test for categorical variables (the proportion of tuberculosis infected mothers, the proportion of CMV infected infants, the proportion of EBV infected infants). Due to a limited sample volume, not all infants were tested for CMV and EBV in Uganda.



T Cell	Visit	Cytokine	Median UK	Median Uganda	Median Uganda in LTBI <sup>a</sup>	Median Uganda in CMV <sup>b</sup>	Median Uganda in EBV <sup>c</sup>	Fold Difference <sup>d</sup>	P <sup>e</sup>	P in LTBI <sup>f</sup>	P in CMV <sup>g</sup>	P in EBV <sup>h</sup>
CD4	10	IFN $\gamma$	0.035	0.022	0.022	0.037	0.023	1.5	0.117	0.137	0.194	0.151
		IL2	0.020	0.013	0.013	0.014	0.007	1.6	0.751	0.959	0.902	0.847
		IL8	0.001	0.002	0.012	0.012	0.004	2.0	0.711	0.798	0.730	0.650
		IL4/5/13	0.026	0.002	0.000	0.004	0.001	13.0	0.655	0.894	0.986	0.922
		TNF $\alpha$	0.016	0.050	0.044	0.056	0.056	3.1	0.033	0.286	0.211	0.105
	52	IFN $\gamma$	0.017	0.006	0.008	0.005	0.005	2.6	<0.001*	0.028	0.001*	0.004*
		IL2	0.141	0.006	0.004	0.004	0.006	24.0	<0.001*	0.003*	<0.001*	<0.001*
		IL8	0.061	0.009	0.028	0.010	0.009	6.8	0.799	0.096	0.885	0.899
		IL4/5/13	0.000	0.010	0.010	0.009	0.020	NA	0.108	0.316	0.028	0.288
		TNF $\alpha$	0.017	0.040	0.036	0.029	0.038	2.3	0.298	0.084	0.526	0.373
CD8	10	IFN $\gamma$	0.000	0.001	0.000	0.003	0.000	NA	0.080	0.191	0.201	0.162
		IL2	0.028	0.000	0.000	0.000	0.000	NA	0.161	0.313	0.328	0.286
		IL8	0.000	0.000	0.000	0.005	0.002	NA	0.400	0.485	0.542	0.473
		IL4/5/13	0.040	0.005	0.013	0.013	0.007	8.0	0.203	0.451	0.430	0.373
		TNF $\alpha$	0.000	0.002	0.000	0.000	0.000	NA	0.793	0.817	0.901	0.652
	52	IFN $\gamma$	0.000	0.000	0.000	0.000	0.001	NA	0.010*	0.062	0.036	0.036
		IL2	0.003	0.000	0.004	0.002	0.002	NA	0.005*	0.032	0.009*	0.010*
		IL8	0.500	0.011	0.033	0.013	0.008	45.5	0.001*	0.004*	0.004*	0.007*
		IL4/5/13	0.001	0.013	0.020	0.008	0.011	16.7	0.024	0.090	0.030	0.094
		TNF $\alpha$	0.002	0.012	0.019	0.000	0.003	5.8	0.369	0.245	0.434	0.475

Higher in the UK

Higher in Uganda

\* P Value Remains Significant After Bonferroni Correction

Table 4. A summary of PPD specific background-corrected median frequencies (%) and p values of cytokines between all infants, CMV uninfected, EBV uninfected, or infants born to non-LTBI mothers. Cytokines were examined in Uganda at 10 (n=19) and 52 weeks (n=92); in the UK at 10 (n=23) and 52 weeks (n=12). None of the UK infants had CMV, or were born to LTBI mothers, and one infant had EBV (not shown). Cytokine levels were acquired using flow cytometry after a 24 hours whole blood stimulation assay. The linear regression was used to test for significance adjusting for mother's age between the UK and Uganda over a single time point. P value  $\leq 0.05$  was considered significant and highlighted in bold. The asterisk indicates that the p-value remains significant after the Bonferroni multiple testing adjustment. The blue colour means that the median cytokine concentration was higher in the UK whereas the red colour indicates that the median cytokine concentration was higher in Uganda. If medians were equal, then the mean concentration was compared. Abbreviations used: United Kingdom (UK), Uganda (UG), LTBI (latent TB infection), CMV (cytomegalovirus), EBV (Epstein-Barr virus), Not Applicable (NA).

<sup>a</sup> Median frequency of cytokines in Uganda in infants born to LTBI negative mothers only

<sup>b</sup> Median frequency of cytokines in Uganda in infants uninfected with CMV

<sup>c</sup> Median frequency of cytokines in Uganda in infants uninfected with EBV

<sup>d</sup> Fold difference between all UK and all Ugandan infants

<sup>e</sup> P values for a comparison between all UK and all Ugandan infants

<sup>f</sup> P values for a comparison between UK and Ugandan infants born to LTBI negative mothers only

<sup>g</sup> P values for a comparison between UK and Ugandan infants uninfected with CMV

<sup>h</sup> P values for a comparison between UK and Ugandan infants uninfected with EBV

Characteristics	UK	Uganda	P value
Mother's Age (Median Years)	35.50	24.00	<b>&lt;0.001</b>
Birth Weight (kg)	3.26	3.15	0.951
Proportion of Boys	12/24	8/24	0.242
Proportion of Tuberculosis Infected Mothers	0/24	8/24	<b>0.002</b>
Proportion of CMV Infected Infants	0/24	2/21	0.122
Proportion of EBV Infected Infants	1/24	5/24	0.053

Table 5: Characteristics of all samples included in the tSNE analysis in Uganda (n=24) and in the UK (n=24). P value  $\leq 0.05$  was considered significant and highlighted in bold. The Mann-Whitney test was used for continuous variables (mother's median age and birth weight) and the Chi-square test for categorical variables (the proportion of tuberculosis infected mothers, the proportion of CMV infected infants, the proportion of EBV infected infants). Due to a limited sample volume not all infants were tested for CMV and EBV in Uganda.

Target gene	Forward Primer	Reverse Primer	Probe
EBV-( <i>BALF5</i> )	CGGAAGCCCTCTGGACTTC	CCCTGTTTATCCGATGGAATG	6FAM-TGTACACGCACGAGAAATGCGCC-BHQ1
HCMV ( <i>UL55</i> )	TGGGCGAGGACAACGAA	TGAGGCTGGGAAGCTGACAT	HEX-TGGGCAACCACCGCACTGAGG-BHQ1

Table S1. Primer/Probe sequences used for EBV and CMV detection

Visit	Cytokine	Median UK	Median Uganda	Fold Difference	P
10	GMCSF	1.6	1.6	1.0	0.193
	IFN $\gamma$	1.6	1.6	1.0	0.100
	IL10	3.8	1.6	2.4	<b>&lt;0.001*</b>
	IL12p40	1.6	1.6	1.0	0.472
	IL13	1.6	1.6	1.0	0.991
	IL17A	1.6	1.6	1.0	0.628
	IL1 $\alpha$	1.6	1.6	1.0	0.074
	IL1 $\beta$	1.6	2.3	1.4	<b>0.005</b>
	IL1RA	4.6	8.2	1.8	<b>0.037</b>
	IL2	1.6	1.6	1.0	0.554
	IL5	1.6	1.6	1.0	0.548
	IL8	383.1	665.5	1.7	<b>0.020</b>
	IP10	126.6	452.0	3.6	<b>&lt;0.001*</b>
	MCP1	4878.1	3667.3	1.3	0.968
	MIP1 $\alpha$	1.6	1.6	1.0	<b>0.015</b>
	MIP1 $\beta$	6.8	8.7	1.3	0.321
	TNF $\alpha$	1.6	2.7	1.7	<b>0.002*</b>
52	GMCSF	1.6	1.6	1.0	0.157
	IFN $\gamma$	1.6	1.6	1.0	<b>0.008</b>
	IL10	1.6	1.6	1.0	0.087
	IL12p40	2.8	1.6	1.7	0.928
	IL13	1.6	1.6	1.0	0.074
	IL17A	1.6	1.6	1.0	0.590
	IL1 $\alpha$	1.6	1.6	1.0	0.139
	IL1 $\beta$	1.6	2.6	1.6	<b>0.014</b>
	IL1RA	18.8	13.5	1.4	0.236
	IL2	1.6	1.6	1.0	0.158
	IL5	1.6	1.6	1.0	0.113
	IL8	259.7	526.7	2.0	<b>0.031</b>
	IP10	106.2	404.8	3.8	<b>&lt;0.001*</b>
	MCP1	3564.8	1176.4	3.0	0.115
	MIP1 $\alpha$	1.6	2.9	1.8	0.127
	MIP1 $\beta$	7.1	13.2	1.9	<b>0.021</b>
	TNF $\alpha$	1.6	3.5	2.2	<b>0.001*</b>

Higher in the UK

Higher in Uganda

\* P value Remains Significant After Bonferroni Correction

Table S2. A summary of median background concentrations (pg/ml) and p values of cytokines between infants. Cytokines were examined in Uganda at 10 (n=108) and 52 weeks (n=205); in the UK at 10 (n=28) and 52 weeks (n=12). Cytokine levels were acquired using the 17-plex Luminex assay after the 6-day whole blood stimulation assay. The linear regression was used to test for significance adjusting for mother's age between the UK and Uganda over a single time point. P value  $\leq 0.05$  was considered significant and highlighted in bold. The asterisk indicates that the p-value remains significant after the Bonferroni multiple testing adjustment. The blue colour means that the median cytokine concentration was higher in the UK whereas the red colour indicates that the median cytokine concentration was higher in Uganda. If medians were equal, then the mean concentration was compared. Abbreviations used: United Kingdom (UK), Uganda (UG).

Visit	Cytokine	Median UK	Median Uganda	Fold Difference	P
10	GMCSF	25.1	263.4	10.5	<b>0.030</b>
	IFN $\gamma$	155.7	414.1	2.7	0.055
	IL10	131.6	73.7	1.8	0.088
	IL12p40	52.6	48.1	1.1	0.710
	IL13	14.8	717.9	48.4	<b>&lt;0.001*</b>
	IL17A	103.4	163.1	1.6	0.104
	IL1 $\alpha$	151.6	68.6	2.2	0.264
	IL1 $\beta$	241.1	463.1	1.9	<b>0.010</b>
	IL1RA	80.3	484.7	6.0	<b>&lt;0.001*</b>
	IL2	1.6	1.6	1.0	0.382
	IL5	1.6	46.9	29.3	<b>&lt;0.001*</b>
	IL8	10616.9	10248.3	1.0	<b>0.047</b>
	IP10	4202.6	10418.2	2.5	<b>0.029</b>
	MCP1	3212.2	4122.1	1.3	0.110
	MIP1 $\alpha$	150.6	2332.3	15.5	<b>0.002*</b>
	MIP1 $\beta$	566.5	4490.4	7.9	<b>0.027</b>
	TNF $\alpha$	42.1	323.8	7.7	<b>&lt;0.001*</b>
52	GMCSF	20.4	115.3	5.7	<b>&lt;0.001*</b>
	IFN $\gamma$	108.9	261.8	2.4	<b>0.026</b>
	IL10	58.3	33.0	1.8	0.748
	IL12p40	59.3	29.2	2.0	0.145
	IL13	5.8	288.7	49.7	<b>&lt;0.001*</b>
	IL17A	40.1	272.8	6.8	<b>0.010</b>
	IL1 $\alpha$	37.2	25.2	1.5	0.390
	IL1 $\beta$	170.1	345.6	2.0	0.266
	IL1RA	51.7	299.5	5.8	<b>0.001*</b>
	IL2	1.6	1.6	1.0	0.505
	IL5	1.9	35.3	18.8	<b>&lt;0.001*</b>
	IL8	10609.2	10385.9	1.0	0.228
	IP10	3318.8	10106.0	3.0	0.692
	MCP1	3236.2	5520.5	1.7	0.294
	MIP1 $\alpha$	52.3	260.9	5.0	<b>0.021</b>
	MIP1 $\beta$	189.8	953.8	5.0	0.167
	TNF $\alpha$	34.1	412.6	12.1	<b>&lt;0.001*</b>

Higher in the UK

Higher in Uganda

\* P value Remains Significant After Bonferroni Correction

Table S3. A summary of PHA-induced background-corrected concentrations (pg/ml) and p values of cytokines between infants. Cytokines were examined in Uganda at 10 (n=108) and 52 weeks (n=205); in the UK at 10 (n=28) and 52 weeks (n=12). Cytokine levels were acquired using a 17-plex Luminex assay after a 6-day whole blood stimulation assay. The linear regression was used to test for significance adjusting for mother's age between the UK and Uganda over a single time point. P value  $\leq 0.05$  was considered significant and highlighted in bold. The asterisk indicates that the p-value remains significant after the Bonferroni multiple testing adjustment. The blue colour means that the median cytokine concentration was higher in the UK whereas the red colour indicates that the median cytokine concentration was higher in Uganda. If medians were equal, then the mean concentration was compared. Abbreviations used: United Kingdom (UK), Uganda (UG).



Population	Visit	Cytokine	Median UK	Median Uganda	Fold Difference	P
CD4	10	IFN $\gamma$	0.007	0.006	1.1	0.673
		IL2	0.310	0.018	17.2	<b>0.001</b>
		IL8	0.053	0.055	1.0	0.553
		IL4/5/13	0.130	0.057	2.3	0.215
		TNF $\alpha$	0.009	0.092	10.0	<b>0.000</b>
	52	IFN $\gamma$	0.025	0.009	2.8	<b>0.001</b>
		IL2	1.265	0.021	61.7	<b>0.006</b>
		IL8	0.165	0.076	2.2	0.783
		IL4/5/13	0.705	0.170	4.1	0.055
		TNF $\alpha$	0.017	0.150	8.9	<b>0.000</b>
CD8	10	IFN $\gamma$	0.003	0.011	4.0	0.458
		IL2	0.015	0.031	2.1	0.462
		IL8	1.330	0.062	21.5	<b>0.003</b>
		IL4/5/13	0.110	0.058	1.9	0.235
		TNF $\alpha$	0.000	0.071	NA	<b>0.000</b>
	52	IFN $\gamma$	0.059	0.010	6.1	<b>0.011</b>
		IL2	0.190	0.027	7.0	<b>0.018</b>
		IL8	9.850	0.074	133.1	<b>0.000</b>
		IL4/5/13	0.755	0.175	4.3	<b>0.022</b>
		TNF $\alpha$	0.007	0.115	16.5	<b>0.000</b>

Higher in the UK

Higher in Uganda

\* P Value Remains Significant After Bonferroni Correction

Table S4. A summary of background median frequencies (%) and p values of cytokines between all infants. Cytokines were examined in Uganda at 10 (n=19) and 52 weeks (n=92); in the UK at 10 (n=23) and 52 weeks (n=12). Cytokine levels were acquired using flow cytometry after a 24 hours whole blood stimulation assay. The linear regression was used to test for significance adjusting for mother's age between the UK and Uganda over a single time point. P value  $\leq 0.05$  was considered significant and highlighted in bold. The asterisk indicates that the p-value remains significant after the Bonferroni multiple testing adjustment. The blue colour means that the median cytokine concentration was higher in the UK whereas the red colour indicates that the median cytokine concentration was higher in Uganda. If medians were equal, then the mean concentration was compared. Abbreviations used: United Kingdom (UK), Uganda (UG), Not Applicable (NA).

Population	Visit	Cytokine	Median UK	Median Uganda	Fold Difference	P
CD4	10	IFN $\gamma$	0.061	0.094	1.5	0.288
		IL2	0.290	0.663	2.3	0.174
		IL8	0.048	0.059	1.2	0.456
		IL4/5/13	0.036	0.060	1.7	0.926
		TNF $\alpha$	0.280	3.933	14.0	<b>0.021</b>
	52	IFN $\gamma$	0.029	0.057	2.0	0.205
		IL2	0.625	0.146	4.3	<b>0.022</b>
		IL8	0.223	0.033	6.7	0.027
		IL4/5/13	0.064	0.171	2.7	0.890
		TNF $\alpha$	0.116	1.489	12.8	0.055
CD8	10	IFN $\gamma$	0.056	0.051	1.1	0.230
		IL2	0.015	0.102	6.9	0.390
		IL8	0.000	0.017	NA	0.765
		IL4/5/13	0.000	0.027	NA	0.530
		TNF $\alpha$	0.038	0.490	12.9	<b>0.039</b>
	52	IFN $\gamma$	0.188	0.163	1.2	0.783
		IL2	0.003	0.021	6.6	0.701
		IL8	1.540	0.016	95.8	<b>&lt;0.001*</b>
		IL4/5/13	0.160	0.106	1.5	0.772
		TNF $\alpha$	0.063	0.475	7.6	0.078

Higher in the UK

Higher in Uganda

\* P Value Remains Significant After Bonferroni Correction

Table S5. A summary of median frequencies (%) and p values of PHA-induced and background corrected cytokines between all infants. Cytokines were examined in Uganda at 10 (n=19) and 52 weeks (n=92); in the UK at 10 (n=23) and 52 weeks (n=12). Cytokine levels were acquired using flow cytometry after a 24 hours whole blood stimulation assay. The linear regression was used to test for significance adjusting for mother's age between the UK and Uganda over a single time point. P value  $\leq 0.05$  was considered significant and highlighted in bold. The asterisk indicates that the p-value remains significant after the Bonferroni multiple testing adjustment. The blue colour means that the median cytokine concentration was higher in the UK whereas the red colour indicates that the median cytokine concentration was higher in Uganda. If medians were equal, then the mean concentration was compared. Abbreviations used: United Kingdom (UK), Uganda (UG), Not Applicable (NA).

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## **5. Profound phenotypic changes following birth and the effect of common early-life infections on Ugandan infant dendritic cells during the first year of life.**

In the previous chapter, I have examined the immune profiles of BCG vaccinated infants in the UK and Uganda and their T cell phenotypes. In this chapter, I aim to dissect the mechanisms that drive BCG immunogenicity in Uganda and look at the role of dendritic cells over time. I also examine how common infections affect phenotypes of dendritic cells, and discuss how these changes could influence the immune response to the BCG vaccine.

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## RESEARCH PAPER COVER SHEET

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### SECTION A – Student Details

Student	Mateusz Hasso-Agopsowicz
Principal Supervisor	Dr Steven Smith
Thesis Title	A STUDY OF FACTORS UNDERLYING BCG IMMUNOGENICITY DIFFERENCES ACROSS COUNTRIES: THE INFLUENCE OF DNA METHYLATION PATTERNS AND ANTIGEN PRESENTING CELLS

**If the Research Paper has previously been published please complete Section B, if not please move to Section C**

### SECTION B – Paper already published

Where was the work published?	
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### SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	Journal of Experimental Medicine
Please list the paper's authors in the intended authorship order:	Mateusz Hasso-Agopsowicz <sup>1</sup> , Grace Nabakooza, Marjorie Nakibuule, John Vianney Tushabe, JiSook Lee, Patrice Mawa, Dorothy Aibo, Joel Serubanja, Emily L Webb, Hazel Dockrell, Alison M Elliott, Steven G Smith, Stephen Cose
Stage of publication	In submission

### SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I received .fcs files and established a data analysis pipeline for the flow cytometry data analysis. I applied compensation, developed a gating strategy and analysed all samples. I prepared a common database with patients characteristics. I analysed patients characteristics. I established a data analysis pipeline for tSNE and SPADE analysis and analysed all samples. I prepared the graphs and wrote the manuscript.
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Student Signature: \_\_\_\_\_ Date: 23/07/2018

Supervisor Signature: \_\_\_\_\_ Date: \_\_\_\_\_



**Profound phenotypic changes following birth and the effect of common early-life infections on Ugandan infant dendritic cells during the first year of life.**

Authors:

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## **Summary**

We report that known dendritic cell (DC) subsets are absent at birth but appear within the first month of life. Cytomegalovirus, Epstein-Barr virus and maternal latent tuberculosis infection are associated with profound changes in DC phenotype. This work provides new insights into DC biology in infancy and the potential role of DCs as mediators of the effects of infection exposure on adaptive immune responses.

## **Abstract**

Dendritic cells (DCs) are key antigen presenting cells that bridge between innate and adaptive immune responses. They include plasmacytoid dendritic cells (pDCs), and classical (c) DC1 and DC2. Few studies have investigated changes in phenotype of DCs over the course of life as well as in response to common pathogens. We have conducted a longitudinal study of Ugandan infants from birth to one year of life and investigated phenotypes of dendritic cells, how they change over time, and how common infections in infancy (cytomegalovirus, Epstein-Barr virus) and exposure to maternal latent tuberculosis infection (LTBI) affect DC phenotype. Using t-Stochastic Neighbour Embedding (tSNE) and Spanning-tree Progression Analysis of Density-normalized Events (SPADE) algorithms we show that pDCs, cDC1 and cDC2 subsets are absent in cord blood but appear within the first month of life; and highlight the profound effects of pathogens on DC phenotypes. These findings are important for understanding the biology of DCs and the potential impact of common infections on adaptive immunity.

## Introduction

Dendritic cells (DC) are highly heterogeneous antigen presenting cells (APCs) that mediate immune functions by presenting antigens to T cells<sup>1</sup> and B cells<sup>2</sup>. As they bridge between innate and adaptive immunity, they are vital mediators of vaccine-induced immune responses<sup>3</sup>. Numerous studies have classified and examined DC populations in murine models<sup>4</sup> but a comprehensive description of human DC ontology, phenotype and function is lacking. The nomenclature of human DCs is complicated; DCs can be classified based on their origin, their location or their function. DC progenitors are released to blood from the bone marrow and differentiate into lymphoid tissue (LT) and non-lymphoid tissue (NLT) DCs *in situ*. LT DCs present lymph and bloodstream antigens to local T cells in lymphoid tissue. NLT DCs migrate from peripheral blood into tissues and then to lymph nodes where they present tissue-derived antigens to T cells<sup>5</sup>. There are three main subsets of NLT DCs in humans. Plasmacytoid DCs (pDC) can be characterised by the expression of CD123 and production of type I Interferons (IFN)<sup>6</sup>. The remaining two subsets are cDC1 and cDC2, which are collectively and interchangeably known as myeloid, conventional or classical DCs (cDC). cDC1 can be differentiated by their expression of BDCA-1, BDCA-3 and CD1c, whereas cDC2 express high levels of CD141<sup>7</sup>. cDC1 are known to prime CD4 T cells<sup>8</sup> and induce differentiation of Th17 and Th2<sup>9</sup> immune responses to extracellular pathogens. In contrast, cDC2 promote Th1 immune responses and cross-present tumour and pathogenic antigens to prime CD8 T cells<sup>10</sup>. Lastly, monocytes have shown the ability to differentiate into a dendritic cell-like phenotype after encountering tissue antigens. These cells, known as monocyte-derived dendritic cells (MDDCs) originate from monocyte precursors and are characterised by the expression of CD14 and CD16 markers<sup>11</sup>. DC phenotypes have been shown to change with age and in response to antigens. DCs from aged subjects had a reduced capacity to prime T cells, aberrant cytokine secretion and decreased response to

foreign antigens<sup>12</sup>. Pathogen encounter stimulates antigen processing and activates DCs by modulating expression of markers including HLA-DR, CD83 and CD86<sup>13</sup>.

In this study, we used visual Stochastic Neighbour Embedding (viSNE) to characterise 50 human DC clusters, each resembling a distinct DC phenotype. We examined the expression of common DC markers for the identified clusters over the first year of life of Ugandan infants. We also investigated how maternal latent tuberculosis infection (LTBI), and common pathogens such as cytomegalovirus (CMV) and Epstein-Barr virus (EBV), affect cluster size and phenotype of these DC subsets. To our knowledge, this is the first study to describe dendritic cells subsets and how they change during the first year of life in an African infant, and how common exposures modify DCs subsets in this setting.

## Methods

### *Ethical approval and informed consent*

Infants were included in the study only when mothers gave consent for participation. This project was approved by the Uganda Virus Research Institute (UVRI) Research and Ethics Committee, the Uganda National Council for Science and Technology (UNCST), and the Research Ethics Committee of the London School of Hygiene and Tropical Medicine (LSHTM).

### *Study design and setting*

This was a nested study within a larger cohort designed to examine the impact of maternal latent TB infection (LTBI) on the infant immune response to the Bacillus Calmette–Guérin (BCG) vaccine. Women who resided within Entebbe Municipality and Katabi sub-county, Wakiso district, Uganda, and who delivered in Entebbe General Hospital, were considered for study inclusion. They were approached for consent at hospital admission and included if they had a singleton pregnancy, were HIV negative (confirmed by Determine, Inverness Medical), the delivery was uncomplicated, and the neonate was healthy with birth weight above 2500g. Cord blood was obtained at delivery and mothers were invited to attend a neonatal clinic one week post-delivery, where maternal BCG scar was measured and a questionnaire completed. Mothers were tested for LTBI using both the T-SPOT.TB assay (Oxford Immunotec) and a tuberculin skin test (TST; 2 tuberculin units, Statens Serum Institut). They were considered LTBI positive when both tests were positive, and LTBI negative when both tests were negative. Mothers with discordant assay results were excluded from the study. Mothers with signs or symptoms of TB disease were excluded from the study and referred for treatment. For the larger study, 284

mother/infant pairs were included. There were 132 mothers with LTBI, and 152 mothers without LTBI.

### *Samples*

Up to 10ml of cord blood was collected at birth, and up to 5ml of peripheral venous blood was obtained from each infant at selected time points. Samples were collected at 0, 1, 4, 6, 10, 14, 24 and 52 weeks of infant's age. To reduce the burden of blood draws from any one child, infants were randomised to give blood at either 1 and 6 weeks, or 4 and 10 weeks. Blood was collected from all infants at birth (cord blood) and 52 weeks. A subset of infants gave blood at 14 and 24 weeks, as these time points were included later into the study. Due to limited blood volume, not all infants had their DC profile examined. For infants where sufficient volume of blood was collected, DNA was extracted, and blood samples were examined for CMV and EBV. Numbers of samples available at 1 and 4 weeks was low and therefore these time points were combined. For all time points, we recruited 284 infants and examined DC profiles of 265 of them. Some infants donated samples at more than one time point, hence we examined a total of 296 DC profiles over eight time points. We examined 115 infants for CMV and EBV infections across time points resulting in a total of 134 samples. All infants were BCG vaccinated at birth with BCG SSI strain.

### *EBV and CMV viral DNA detection from whole blood using droplet digital PCR (ddPCR) assay*

DNA was extracted from 200 µl whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction, in 100 µl of the elution buffer. The ddPCR reaction mix contained 10 µl ddPCR™ Supermix for Probes (Bio-Rad Laboratories, Hercules, USA), 10 µl of template DNA, 1.1 µl of 20 x

primers (final concentration 900nM) and probe (final concentration 250 nM) mixture for EBV and CMV (Table S1). 20 µl of the mixture was partitioned in oil-in-water with 70 µl of droplet generator oil using a QX-100 droplet generator (Bio-Rad). The generated droplets (around 40 µl) were then transferred to a ddPCR™ 96-Well Plates (Bio-Rad) and heat-sealed with PCR plate heat seal for 5 sec at 170 °C using a PX1™ PCR Plate Sealer (Bio-rad). PCR amplification was performed with the following thermal cycling: 95 °C for 10 min, 40 cycles consisting of 94 °C for 30 sec (denaturation) and 60 °C for 1 min (extension), followed by 98°C for 10 min and holding at 12 °C. Droplets (positive and negative) were then analyzed using a QX100 droplet reader (Bio-rad) and the target DNA concentration was calculated and presented as copies/µl in the reaction mixture using QuantaSoft software (Bio-Rad). DNA samples were tested in duplicates.

#### *Immune cell phenotyping and flow cytometry*

Two hundred µl of heparinised whole blood was spun at 1000g for 5 minutes, and plasma was removed and stored. FACS Lysing Solution (BD Biosciences, 349202) reconstituted in distilled water was added to cells for 15 minutes at room temperature to lyse red blood cells. Cells were spun at 400g for 5 minutes and washed twice with phosphate-buffered saline (PBS). They were stained with a viability dye (LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit, L34957) for 20 minutes, in the dark at 4°C; washed with FACS buffer (1XPBS, 5% FBS, 0.05% Sodium Azide) and stained with FcR blocking antibody (14-9161-73, eBiosciences) for 10 minutes in the dark at 4°C. Cells were washed twice with FACS buffer and stained for 30 mins, in the dark at 4°C with a cocktail of antibodies containing: HLA-DR BV570 (#307637, Biolegend, clone L243), CD3 FITC (#344804, BioLegend, clone SK7), CD19 FITC (BD Pharmingen, #555412, clone HIB19), CD56-FITC (340410, BD Bioscience, clone NCAM16), CD14 APC-H7 (560180, BD Pharmingen™, clone MφP9), CD16 Alexa

Fluor® 700 (557920, BD Pharmingen™, clone 3G8), CD11c BV605 (563929, BD Horizon™, clone B-ly6), CD141 PE (559781, BD Pharmingen™, clone 1A4), CD123 BV650 (563405, BD Horizon™, clone 7G3), CD40 BV421 (563396, BD Horizon™, clone 5C3), CD70 PE-CF594 (562484, BD Horizon, clone Ki-24), CD83 PE-Cy™7 (561132, BD Pharmingen™, clone HB15e), CD86 PE-Cy™5 (555659, BD Pharmingen™, clone 2331 (FUN-1)), CD1c APC (130-090-903, MACS Biotec Miltenyi, clone AD5-8E7). After staining, samples were washed with FACS buffer and stored at 4°C in the dark in PBS with 1% paraformaldehyde (PFA) to be acquired within 24 hours using a BD LSRII Flow Cytometer and obtaining a minimum of 200,00 events.

#### *Flow cytometry data analysis*

Cells were gated for DC phenotype. Dead cells, doublets, monocytes (CD14+), T Cells (CD3+), B Cells (CD19+) and Natural Killer Cells (NK Cells, CD56+) were excluded, and cells that were highly positive for HLA-DR were included. Within that population, various markers that define dendritic cell phenotypes were measured. The full gating strategy is described in Figure 1. The “Downsample” plugin available in FlowJo was used to select 1000 most representative cells per sample within the final gate. The downsampled samples were concatenated, and t Stochastic Neighbour Embedding (tSNE) algorithm was used to reduce data dimensionality using the following parameters: perplexity 40-100; iterations 1000, Eta learning rate 200 and Theta 0.5. Samples were exported to Cytobank ([www.cytobank.org](http://www.cytobank.org)), where Spanning-tree Progression Analysis of Density-normalized Events (SPADE) was used to identify distinct cell clusters from previously calculated tSNE parameters. The viSNE algorithm was used to analyse phenotypes of dendritic cells.



The viSNE algorithm separated cells based on their marker expression and SPADE algorithm identified 50 distinct DC clusters with a minimum of 50 cells per cluster. A cluster is a group of cells with a similar phenotype. It contains a number of cells (cluster size), which can change depending on condition and/or sample characteristics. These clusters were overlayed onto a viSNE plot. The expression of markers (CD1c, CD14, CD141, CD83, CD16, CD86, CD70, CD40, CD11c and CD123) for all cells was used to assign cell ontology following established guidelines<sup>14</sup>. Despite excluding CD14+ cells during the gating process, we included this marker when naming DC subsets, as CD14- cells continue to express varying levels of CD14. Samples from different infants were available for each sub-analysis (DCs phenotypes over time, the effect of EBV infection, the effect of CMV infection and the effect of maternal LTBI). viSNE and SPADE calculations were performed independently for each sub-analysis and thus plots and ontology differ.

### *Statistical Analysis*

The linear regression was used to examine whether continuous characteristics (mother's age, number of pregnancies, a total number of born children, birth weight) vary between samples available at different time points. The logistic regression was used to measure if categorical characteristics (the proportion of boys, the proportion of tuberculosis latently infected mothers, the proportion of mothers with BCG scar, the proportion of CMV infected infants, the proportion of EBV infected infants) of samples vary between time points. For a comparison of sample characteristics between CMV infected and uninfected, EBV infected and uninfected and infants born to mothers with and without LTBI, the Mann-Whitney test was used for continuous and the Chi-squared ( $X^2$ ) for categorical sample characteristics. Stata/SE 15.1 was used.

## Results

### *Sample characteristics*

Infants had a median birth weight of 3.2kg, 50% (148/296) were male, 39% (94/242) were born to mothers with latent TB infection, 32% (41/134) had CMV, and 14% (19/134) had EBV (Table 1).

### *Known dendritic cell subsets are absent at birth but appear during the neonatal period*

Samples from 70 infants were analysed, stratified over seven time points (0, 1 and 4, 6, 10, 14, 24, 52weeks; 10 samples per time point). Infants' and mothers' characteristics did not differ across time points (Supplementary Table S2).

We first analysed the sizes of DC clusters and how these change over time. Of the 50 DC clusters identified 1-20 and 22-27 are present in blood at birth (Figure 2A). In contrast, known DC phenotypes such as pDCs (clusters 21, 40 and 43), cDC1 (clusters 42, 44), cDC2 (clusters 29, 35, 36, 39, 46-49) and MDDC (clusters 31 and 41) are absent in blood at birth but appear within the neonatal period, with full reconstitution of mature DC subsets by 10 weeks of life (Figure 2B). Most pDCs appear at 6-10 weeks of life (clusters 21, 40, 45) and most cDC2 (clusters 35, 48-49) appear at 6 weeks of life followed by cDC1 cluster 44 and others (Figure 2B, green squares).

Having looked at changes in cluster size over time, we then proceeded to analyse the median fluorescence intensity (MFI) of markers in DC clusters over time. We assigned ontology to 16/50 (32.00%) clusters of DCs based on their median marker expression

(Figure 3A) and looked at a fold change of MFI over time in all clusters. We found an overall increase between 0 to 1 and 4 weeks of life in marker expression for the majority of clusters (Figure 3B). There was a consistent increase in CD1c and CD141 expression, and some downregulation of CD16 at 1 and 4 weeks of life. The expression pattern for other markers does not appear to follow a consistent pattern over time. Interestingly, the expression of markers in clusters 1-20 and 22-27 increases at 6 and 10 weeks and then decreases at 14 and 24 weeks, slightly increasing again at 52 weeks of life. In contrast, the marker expression of clusters 21 and 28-49 that contain known DC subsets remains unchanged throughout the first year of life. This data suggests that the DC subsets that are present at birth are likely immature, but rapidly mature into known DC subsets in the first few weeks of life.

#### *Maternal LTBI expands key DC populations in the early weeks of life*

We investigated how maternal LTBI alters DC cluster size and phenotype. We analysed 48 samples from 24 infants who were born to mothers with, and 24 who were born to mothers without a LTBI, at 6, 14 and 52 weeks. Infants' and mothers' characteristics did not differ with maternal LTBI status (Supplementary Table S3). We stratified viSNE plots by time and maternal LTBI status (Figure 4A). We then determined the size of each cluster and calculated the fold change of cluster size for all clusters (Figure 4B). We report a notably increased cluster size for MDDC (cluster 32), cDC2 (clusters 41 and 48) and cDC1 (cluster 49) in infants that were born to mothers with LTBI, at all time points (Figure 4B, 1<sup>st</sup> column). In contrast, the size of clusters 1-30 is similar or moderately lower in infants born to mothers with LTBI at 6 and 14 weeks of life (Figure 4B). Based on the MFI of markers, we assigned ontology to 12/50 (24.00%) clusters (Figure 5A) and calculated fold change of MFI between infants born to mothers with and without a LTBI. We report increased expression of CD14 and CD16 markers in clusters 6, 15, 17, 19, and higher overall expression of

markers in MDDC cluster 32 in infants born to mothers with a LTBI. However, for clusters of other known DC subsets (29,30, 34-50), we report no change in marker expression associated with the exposure to LTBI (Figure 5B). Taken together, this data suggests that infants born to mothers with LTBI are likely exposed to *M.tb* antigens *in utero*, and that this exposure markedly increases MDDC populations in the early weeks of life.

#### *CMV infection delays development of key DC populations*

We examined the impact of CMV infection DC cluster size and phenotype, analysing 49 samples, 25 from CMV uninfected and 24 from CMV infected infants, at 6, 10, 14, 24 and 52 weeks of age. Infants' and mothers' characteristics did not differ by CMV infection status (Supplementary Table S4). We stratified viSNE plots over time and CMV infection status (Figure 6A) and determined cluster sizes. We then calculated fold change of cluster sizes between CMV uninfected and CMV infected infants for a given time point (Figure 6B). We report moderately lower cluster size of MDDC (cluster 34), CD11c/CD86 DCs (clusters 35 and 36), cDC2 (clusters 42 and 43) and other clusters (30, 46 and 48) in CMV infected infants at all time points (Figure 6B, 1<sup>st</sup> column). The highest fold change in cluster size is mostly at 6 weeks of life and remains up to the 24<sup>th</sup> week of life. In contrast, clusters 2-29, are larger in CMV infected infants at 6 and 10 weeks of life, but are reduced in these infants at 14 and 24 weeks of life (Figure 6B, columns 2-5). We then analysed MFI of markers, assigning ontology to 14/50 (28.00%) of clusters (Figure 7A) and calculated the fold change of MFI between CMV uninfected and CMV infected infants in all clusters at all time points (Figure 7B). Except for cluster 37, where we see a lower marker expression in CMV infected infants, we report no overall difference of marker expression with CMV infection. In summary, this data indicates that CMV infection delays the development of known dendritic cell populations, but which eventually

recover to a similar distribution of clusters in infants with and without CMV infection at 52 weeks.

*EBV infection reduces DC subsets known to be important in initiating anti-viral immune responses.*

We investigated how EBV infection affects DC subsets and their phenotype. The majority of infants (18/19, 94.7%) across all time points were EBV infected by the 52<sup>nd</sup> week of life. As only one infant was EBV infected and younger than 52 weeks (table 1), and viSNE analysis requires an equal number of samples across time points, we limited the analysis to a single time point of 52 weeks. We analysed 32 samples, 16 from infants that were infected with EBV and 16 from uninfected infants. Infants' and mothers' characteristics did not differ with EBV infection status (Supplementary Table S5). We stratified viSNE plots based on the EBV infection status (Figure 8A) and determined the size of each cluster. We then looked at a fold change of cluster size between EBV non-infected and EBV infected infants (Figure 8B). We found reduced cluster sizes in EBV infected infants for pDCs (clusters 8, 24, 33, 39, 40 and 50), cDC1 (clusters 36, 37, 39 and 45), cDC2 (clusters 18, 32, 33, 36, 37 and 39) and MDDC (clusters 12 and 38). These changes are reflected in viSNE plots where we see a decrease in the colour intensity of clusters 33 (cadet blue), 45 (electric blue) and the presence of cluster 41 (cinnamon brown) in EBV infected infants (Figure 8A). We then analysed the MFI of markers for all clusters, assigned ontology to 21/50 (42.00%) clusters (Figure 8C) and calculated the fold change of MFI in all clusters between EBV uninfected and EBV infected infants. We found an overall decrease in marker expression in EBV infected infants. The downregulation of markers was most noticeable for CD14 and CD16, markers associated with an MDDC phenotype. Taken together, these results indicate that EBV infection reduces cluster size and

downregulates marker expression of DC subsets known to be important in initiating anti-viral immune responses.

## Discussion

Following birth, a nascent and underdeveloped immune system begins to mature, with immune cells appearing, differentiating and developing over time and after antigen exposure. In this study, we examined infant DC phenotypes over time directly *ex vivo*, and analysed the impact of maternal LTBI, and infant CMV and EBV infection on these developing subsets.

We used flow cytometry combined with viSNE and SPADE techniques to analyse DC populations. The advantages of viSNE and SPADE over a standard biaxial analysis include reduced dimensionality of data while retaining single cell resolution, without an assumption of a linear relationship<sup>15</sup>. These approaches allow us to look at more markers simultaneously and aid identification of new cell populations.

Based on the number of examined phenotypic markers and their expression, the viSNE analysis identified 50 distinct DC clusters. We report clusters whose marker expression corresponds to previously identified subsets, based on the current literature (pDCs, cDC1, cDC2 and MDDC). We also report novel DC clusters that do not express the full array of phenotypic markers (1-20, 22-27), however, they have an intermediate expression of CD40, CD11c and low expression of CD123. We have shown here that all DC cluster sizes change over time and that fully developed pDC, cDc1 and cDc2 phenotypes are absent in cord blood, but appear within the first month of life. Previous studies have investigated frequencies of DCs in infancy, but none have focussed on the first weeks of life, or indeed at multiple time points throughout the first year of life. Studies have reported that frequencies of both pDC and cDC subsets increase with age from 6 months to 5 years of life<sup>16</sup>; that CD11b+ cells are absent in neonatal lungs but not in adults<sup>17</sup>; and that infants have significantly fewer pDC and cDC as a proportion of peripheral blood mononuclear cells when compared

to adults<sup>18</sup>. In adulthood, levels of pDC but not cDC decrease with age<sup>19</sup>. Two studies find that in the UK and Spanish infants, pDCs and cDCs are present in cord blood but in a much reduced frequency<sup>20</sup>, and with a lower marker expression<sup>21</sup>. They could be merely highlighting populations that were identified in our study as clusters 1-20 and 22-27, with a low CD123 expression; identifying cells with medium HLA-DR expression (which were excluded from this analysis due to established ontology guidelines<sup>14</sup>); or reflecting differences between European and African infants. These observations (both published and reported in this article) could be explained by the development of DCs over time from DC precursors. Studies suggest high frequencies of hematopoietic CD34+ stem cells in cord blood, a vital source of DCs in infancy<sup>22,23</sup>. DC development occurs over time and is also induced by antigen exposure, both environmental and vaccine-derived. We also report that the expression of markers for identified clusters is markedly increased in the neonatal period. This suggests that DC subsets in cord blood have an immature profile, in agreement with the literature that reports cord blood DC subsets to express lower levels of MHC-II, ICAM-1, CD80 and CD86<sup>24</sup>. After the 4<sup>th</sup> week of life, we observe an increase in marker expression of unidentified clusters (1-20, 22-27) up to week 10 and a decrease from week 10 onwards. Despite overall low marker expression, clusters 1-20 and 22-27 are phenotypically dynamic and distinct from identified DC clusters. These DCs may be DC precursors that differentiate into known DC populations throughout the first 10 weeks of life. Alternatively, they may be novel DC populations that are active in the neonatal period and disappear as the immune system develops with age. Further functional studies will be needed to determine whether they are indeed an immature subset, or in fact a new DC subset. Importantly, developed DCs only migrate in blood when transporting antigens from tissues to lymph nodes. Hence, blood-circulating DCs are not considered to be fully representative of tissue resident DCs, which may have a different profile and development dynamics. We acknowledge that this is a limitation of this study. In summary, we have identified 50 distinct DC clusters and



show that they are dynamic over the first year of life. We emphasise the absence of pDC, cDC1 and cDC2 in cord blood.

Having identified DC clusters and examined their qualitative and quantitative changes over time, we examined the effect of maternal LTBI, and of two common pathogens CMV and EBV, on the infant DC profile.

LTBI affects a quarter of the world's population<sup>25</sup>. *Mycobacterium tuberculosis*, the intracellular pathogen responsible for tuberculosis (TB) infection, mainly resides in macrophages but has also been shown to infect DCs<sup>26</sup>. Mycobacterial antigens can cross the placenta in murine models<sup>27</sup> potentially leading to mycobacterial exposure *in utero* and an altered, sensitised immune profile of the fetus<sup>28</sup>. We hypothesised that infants born to mothers with a LTBI would have an altered DC profile that may affect their response to BCG vaccination, which is given at birth in this setting. We report an increase in cluster sizes of cDC1, cDC2 and MDDC, but not pDC, in infants born to mothers with a LTBI. We also highlight an increased expression of CD14 and CD16 markers in unidentified DC clusters in infants born to mothers with a LTBI. Previous studies have examined the effect of maternal LTBI on the infants' immune responses. We have previously found that frequencies of PPD specific IFN $\gamma$ , tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) and total cytokine (IFN $\gamma$ , IL2, TNF $\alpha$ ) expressing CD4+ T cells were lower at 1 week post-BCG vaccination in infants born to mothers with a LTBI<sup>29</sup>. Conversely, others have reported no differences in BCG specific immune responses in infants exposed to maternal *M. tb*<sup>30</sup>, however, the study design differed between the two groups, and may explain the differences that were reported. Our current study gives evidence for modulation of an infant's DC phenotype after (likely) mycobacterial exposure *in utero*. Importantly, changes in DC numbers could be one of the mechanisms that may explain observed differences in immune responses

measured in the same infants<sup>29</sup>. The term BCG masking has been used before to describe this effect<sup>31</sup>. *In utero* sensitisation may confer some level of protection and reduced early response to the BCG vaccine, as observed in our previous study<sup>29</sup>. This finding has significant implications for BCG vaccine immunity and effectiveness and may help to explain the variable efficacy of BCG observed across the globe.

Human cytomegalovirus, also known as human herpesvirus-5 (HHV-5), is a member of the  $\beta$ -herpes virus family. Almost 100% of adults in Sub-Saharan Africa are CMV positive<sup>32</sup> and in Uganda, ~75% of infants under one year of age have CMV-specific IgG antibodies<sup>33</sup>. DCs act as a source of primary lytic infection as well as reactivation from CMV latency<sup>34</sup>. We examined how CMV infection affects DC subsets and found that CMV infected infants have delayed development and reduced sizes of DC clusters that correspond to known DC phenotypes, compared to their uninfected counterparts. We observed larger DC clusters in CMV uninfected infants in 13/50 clusters, including cDC1, MDDC, cDC2 and CD11c DCs with high CD86 expression. This agrees with previous studies that show reduced cellular differentiation of CMV-infected DCs. Indeed, CMV gene UL111A encodes viral homologs of interleukin 10 (cmvIL-10) that inhibit the differentiation of CD34+ DC progenitors (and known CMV reservoirs) to mature DC subsets<sup>35</sup>. We show no differences in the cluster size of pDCs, in agreement with the literature confirming that blood pDCs are resistant to CMV infection *in vitro*<sup>36</sup>. We looked at phenotypic changes of identified DC phenotypes but observed no differences in marker expression for all clusters regardless of the CMV status. Whereas previous studies describe how CMV infection impairs DC function by lowering their expression of CD80, HLA-DR, CD86 and CD83 after viral reactivation or infection *in vitro*<sup>37</sup>, we did not observe this immune downregulation in latently infected infants *ex vivo*. Our study is the first longitudinal analysis of CMV infection *ex vivo*, and further studies should be conducted to explore the mechanisms of latent and active CMV infection and its timing on DC phenotype.

Epstein-Barr Virus is one of the most ubiquitous viruses in humans, reaching an estimated worldwide seroprevalence of 90%<sup>38</sup>. It promotes malignant differentiation of B cells in immunosuppressed hosts<sup>39</sup>, drives innate immune dysregulation<sup>40</sup>, regulates CD8+ NKT cell development<sup>41</sup> and is associated with reduced vaccine antibody responses in infants<sup>42</sup>. We quantified DC cluster sizes and measured their phenotypes in EBV infected and non-infected infants. We report that EBV infection markedly decreases all three main clusters of DCs: pDCs, cDC1 and cDC2. We also highlight that EBV infection decreases the expression of surface markers, especially CD14 and CD16, in the majority of identified clusters. In humans, the recognition of EBV antigens is modulated by Toll-like receptor 2 (TLR2), and 3 and the central role of DCs<sup>43</sup> is thought to be in T cell priming of EBV-specific protective T cell responses<sup>44</sup>. The central role of DCs in EBV protective immunity is highlighted by studies in humanised, non-obese diabetic severe combined immune deficiency (NOD-SCID) mice, a model for human EBV infection. They show increased mortality in pDC depleted mice from disseminated EBV infection and improved survival in pDC enriched mice, indicating a critical role of pDC in anti-EBV immune responses<sup>45</sup>. We did not observe an increase in mortality of EBV infected infants, suggesting that pDC levels are sufficient to mediate a healthy state; or that the role of pDC and protection against EBV does not correlate well between humans and NOD-SCID mice; or that we were merely not powered to detect mortality in this study. In humans, a study reports a loss of all DC subsets during Epstein-Barr virus-associated acute infectious mononucleosis *in vivo*<sup>46</sup>. Lastly, studies say that EBV infection impairs the differentiation of monocytes to MDDC phenotype, as defined by decreased CD14 and CD16 expression<sup>47,48</sup>. Taken together, these studies highlight the role of DCs in EBV infection and are consistent with our findings. Our study is the first to examine the effect of the EBV infection on an infants' DC phenotype directly *ex vivo*. In addition to the available literature, we find that EBV infection markedly decreases cluster size

and downregulates marker expression of DC populations that are known to mediate anti-viral immune responses.

This study is the first comprehensive, longitudinal and cross-sectional analysis of infant blood DC phenotypes. We used state-of-the-art techniques to analyse and identify unique populations of DCs. We show that infant DC populations change over time and in response to CMV, EBV and maternal LTBI. These findings highlight how dynamic DCs are and challenges our perception of typical DC populations and their profiles. This study highlights that this dynamic milieu of DCs should be considered when designing new vaccines. This is an early discovery research study and further studies need to be conducted to examine the impact of DC development on vaccine efficacy in early infancy and its implications for vaccinologists. Additionally, we must consider the prevalence of common pathogens such as CMV and EBV or stress the importance of vaccine design for these pathogens. Future studies should include a comprehensive analysis of changes in DC phenotypes and the effect of common pathogens beyond one year of life.

## Figures

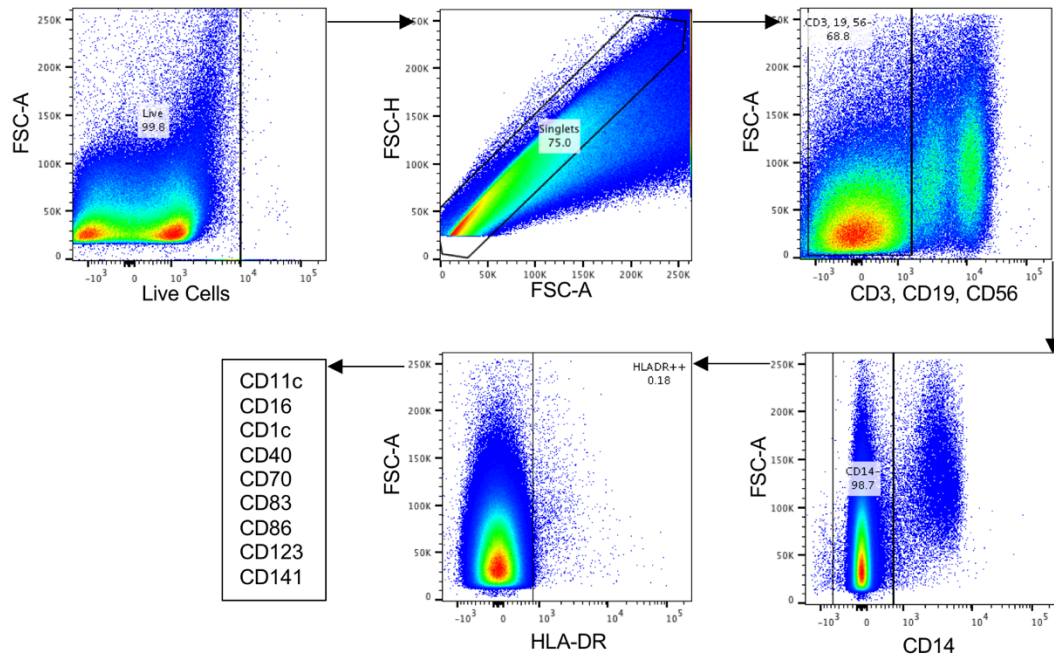


Figure 1. Flow cytometry gating strategy used for the identification of dendritic cells. Live and single cells were included. CD3, CD19, CD56 and CD14 cells were excluded from the analysis. Only cells with a high expression of HLA-DR were included in the analysis.

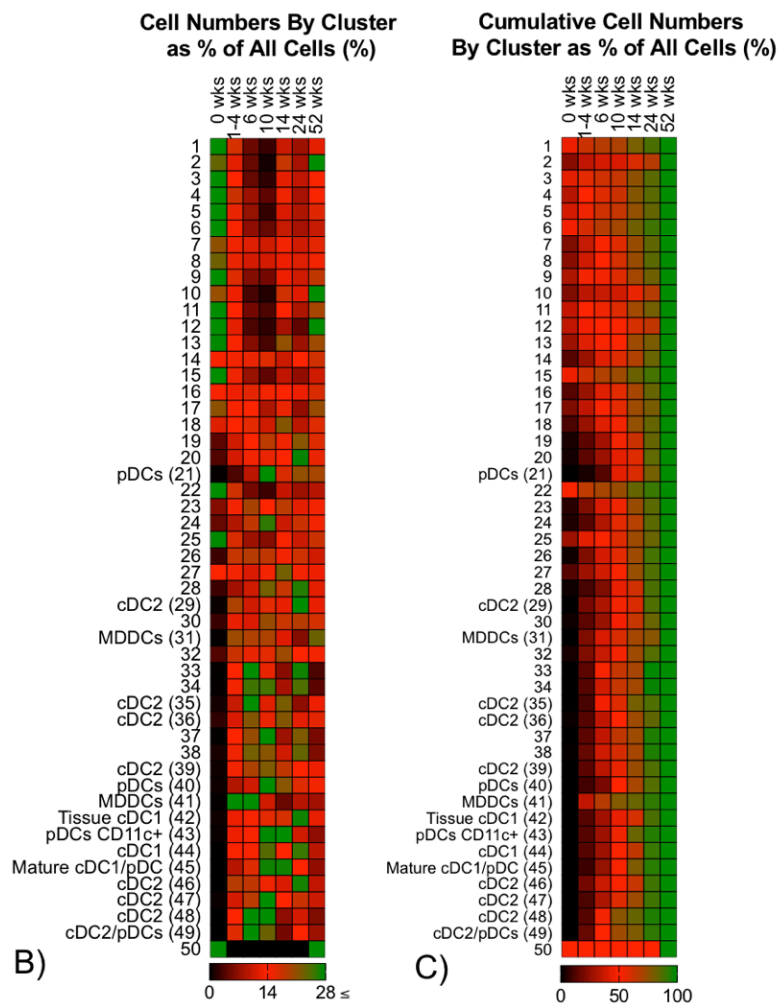
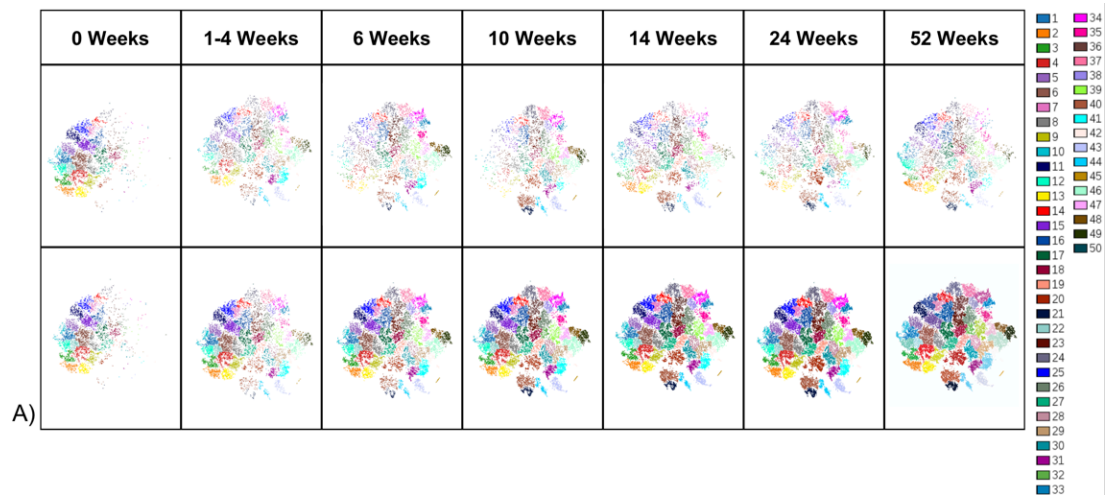


Figure 2. Changes in sizes of clusters of dendritic cell phenotypes over time. Different clusters are represented by the associated colour palette (A). The first row indicates cell clusters that appeared at a given time point. The second row indicates a cumulative number of cells for each cluster over time (A). Quantified numbers of cells in each cluster that appear at a given time point as a % of all cells (B). Black colour indicates that near 0% of cells appeared in blood at a particular time point; bright red indicates that around 14% of cells appeared in a given time point; green indicates that 28% or more of DCs appeared in a given time point (B). A quantified cumulative number of cells for each cluster over time (C). The black colour indicates that near 0% of cells were present up to particular time point; bright red indicates that 50% of cells were present in blood up to a given time point; green indicates that 100% of cells were present up to a given time point (C). All infants were 0, 1 and 4, 6, 10, 14, 24 and 52 weeks old, n=70 (10 samples per time point). Abbreviation used: wks (weeks), pDC (plasmacytoid dendritic cell), cDC1 (classical dendritic cell 1), cDC2 (classical dendritic cell 2), MDDC (monocyte-derived dendritic cell).

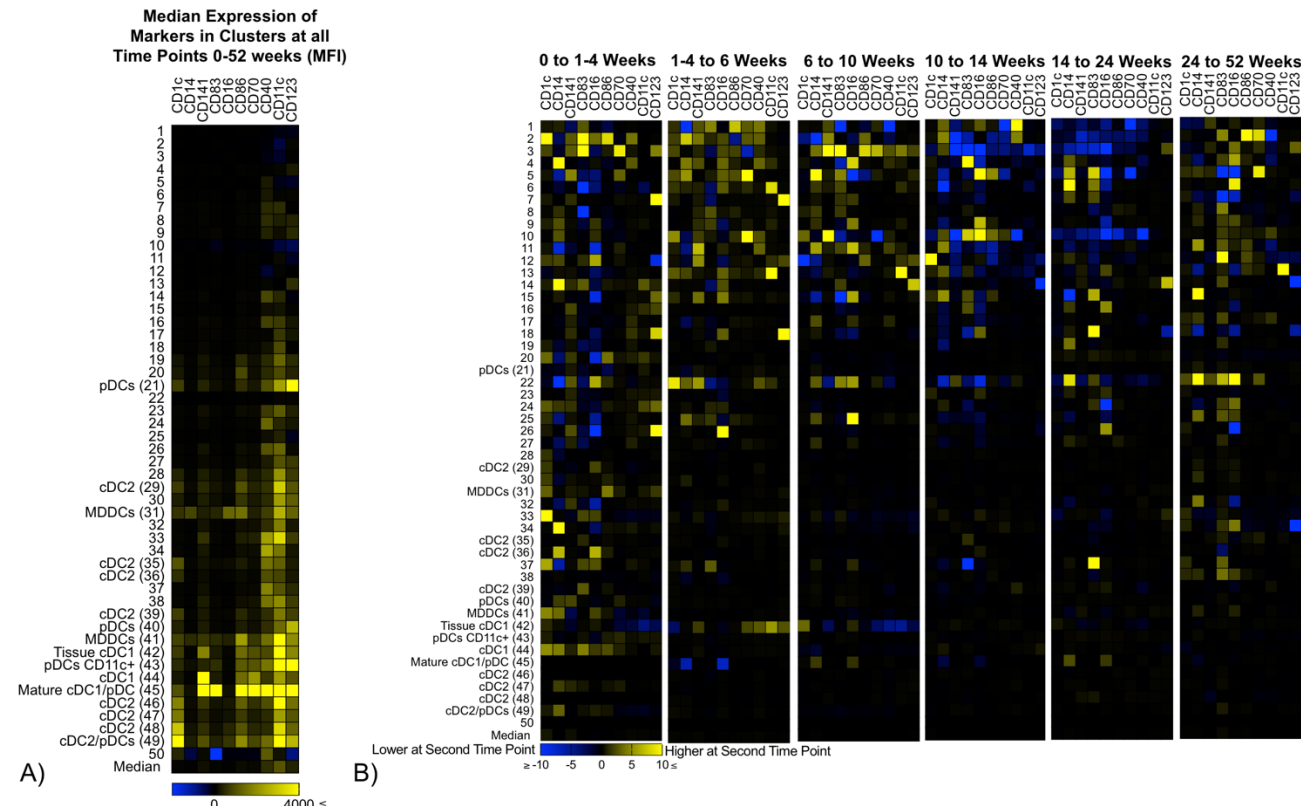


Figure 3. An overview of median expression (MFI) of markers in clusters in cells at all time points 0 to 52 weeks (A). A fold change difference of median expression of markers in clusters between measured time points (B). All infants were 0, 1 and 4, 6, 10, 14, 24 and 52 weeks old, n=70 (10 samples per time point). Abbreviation used: wks (weeks), pDC (plasmacytoid dendritic cell), cDC1 (classical dendritic cell 1), cDC2 (classical dendritic cell 2), MDDC (monocyte-derived dendritic cell), MFI (median fluorescence intensity).





Figure 4. Changes in sizes of clusters of dendritic cells over time and LTBI maternal status. Different clusters are represented by the associated colour palette (A). Fold change of cluster size between maternal LTBI non-exposed and maternal LTBI exposed cells across time points (B). Red colour indicates that there were more cells in infants exposed to maternal LTBI, the green colour indicates that there were more cells in infants not exposed to maternal LTBI. Infants were 6, 10, and 52 weeks old, n=48 (24 LTBI+ and 24 LTBI-). Abbreviation used: pDC (plasmacytoid dendritic cell), cDC1 (classical dendritic cell 1), cDC2 (classical dendritic cell 2), MDDC (monocyte-derived dendritic cell), LTBI (latent tuberculosis infection).

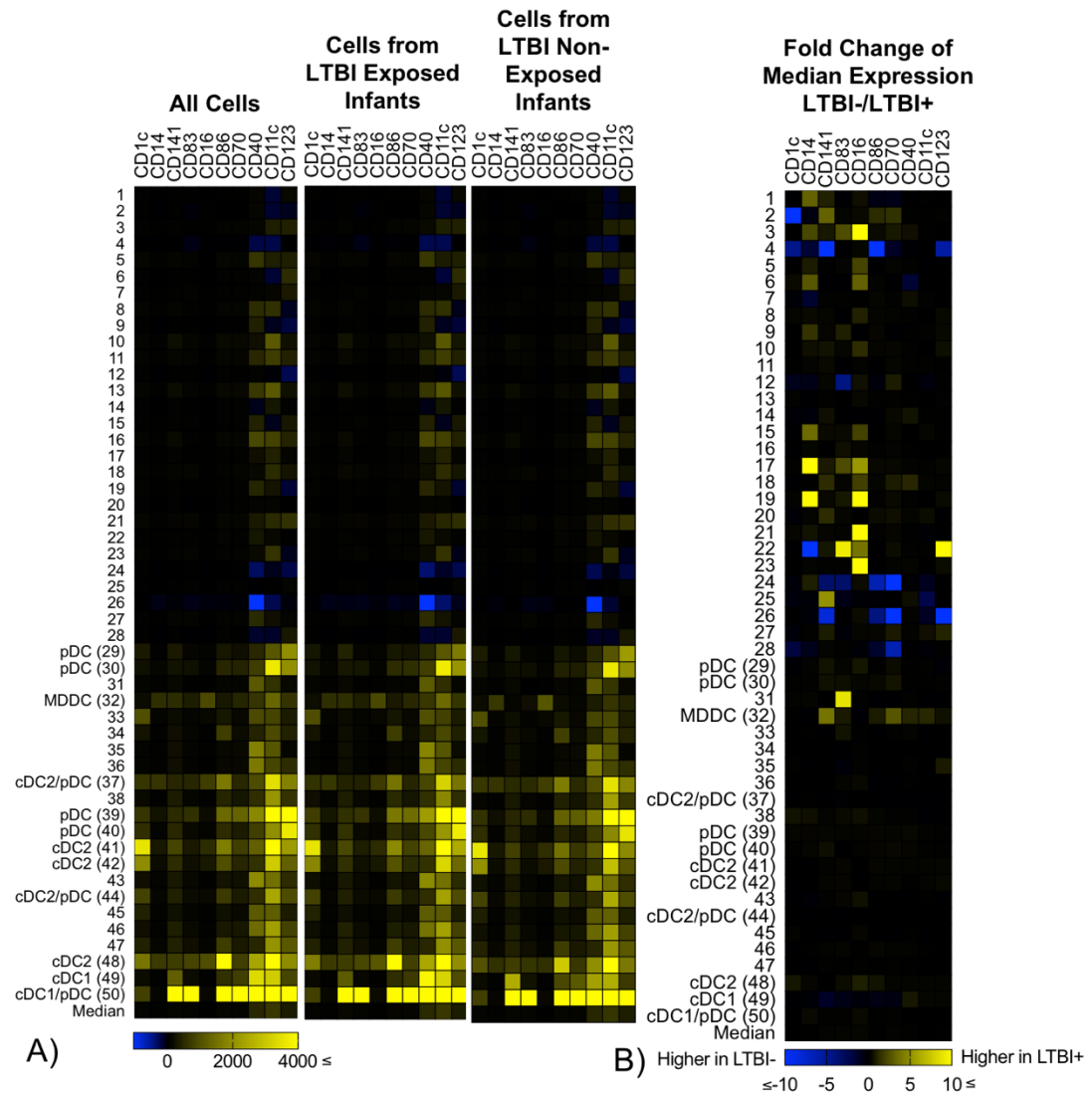


Figure 5. An overview of median expression (MFI) of markers in clusters in all cells and over maternal LTB infection status (A). A fold change difference of median expression of markers in clusters between maternal LTBI exposed and non-exposed infants (B). The yellow colour indicates higher marker expression in LTBI exposed infants, the blue colour indicates higher marker expression in LTBI non-exposed infants. Infants were 6, 10, and 52 weeks old,  $n=48$  (24 LTBI+ and 24 LTBI-). Abbreviation used: pDC (plasmacytoid dendritic cell), cDC1 (classical dendritic cell 1), cDC2 (classical dendritic cell 2), MDDC (monocyte-derived dendritic cell), LTBI (latent tuberculosis infection).

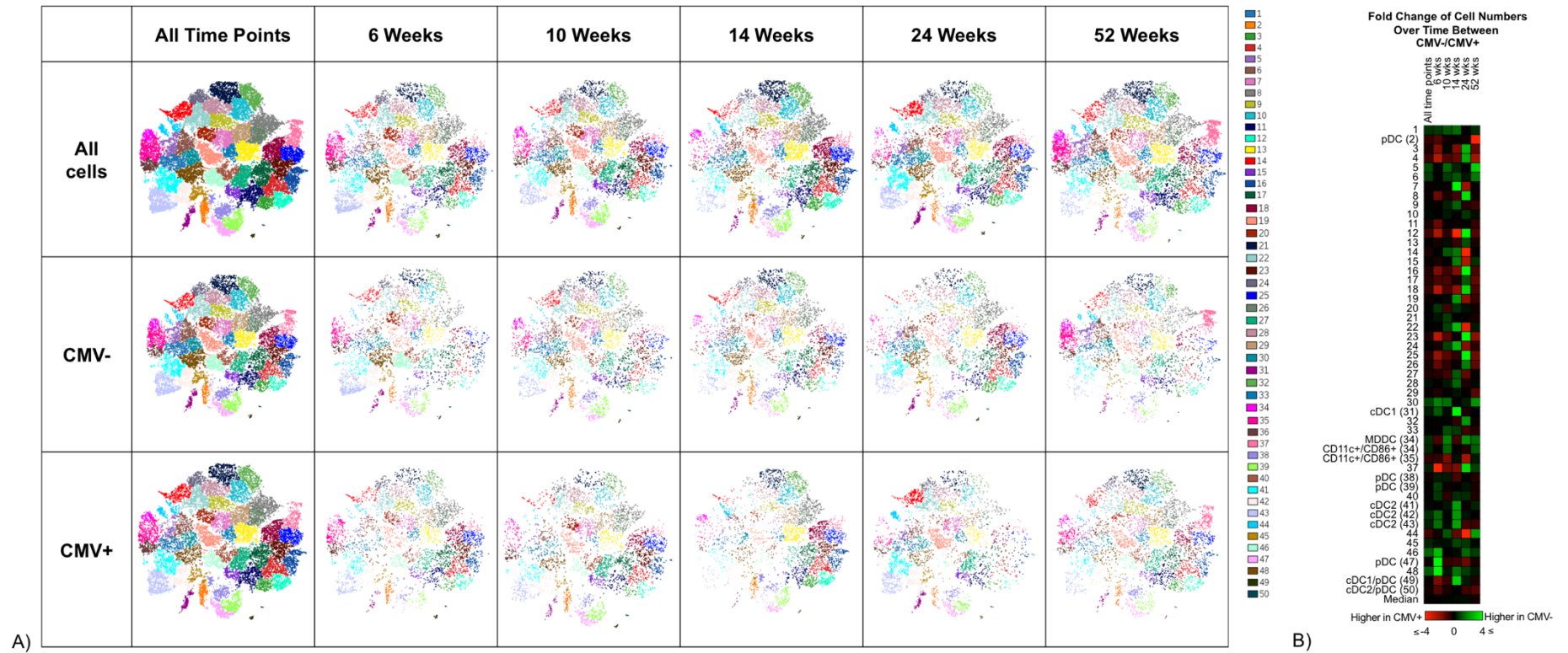


Figure 6. Changes in clusters size of dendritic cell over time and CMV infection status. Different clusters are represented by the associated colour palette (A). Fold change of cluster sizes between CMV non-infected and CMV infected infants across time points (B). The red colour indicates that there were more cells in the CMV infected infants, the green colour indicates that there were more cells in the CMV non-infected infants. Infants were 6, 10, 14, 24 and 52 weeks old, n=49 (24 CMV+ and 25 CMV-). Abbreviation used: wks (weeks), pDC (plasmacytoid dendritic cell), cDC1 (classical dendritic cell 1), cDC2 (classical dendritic cell 2), MDDC (monocyte-derived dendritic cell), CMV (cytomegalovirus).

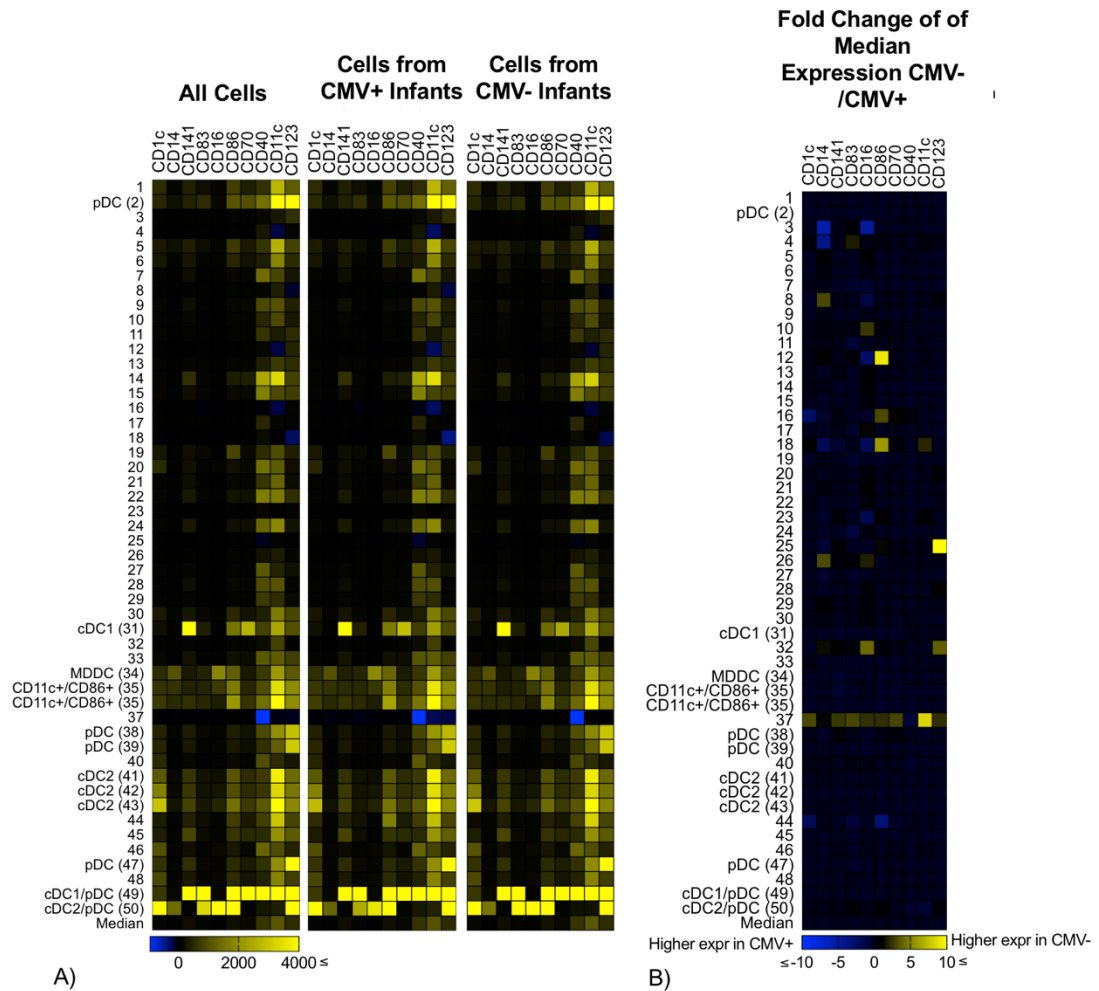


Figure 7. An overview of median expression (MFI) of markers in clusters in all cells and over the CMV infection status (A). A fold change of median expression of markers in clusters between the CMV non-infected and the CMV infected infants (B). The yellow colour indicates higher marker expression in the CMV non-infected infants, the blue colour indicates higher marker expression in the CMV infected infants. Infants were 6, 10, 14, 24 and 52 weeks old, n=49 (24 CMV+ and 25 CMV-). Abbreviation used: pDC (plasmacytoid dendritic cell), cDC1 (classical dendritic cell 1), cDC2 (classical dendritic cell 2), MDDC (monocyte-derived dendritic cell), MFI (median fluorescence intensity), CMV (cytomegalovirus).

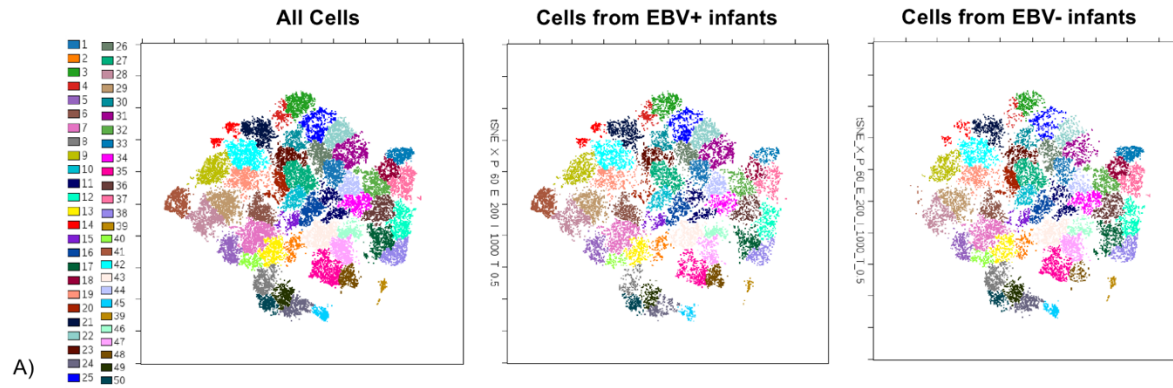
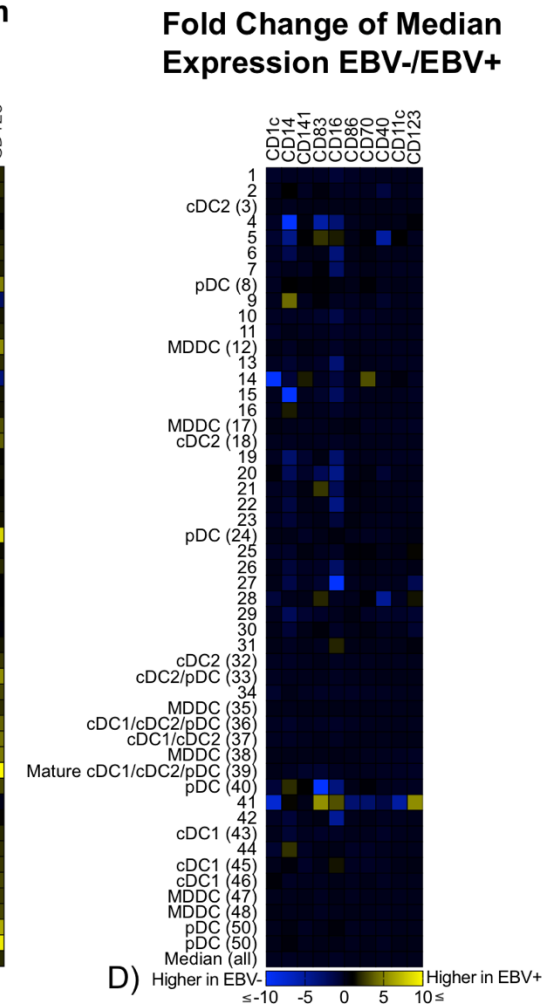
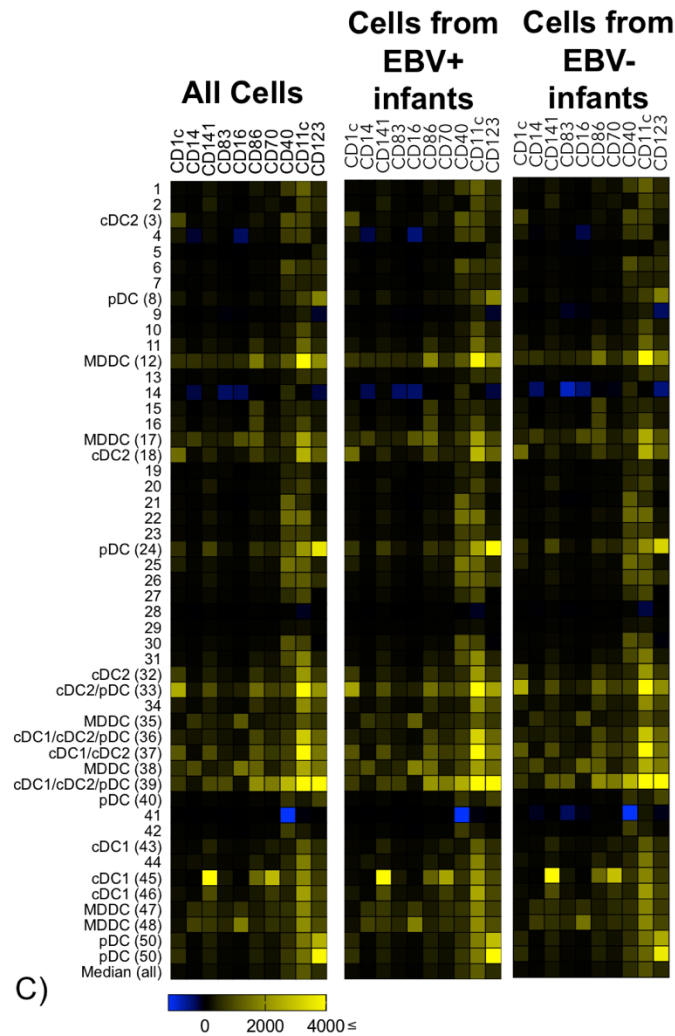
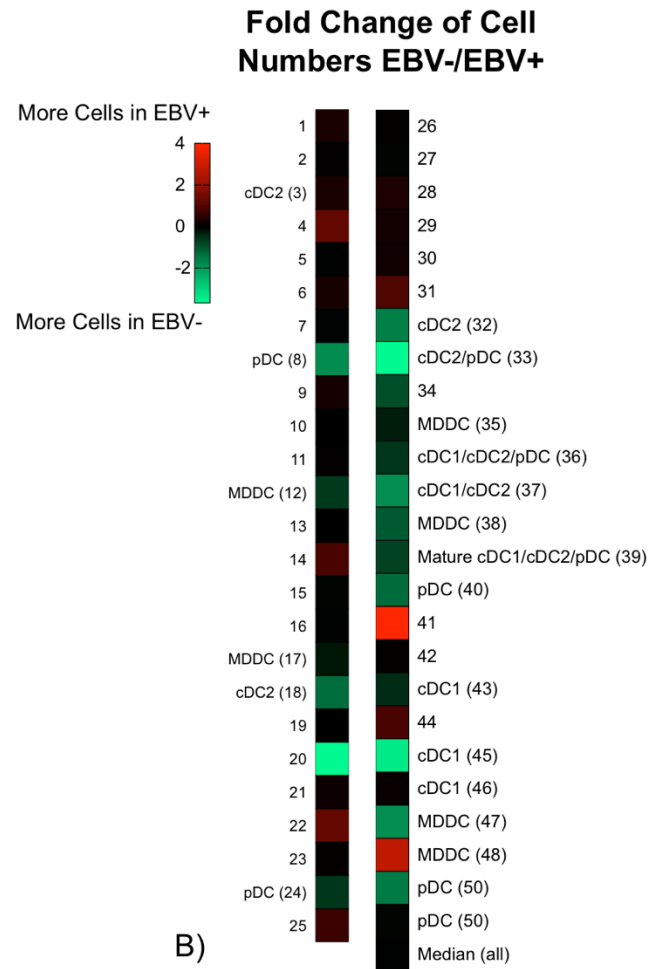


Figure 8. Changes in cluster size of dendritic cell phenotypes over the EBV infection status (A). Different clusters are represented by the associated colour palette (A). Fold change cluster size between the EBV non-infected and the EBV infected infants (B, next page). The red colour indicates that there were more cells in a cluster in the EBV infected infants, the green colour indicates that there were more cells in a cluster in the EBV non-infected infants. Median expression of markers in clusters in all cells and over the EBV infection status (C, next page). Fold change of median expression of markers in clusters between the EBV non-infected and the EBV infected cells (D, next page). The yellow colour indicates higher marker expression in the EBV infected cells, the blue colour indicates higher marker expression in the EBV non-infected cells. All infants were 52 weeks old, n=32 (16 per EBV status). Abbreviation used: pDC (plasmacytoid dendritic cell), cDC1 (classical dendritic cell 1), cDC2 (classical dendritic cell 2), MDDC (monocyte-derived dendritic cell), EBV (Epstein-Barr Virus).







## Tables

Table 1: Characteristics of infants and their mothers from whom samples were collected over time. Note that some data was missing and/ or infection testing was not performed. Values in brackets indicate the standard deviation (SD).

Characteristic	Total	0 weeks	1 week	4 weeks	6 weeks	10 weeks	14 weeks	24 weeks	52 weeks
Number of Samples	296	127	5	8	20	15	18	15	88
Mother's Age (Median Years)	24 (4.57)	24 (4.87)	26 (4.55)	24 (1.39)	22 (3.91)	22 (2.61)	25.5 (3.65)	24 (5.87)	25 (4.61)
Total Number of Pregnancies (Median)	2 (1.48)	2 (1.46)	4 (1.87)	2 (0.74)	2 (1.14)	2 (0.99)	2.5 (1.17)	2 (1.72)	2 (1.68)
Total Number of Born Children (Median)	2 (1.39)	2 (1.41)	1 (1.64)	2 (0.89)	2 (1.01)	2 (1.03)	2 (1.25)	2 (1.50)	2 (1.52)
Birth Weight (kg)	3.2 (0.41)	3.29 (0.38)	3.5 (0.54)	3.45 (0.47)	3.15 (0.33)	3.2 (0.52)	3.35 (0.43)	3.1 (0.34)	3.2 (0.43)
The Proportion of Boys	148/296	66/127	1/5	4/8	9/20	7/15	8/18	11/15	42/88
The proportion of Tuberculosis Latently Infected Mothers	94/242	25/73	2/5	1/8	8/20	5/15	8/18	10/15	35/88
The proportion of Mothers with BCG scar	208/293	97/127	2/5	6/7	15/20	11/14	10/18	10/15	57/87
The proportion of CMV infected infants	41/134	0/5	1/2	1/7	5/16	4/12	10/16	6/11	14/65
The proportion of EBV infected infants	19/134	0/5	0/2	0/7	0/16	0/12	1/16	0/11	18/65

Table S1. Primer/Probe sequences used for the detection of EBV and CMV.

Target gene	Forward Primer	Reverse Primer	Probe
<b>EBV(<i>BALF5</i>)</b>	CGGAAGCCCTCTGGACTTC	CCCTGTTTATCCGATGGAATG	6FAM-TGTACACGCACGAGAAATGCGCC-BHQ1
<b>CMV (<i>UL55</i>)</b>	TGGGCGAGGACAACGAA	TGAGGCTGGGAAGCTGACAT	HEX-TGGGCAACCACCGCACTGAGG-BHQ1

Table S2: Characteristics of infants and their mothers from whom samples were collected over time and included in the analysis of dendritic cell phenotypes over time, n=70, ten samples per time point. The linear regression was used to measure if continuous variables (mother's median age, the total number of pregnancies and birth weight) change over time. The logistic regression was used to see if categorical variables (the proportion of boys, the proportion of tuberculosis latently infected mothers, the proportion of malaria positive mothers during pregnancy, the proportion of mothers with BCG scar, the proportion of CMV infected infants, the proportion of EBV infected infants) change over time. Note that some data was missing and/ or infection testing was not performed.

Characteristic	0 weeks	1 & 4 weeks	6 weeks	10 weeks	14 weeks	24 weeks	52 weeks	P Value
<b>Mother's Age (Median Years)</b>	24	24	22	22.5	25.5	24	25.5	0.190
<b>Total Number of Pregnancies (Median)</b>	2.5	2	2.5	2	2.5	2	1.5	0.376
<b>Total Number of Born Children (Median)</b>	2	2	2	2	2	2	1.5	0.823
<b>Birth Weight (kg)</b>	3.175	3.45	3	3	3.6	3.15	3.1	0.112
<b>The proportion of Boys</b>	5/10	2/10	3/10	5/10	5/10	7/10	5/10	0.265
<b>The proportion of Tuberculosis Latently Infected Mothers</b>	1/6	3/10	3/10	3/10	5/10	8/10	5/10	0.087
<b>The proportion of Mothers with BCG scar</b>	9/10	6/10	7/10	7/10	7/10	6/10	5/10	0.105
<b>The proportion of CMV infected infants</b>	0/1	1/6	2/9	3/8	5/9	5/8	2/6	0.416
<b>The proportion of EBV infected infants</b>	0/1	0/6	0/9	0/8	1/9	0/8	1/6	0.165

Table S3: Characteristics of infants and their mothers from whom samples were collected over time and included in the analysis of dendritic cell phenotypes over the maternal LTBI status, n=48, 24 samples from infants born to LTBI mothers and 24 from infants born to non-LTBI mothers. The Mann-Whitney test was used to measure if continuous variables (mother's median age, the total number of pregnancies and birth weight) varied by maternal the LTBI status. The Chi<sup>2</sup> test was used to see if categorical variables (the proportion of boys, the proportion of malaria positive mothers during pregnancy, the proportion of mothers with BCG scar, the proportion of EBV infected infants, the proportion of CMV infected infants) differed by the maternal LTBI status. Note that some data was missing and/ or infection testing was not performed.

<b>Characteristic</b>	<b>LTBI+</b>	<b>LTBI-</b>	<b>P Value</b>
<b>Mother's Age (Median Years)</b>	24	23.5	0.2462
<b>Total Number of Pregnancies (Median)</b>	3	2	0.1870
<b>Total Number of Born Children (Median)</b>	3.5	2	0.0537
<b>Birth Weight (kg)</b>	3.35	3.1	0.1355
<b>The proportion of Boys</b>	9/24	12/24	0.383
<b>The proportion of Mothers with BCG scar</b>	13/24	19/24	0.066
<b>The proportion of CMV infected infants</b>	6/19	10/20	0.242
<b>The proportion of EBV infected infants</b>	2/19	2/20	0.957

Table S4: Characteristics of infants and their mothers from whom samples were collected over time and included in the analysis of dendritic cell phenotypes over CMV infection status, n=49, 24 samples from infected and 25 from non-infected CMV infants. The Mann-Whitney test was used to measure if continuous variables (mother's median age, the total number of pregnancies and birth weight) change over the CMV infection status. The Chi<sup>2</sup> test was used to see if categorical variables (the proportion of boys, the proportion of tuberculosis latently infected mothers, the proportion of malaria positive mothers during pregnancy, the proportion of mothers with BCG scar, the proportion of EBV infected infants) change over the CMV infection status. Note that some data was missing and/ or infection testing was not performed.

<b>Characteristic</b>	<b>CMV+</b>	<b>CMV-</b>	<b>P Value</b>
<b>Mother's Age (Median Years)</b>	24	22	0.2318
<b>Total Number of Pregnancies (Median)</b>	2	2	0.0845
<b>Total Number of Born Children (Median)</b>	2	2	0.2272
<b>Birth Weight (kg)</b>	3.3	3.05	0.1343
<b>The proportion of Boys</b>	10/25	10/24	0.062
<b>The proportion of Tuberculosis Latently Infected Mothers</b>	12/25	14/24	0.656
<b>The proportion of Mothers with BCG scar</b>	16/24	19/25	0.470
<b>The proportion of EBV infected infants</b>	1/24	1/25	0.976

Table S5: Characteristics of infants and their mothers from whom samples were collected over time and included in the analysis of dendritic cell phenotypes over the EBV infection status, n=32, 16 samples from infected and 16 from non-infected EBV infants. The Mann-Whitney test was used to measure if continuous variables (mother's median age, the total number of pregnancies and birth weight) change over the EBV infection status. The Chi<sup>2</sup> test was used to see if categorical variables (the proportion of boys, the proportion of tuberculosis latently infected mothers, the proportion of malaria positive mothers during pregnancy, the proportion of mothers with BCG scar, the proportion of CMV infected infants) change over EBV infection status. Note that some data was missing and/ or infection testing was not performed.

<b>Characteristic</b>	<b>EBV+</b>	<b>EBV-</b>	<b>P Value</b>
<b>Mother's Age (Median Years)</b>	26	25	0.5252
<b>Total Number of Pregnancies (Median)</b>	3	2	0.2239
<b>Total Number of Born Children (Median)</b>	3	2	0.2136
<b>Birth Weight (kg)</b>	3.135	3.4	0.1397
<b>The proportion of Boys</b>	9/16	7/16	0.480
<b>The proportion of Tuberculosis Latently Infected Mothers</b>	4/16	7/16	0.264
<b>The proportion of Mothers with BCG scar</b>	11/15	10/16	0.519
<b>The proportion of CMV infected infants</b>	3/16	7/16	0.127

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## **6. Whole blood DNA methylation landscape of UK and Ugandan infants.**

In chapter four, I established protocols for the analysis of the DNA methylation, and in chapter five, I examined the immunogenicity of the BCG vaccine in the UK and Uganda. Here, I utilise the developed data analysis pipeline and examine DNA methylation patterns of UK and Ugandan infants. I also discuss how DNA methylation profiles could explain the variations in BCG immunogenicity.

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## RESEARCH PAPER COVER SHEET

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### SECTION A – Student Details

Student	Mateusz Hasso-Agopsowicz
Principal Supervisor	Dr Steven Smith
Thesis Title	A STUDY OF FACTORS UNDERLYING BCG IMMUNOGENICITY DIFFERENCES ACROSS COUNTRIES: THE INFLUENCE OF DNA METHYLATION PATTERNS AND ANTIGEN PRESENTING CELLS

**If the Research Paper has previously been published please complete Section B, if not please move to Section C**

### SECTION B – Paper already published

Where was the work published?	
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### SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	Scientific Reports
Please list the paper's authors in the intended authorship order:	Mateusz Hasso-Agopsowicz, John Vianney Tushabe, JiSook Lee, Patrice Mawa, Dorothy Aibo, Joel Serubanja, Emily Webb, Alison Elliott, Stephen Cose, Hazel Dockrell, Steven G Smith
Stage of publication	Preparation for submission

### SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I processed samples collected from UK infants. I extracted DNA from samples collected from UK infants. I received DNA collected from samples from Ugandan infants. I prepared library for the DNA methylation analysis. I sequenced samples using the MiSeq assay. I analysed FASTA files using a previously established workflow. I prepared the graphs and wrote the manuscript.
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Student Signature: \_\_\_\_\_ Date: 23/07/2018

Supervisor Signature: \_\_\_\_\_ Date: \_\_\_\_\_

## **Whole blood DNA methylation landscape of UK and Ugandan infants.**

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## Summary

**Introduction:** Epigenetic modifications have shown to regulate innate and adaptive immune responses and mediate vaccine-induced immunity. Bacillus Calmette–Guérin (BCG) has variable protection against pulmonary tuberculosis (TB) and induces different immune responses across countries. The vaccine works well in the UK and produces a strong T helper like 1 (TH1) response, but in African countries, it stimulates a TH2 response and has a variable efficacy.

**Hypothesis & Methods:** We hypothesised that DNA methylation could drive sustained differences in BCG immunogenicity and compared DNA methylation patterns between UK and Ugandan infants in the first year of life. We measured DNA methylation using reduced representation bisulphite sequencing (RRBS) and compared DNA methylation patterns between BCG vaccinated UK and Ugandan infants at 10 and 52 weeks of life, focusing on immune pathways.

**Results and Conclusions:** We found that genes of the B cell activation pathway, as well as vital immune regulators NFATC1 and SOCS5 have higher methylation in Uganda at 10 weeks of life. We report that at 10 weeks, the integrin pathways, TGF-beta signalling and key interferon and T cell activation signalling genes JAK1 and JAK2 are hypermethylated in the UK. We report genes and pathways that should be considered in mechanisms of vaccine-induced immunity.



## Introduction

Vaccines are the best life-saving health intervention to date, but they differ in efficacy across countries and populations, and the efficacy tends to be lower in regions near the equator or in low-income countries<sup>1–3</sup>. This is particularly the case for rotavirus<sup>1</sup> and pneumococcal<sup>3</sup> vaccines as well as the Bacillus Calmette–Guérin<sup>2</sup> (BCG) vaccine against tuberculosis (TB). We have previously reported striking differences in BCG immunogenicity between the United Kingdom (UK) and African populations<sup>4,5</sup>. UK infants produced twelve-fold higher levels of interferon  $\gamma$  (IFN $\gamma$ ) and six-fold higher levels of interleukin 2 (IL2), whereas Malawian infants produced thirty-fold higher levels of IL13 and eleven-fold higher levels of IL5<sup>5</sup>. We proposed that these immunogenicity differences could explain lower vaccine effectiveness in low-income countries. Transfer of placental antibodies, nutritional status, exposure to environmental mycobacteria, co-infections with other pathogens such as helminths or malaria parasites, have all been proposed as potential mechanisms to cause variations in vaccine efficacy, effectiveness and immunogenicity<sup>6</sup>. Whereas the causes of variations in vaccine efficacy and immunogenicity are widely discussed, the molecular mechanisms that drive these differences are less often considered. Numerous host molecular mechanisms could contribute to explaining observed differences in vaccine responses: genetics, epigenetics, gene transcription and translation, and post-translational protein formation and modification. Genetic studies report a high degree of gene heritability involvement in the response to vaccines<sup>7</sup>; that 76% of immune traits show a predominantly heritable influence<sup>8</sup>; and a growing body of research highlights the contribution of transcriptomes in the regulation of vaccine immune responses<sup>7</sup>.

Epigenetic modifications are heritable regulators of gene expression that do not alter the DNA sequence<sup>9</sup>. They work by modifying the binding of transcription factors by

either limiting chromatin access and reducing gene expression, or conversely, increasing chromatin access and allowing gene expression and subsequent protein translation<sup>10</sup>. Epigenetic modifications can also affect post transcription proteins and affect mRNA stability<sup>10</sup>. The three most discussed epigenetic mechanisms include DNA methylation, histone methylation and acetylation and micro RNAs (miRNAs). In the context of vaccines, studies have highlighted that DNA methylation was correlated with gene expression of multiple genes after influenza vaccination<sup>11</sup>; and several differentially methylated genes were identified in low and high responders to Hepatitis B vaccine<sup>12</sup>. Epigenetic modifications mediate multiple immune processes. Increased production of tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) and interleukin 6 (IL6), as well as expression of toll-like receptor 4 (TLR4) was associated with increased trimethylation of lysine 4 at histone 3 (H3K4me3) after BCG vaccination of Dutch adults<sup>13</sup>. High DNA methylation of the IL4 gene was observed in T Helper 1 (TH1) T cells whereas high methylation of the IFN $\gamma$  gene was reported in TH2 T cells. Lastly, miRNA-21 targets IL12 mRNA and inhibits its translation and subsequently impairs TH1 development<sup>14</sup>; miRNA-29 inhibits the translation of IFN $\gamma$  mRNA and impairs the immune response to Mtb<sup>15</sup>; and other miRNAs were implicated to play a role in the immunity against Mtb<sup>16,17</sup>.

Having observed disparate immune responses to BCG vaccination in UK and African populations, we decided to investigate potentially underlying molecular mechanisms that drive sustained differences in BCG immunogenicity and subsequent BCG efficacy. This study investigates differences in DNA methylation between UK and Ugandan infants, specifically focusing on immune genes and pathways. We hypothesise that DNA methylation patterns mirror previously observed differences in BCG immunogenicity. Specifically, we hypothesise that regulatory and TH2 genes

have lower methylation in Uganda whereas TH1 genes have lower methylation in the UK.

## Methods

### *Ethical approval and informed consent*

Infants were only included in the study if their mothers gave written, informed consent for participation. This project was approved by the Research Ethics Committee of the London School of Hygiene and Tropical Medicine (LSHTM) (#8720), the Uganda National Council for Science and Technology (UNCST), (#Hs 1526), and the Uganda Virus Research Institute (UVRI) Research and Ethics Committee (#GC/127/14/02/432).

### *Study Participants*

This study was nested into a larger trial that examined the effect of maternal latent tuberculosis infection (LTBI) on the infant's immune response to the BCG vaccination. Mothers and their infants were enrolled in the study if they were HIV negative; had a singleton pregnancy with a healthy neonate above 2500g; delivered in Entebbe General Hospital, Entebbe, Uganda; or North Middlesex Hospital, London, United Kingdom. In Uganda, mother's tuberculosis infection was examined using T-SPOT.TB assay (Oxford Immunotec) and a tuberculin skin test (TST; 2 tuberculin units, Statens Serum Institut); and mothers were included only if both tests showed concordant results. In the UK, the TB infection status was measured using T-SPOT.TB assay (Oxford Immunotec). All infants were BCG vaccinated at birth with BCG SSI strain. In Uganda, up to 5ml of peripheral venous blood and up to 10ml of cord blood was taken at birth, 1, 4, 6, 10, 14, 24 and 52 weeks. In the UK, up to 5ml of blood was taken at 10 and 52 weeks. To allow comparison between samples, for this analysis only samples from 10 and 52 weeks infants were included.

*EBV and CMV viral DNA detection from whole blood using droplet digital PCR (ddPCR) assay*

DNA was extracted from 200µl whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) in 100µl elution buffer and according to manufacturer's guidelines. The ddPCR reaction mixture included the probe mixture (final concentration 250 nM) for Epstein-Barr virus (EBV) and cytomegalovirus (CMV) (table S1), 10 µl ddPCR™ Supermix for Probes (Bio-Rad Laboratories, Hercules, USA), 10 µl of template DNA, and 1.1 µl of 20 x primers (final concentration 900nM). 20 µl of the mixture was partitioned in oil-in-water with 70 µl of droplet generator oil using a QX-100 droplet generator (Bio-Rad). The generated droplets (around 40 µl) were then heated for 5 sec at 170 °C using a PX1™ PCR Plate Sealer (Bio-rad). PCR amplification was performed with the following thermal cycling: 95 °C for 10 min, 40 cycles consisting of 94 °C for 30 sec (denaturation) and 60 °C for 1 min (extension), followed by 98°C for 10 min and holding at 12 °C. Droplets were analyzed using a QX100 droplet reader (Bio-rad) and the target DNA concentration was calculated using QuantaSoft software (Bio-Rad). All samples were tested in duplicates.

*DNA extraction, methylation, and sequencing*

DNA was extracted from 200µl of whole blood using DNeasy Blood & Tissue Kit (Qiagen), the quality was checked with NanoDrop (Thermo Fisher Scientific) and the concentration with Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific). The DNA library was prepared using the reduced representation bisulphite sequencing (RRBS) method with the Premium Reduced Representation Bisulphite Sequencing Kit (Diagenode) following the manufacturer's instructions. The concentration of the DNA library was measured again with Qubit dsDNA Assay Kit and size examined with the High Sensitivity DNA Analysis Kit (Genomics Agilent) using Bioanalyzer 2100

(Genomics Agilent). For sequencing, the DNA library was denatured with 0.2 M NaOH and concentration adjusted to 16 pM. A positive control PhiX v3 library (Illumina) was added at 12.5pM. Samples were sequenced using the Miseq Reagent Kit v3 (Illumina), running at 51 cycles per run with single end reads.

#### *Data processing and analysis*

FASTA files with a DNA sequence were generated as the final DNA sequencing output. They were trimmed with TrimGalore (v0.4.3) and Cutadapt software with a quality cut-off of 30 Phred score. The quality of the samples was confirmed with the FastQC software (v0.11.5). A *homo sapiens* genome reference post-bisulphite treatment was prepared using the GrCh38p7 assembly and Bismark Genome Preparation (v0.16.3) and Bowtie2 (v2.2.9) software. Trimmed FASTA sequences were mapped to the reference genome using Bismark (v0.16.3) and Bowtie2. The methylation of DNA sequences was analysed using SeqMonk (v1.41.0) and Rstudio (3.4.2) software. Contig methylation probes were generated with a 10-fold depth cut-off, duplicate reads were ignored, and probes closer than 500bp merged. Probes were quantified using read count quantitation and only probes sequenced at least ten times were included. The methylation of probes was quantified using bisulphite methylation pipeline embedded in the SeqMonk software. To eliminate gender bias chromosomes X and Y were excluded from the analysis. To identify differentially methylated regions (DMRs) logistic regression with multiple testing adjustment was conducted for the following groups of probes: UK all time points vs Uganda all time points; UK at 10 weeks vs Uganda at 10 weeks; UK at 52 weeks vs Uganda at 52 weeks; Uganda at 10 weeks vs Uganda at 52 weeks; UK at 10 weeks vs UK at 52 weeks. A probe was considered to be a DMR when the adjusted p value was  $\leq 0.05$  and the minimum average difference in methylation between comparison groups was 5%. To identify differentially methylated genes (DMGs), a gene feature report of

DMRs was conducted, i.e. a list of genes, which contain DMRs was produced. We refer to genes or DMRs that had higher methylation as “hypermethylated”, and genes or DMRs that had lower methylation as “hypomethylated”.

The datasets generated and/or analysed as part of the current study are available from the corresponding author on reasonable request.

## Results

### *Sample characteristics*

We included 62 Ugandan infants (16 at 10 weeks, 46 at 52 weeks) and 43 UK infants (31 at 10 weeks, 12 at 52 weeks). None of the samples were paired in Uganda and 11/12 samples were paired in the UK. Infants recruited for this study had a similar median birth weight across countries (3.31kg in the UK, 3.14kg in Uganda ( $p=0.095$ )) and a similar proportion of infants were male (51% in the UK, 48% in Uganda). There were more CMV ( $p=0.001$ ) and EBV ( $p=0.002$ ) infected infants in Uganda than in the UK. In Uganda, more mothers had LTBI during pregnancy ( $p=0.023$ ), and their median age at delivery was lower than in the UK (34 years in the UK, 24 years in Uganda,  $p<0.001$ ) (table 1).

### *Identification of longitudinal and inter-population differences in the methylation of DNA regions and genes*

We aimed to compare DNA methylation of genomic regions in infants at 10 and 52 weeks of age in the UK and Uganda. Firstly, we compared DNA methylation between all UK and Ugandan infant samples, irrespective of time point. We found that only 37 probes were identified as DMRs with a minimum difference of 5% DNA methylation between groups (figure 1A). We then conducted a further four comparisons: UK and Ugandan infants at 10 weeks of age (figure 1B); Ugandan infants between 10 and 52 weeks of age (figure 1C); UK and Ugandan infants at 52 weeks of age (figure 1D); UK infants between 10 and 52 weeks of age (figure 1E). Altogether we have found 2073 DMRs that were located within 2783 DMGs (figure 2, table S2) of which 2251 were unique DMGs which were differentially methylated in only one comparison. We report several DMGs which were differentially methylated across multiple



comparisons (table S2). Notably, paired box protein (*PAX6*), a regulator of transcription and cell development after birth<sup>18</sup> was found to be differentially methylated across five comparisons. The *MSLN* gene was found to be differentially methylated across four comparison groups. *MSLN* encodes megakaryocyte potentiating factor, a cytokine that can induce colony formation of bone marrow megakaryocytes. *MSLN* also encodes mesothelin that is suspected to function as a cell adhesion protein<sup>19</sup>. *SMAD3*, another gene of interest was found to be differentially methylated across four comparisons. *SMAD3* is a ubiquitous intracellular signal transducer and transcriptional modulator activated by transforming growth factor  $\beta$  (TGF- $\beta$ )<sup>20</sup>.

#### *Hierarchical clustering reveals different methylation patterns of identified DMRs*

We conducted a t-distributed stochastic neighbour embedding (tSNE) DNA methylation analysis of all samples. We did not observe a clear separation of samples based on origin or time point (figure S1). We then conducted a hierarchical clustering analysis of all samples and found moderate differences in DNA methylation across DMRs and between comparisons (figure 3).

#### *Differentially methylated genes are part of 111 biological pathways.*

We then proceeded with the analysis of DMGs. We conducted a Panther Pathway Analysis (PPA) for 2783 identified genes. We found that DMGs were involved in a total of 111 pathways involved in multiple biological processes including immune, physiological, metabolic and cellular processes as well as signalling, disease and other pathways (figure 4 and 5). Altogether, we conducted four comparisons (two longitudinal comparisons within each country and two cross-country comparisons at

both time points) and highlighted DMGs that were hypermethylated in each arm of the comparison (table S3).

#### *Comparison of DMGs between UK and Uganda at 10 or 52 weeks of life*

We analysed DMGs between UK and Uganda at both time points. We found the following DMGs to have higher methylation in UK infant 10 week samples: two DMGs in the B cell activation pathway (*SDK1*, *MAPK12*); five DMGs of inflammation mediated by chemokine and cytokine signalling (*ARPC2*, *ARPC1A*, *ARPC1B*, *JAK2*, *ARPC5L*); eight DMGs in the integrin signalling pathway (*ARPC2*, *ARPC1A*, *PIK3R1*, *ARPC1B*, *SH3D21*, *PARVB*, *ARPC5L*, *LAMB1*); three DMGs in the interferon-gamma or JAK/STAT signalling pathways (*JAK1*, *JAK2*, *MAPK12*); a T cell activation pathway DMG, *PIK3R1*; and the TGF-beta signalling pathway DMGs (*SMAD4*, *SMAD3*, *MAPK12*). In contrast, we found that in Uganda the following DMGs had higher methylation in infant 10 week samples: five DMGs of the B cell activation pathway had higher methylation (*ITPR3*, *MAPK11*, *PPP3CB*, *MAP3K3*, *NFATC1*); five DMGs in inflammation mediated by chemokine or cytokine signalling (*ITPR3*, *SOCS5*, *MYH10*, *CAMK2G*, *NFATC1*); three DMGs in the integrin signalling pathway (*MAP3K3*, *ELMO2*, *ASAP1*); two DMGs in the interferon-gamma signalling pathway (*SOCS5*, *MAPK11*); two DMGs in the T cell activation pathway (*PPP3CB*, *NFATC1*); and one DMG *MAPK11* in the TGF-beta signalling pathway.

At 52 weeks of life, *NFATC1* was hypermethylated in the B or T cell activation pathways in the UK; three DMGs in the inflammation mediated by chemokine or cytokine signalling (*NFAT5*, *NFATC1*, *GRK6*); and two DMGs (*GDF7* and *SMAD3*) in the TGF-beta signalling pathway, all had higher methylation in the UK than Uganda at 52 weeks. In Uganda, two DMGs (*IFNGR2*, *CISH*) of the inflammation mediated by chemokine and cytokine signalling or interferon-gamma signalling pathways were

hypermethylated; and one DMG *MEGF9* of the integrin signalling pathway, all had higher methylation in Uganda at 52 weeks (figure 4).

#### *Comparison of DMGs between 10 and 52 weeks of life in the UK or Uganda*

The longitudinal analysis of 10 and 52 week infant samples found a total number of 1055 DMRs and 1423 DMGs in the UK, and 157 DMRs and 223 DMGs in Uganda, suggesting that changes in DNA methylation in Uganda over time are less pronounced. We report that more genes in the B cell activation pathway were hypermethylated at 10 (*SOS2*, *PPP3CC*, *BRAF* in the UK; *VAV3* in Uganda) than at 52 weeks (*MAPK11*, *NFATC1* in the UK; none in Uganda), both in the UK and Uganda (figure 4). We found 15 DMGs to be differentially methylated between 10 and 52 weeks in the UK (*ARPC1A*, *ARPC1B*, *PLCD1*, *RRAS*, *BRAF*, *PLCB4*, *IFNGR2*, *GNAQ* hypermethylated at 10 weeks; *CCRL2*, *PRKCE*, *MAP3K4*, *PAK1*, *CCR6*, *NFATC1*, *GRK6* hypermethylated at 52 weeks) in the inflammation mediated by chemokine and cytokine signalling pathway. In contrast, in Uganda we found only one *APRC4* DMG to be hypermethylated at 52 weeks. We found 17 DMGs to be differentially methylated in the integrin signalling pathway between 10 and 52 weeks in the UK (*SOS2*, *APRC1A*, *APRC1B*, *SH3D21*, *RRAS*, *BRAF*, *MEGF9*, *VASP*, *COL4A1*, *LAMB1*, *ARF1*, *COL4A2*, *BCAR1* were hypermethylated at 10 weeks; *MAP3K4*, *ITGB8*, *RHOB*, *MAP2K3* were hypermethylated at 52 weeks). In Uganda, one DMG, *SH3D21*, was hypermethylated at 52 weeks. In the UK, we found three DMGs to be differentially methylated in the interferon-gamma signalling pathway (*IFNGR2* was hypermethylated at 10 weeks; *MAPK11* and *SOCS2* were hypermethylated at 52 weeks); five in the interleukin signalling pathway (*SOS2*, *BRAF*, *IL5RA* were hypermethylated at 10 weeks; *MAP3K4*, *STAT5A* were hypermethylated at 52 weeks); and one (*STAT5A* was hypermethylated at 52 weeks) in the JAK/STAT signalling pathway. None of these pathways had identified DMGs in

Uganda. In Uganda, one T cell activation gene was hypermethylated at 10 (VAV3) than at 52 (none) weeks. VAV3 is a gene encoding protein that regulates nuclear factor of activated T cells (NFAT) and subsequent T cell activation<sup>21</sup>.

In the UK, we also observed changes in the number of hypermethylated DMGs in physiological pathways. Notably, the thyrotropin-releasing hormone receptor signalling pathway and the angiogenesis pathway had increased numbers of hypermethylated DMGs over time (from one to six for the thyrotropin pathway, and from seven to twelve for the angiogenesis pathway). In the UK, we have also found cellular signalling and processes pathways that decreased in the number of hypermethylated DMGs over time. Two heterotrimeric G-protein signalling pathways and histamine H1 receptor-mediated signalling pathway decreased in a number of hypermethylated DMGs over time, suggesting an increased G-protein and H1 mediated signalling (figure 5).

Taken together, we report numerous pathways in which DMGs were identified between countries and within countries at different time points. In Uganda, we observed a higher number of DMGs in B cell activation pathways, as well as increased methylation of key immune genes such as *SOCS5* and *NFATC1*. In the UK, we observe increased methylation of genes involved in integrin and TGF-beta signalling, as well as key genes involved in T cell activation such as *JAK1* and *JAK2*.

## Discussion

Vaccines induce disparate immune responses across the globe giving rise to uneven efficacy against diseases. Here, we investigate whether DNA methylation patterns differ between the UK and Uganda. We focus on immune pathways, of which different methylation could help to explain observed variations in vaccine responses between high and low-income countries.

We first compared DMGs between UK and Uganda at 10 weeks of life. We found 111 pathways of which genes were identified as DMGs. We report that there are more DMGs that are hypermethylated in Uganda than in the UK in the B cell activation pathway. *MAPK11*, *MAP3K3*, *ITPR3* and *NFATC1* were all hypermethylated in Uganda. *MAPK11* and *MAP3K3* play a ubiquitous role in the modulation of immune responses, by phosphorylation and activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B)<sup>24,25</sup>, an important protein complex that controls inflammation, cell proliferation and maturation<sup>26</sup>. In the context of B cells, NF- $\kappa$ B regulates B cell development, maintenance and function<sup>27</sup>; *ITPR3* mediates intracellular calcium release, which indirectly affects immune responses<sup>28</sup>; *NFATC1* mediates activation, proliferation and differentiation of T cells, lymphoid and non-lymphoid cells, and induces cytokine expression<sup>29,30</sup>. Higher methylation of these genes in Uganda could indicate lower gene expression and subsequently impaired B cell activation and function. Of note, *MAPK11*, *MAP3K3*, *ITPR3* and *NFATC1* are not exclusively involved in B cell pathways, and higher methylation of these genes in Uganda could ubiquitously affect immune responses to pathogens and vaccines. *NFATC1* is also involved in the inflammation mediated by chemokine and cytokine signalling and T cell activation pathway, and *ITPR3* is involved in the inflammation mediated by the chemokine and cytokine signalling pathway. However, the increased methylation of *NFATC1* observed at 10 weeks in Uganda, is not apparent at 52

weeks, and both *NFATC1* and *NFAT5* were hypermethylated in the UK at 52 weeks. These findings could indicate that the NFAT immune mediation is more prominent in the UK than Uganda at 10 but not at 52 weeks, where NFAT mediation of immune responses is more evident in Uganda.

Laminin Subunit Beta 1 (*LAMB1*) and four ARPC genes were hypermethylated in the UK at 10 weeks. ARPC genes are involved in the integrin or inflammation signalling pathways, and they encode for an Arp2/3 protein complex, which is critical in driving integrin-dependent processes such as actin assembly and cellular protrusion<sup>31</sup>. *LAMB1* is a laminin protein-encoding gene, which plays a role in cell binding via high-affinity receptors<sup>32</sup>. In Uganda, we observed hypermethylation of *ITPR3* (intracellular calcium modulator), *CAM2KG* (calcium-dependent protein kinase)<sup>33</sup>, *NFATC1* (cytokine inducer), *SOCS5* (inhibitor of STAT signalling)<sup>34</sup> and *MYH10* (conventional myosin formation)<sup>35</sup>. These differences were diminished by 52 weeks of life, *NFATC1* became hypermethylated in the UK, and a new gene *CISH*, a cytokine-induced STAT inhibitor<sup>36</sup>, became hypermethylated in Uganda. This suggests that at 10 weeks integrin-mediated pathways are more methylated in the UK than Uganda, suggesting that in Uganda, these pathways and integrin processes are more active. The picture for inflammation pathways and genes is less clear as the Arp2/3 complex was hypermethylated in the UK and some key cytokine-inducing genes but also some inflammation inhibitors were hypermethylated in Uganda.

Analysis of the T cell activation, interferon-gamma or interleukin or JAK/STAT signalling pathways revealed that both *JAK1* and *JAK2* were hypermethylated in the UK at 10 weeks, whereas *NFATC1* and *SOCS5* were hypermethylated in Uganda. This suggests that at 10 weeks *JAK1* and *JAK2* play a smaller role in the mediation of T cell activation and IFN $\gamma$  as they do in Uganda (perhaps highlighting a role for

*JAK3*), and that *SOCS5* plays a limited role in the regulation of these immune responses in Uganda.

Analysis of the TGF-beta regulatory pathway revealed hypermethylation of *SMAD3* and *SMAD4* at 10 weeks, and *GDF7* and *SMAD3* at 52 weeks in the UK. SMAD proteins mediate TGFβ induced intracellular signalling that regulates cellular processes such as differentiation, proliferation and cytokine activation<sup>37</sup>; the *GDF7* gene encodes a TGFβ ligand<sup>38</sup>. This suggests that the role of TGFβ in the regulation of immune responses in the UK is limited when compared to Uganda. In the UK, other mechanisms of immune regulation might be more prominent, including IL10 or regulatory T cell (Treg)-mediated regulation.

We also highlight non-immune pathways of which DMGs were hypermethylated in the UK or Uganda. At 10 weeks, the PI3 kinase, NOTCH and GABA-B receptor II signalling pathways had more hypermethylated DMGs in the UK. All of these pathways could indirectly affect immune responses. Previous studies show that PI3 kinases regulate cell growth and survival and are involved in T cell contact with antigen presenting cells (APCs)<sup>39</sup>; GABA receptors mediate immunomodulatory functions in T cells<sup>40</sup>; and NOTCH signalling mediates T cell immune responses<sup>41</sup>. At 52 weeks, the WNT pathway had more hypermethylated DMGs in the UK. Proteins of the WNT pathway are known to increase both the survival of Tregs<sup>42</sup> and IL12 production by APCs, thereby driving TH1 differentiation<sup>43</sup>.

When we looked at pathways and DMGs between 10 and 52 weeks in the UK and Uganda, we found that there were more DMGs over time in the UK (1424) than Uganda (223). This suggests that the dynamics of DNA methylation are more pronounced in the UK within the first year of life and that whereas the 10 weeks to 52

weeks window represents a time of changing DNA methylation content in the UK, this is less so in Uganda where any DNA methylation changes either happen earlier than 10 weeks or later than 52 weeks of age. Both in the UK and Uganda, we found more DMGs of the B cell activation pathway to be hypermethylated at 10 weeks. This suggests that over time, as the DNA methylation of the B cell pathway decreases, the B cell pathway becomes more active, possibly leading to an increase in antibody production and cytokine signalling. This activation of the B cell pathway could likely be driven by exposure to environmental pathogens as well as vaccines. We also found that there were more hypermethylated DMGs in the integrin signalling pathway at 10 vs 52 weeks. Integrin signalling regulates processes such as cell survival, growth, proliferation, motility, spreading, migration, and other<sup>44</sup>, and our finding suggests that they could be more active at 52 than 10 weeks of life.

We also analysed the data for differential methylation of genes and DMRs of non-immune pathways and how they change over time in the UK. Interestingly, we report an increase in hypermethylated DMGs in the thyrotropin and angiogenesis pathways over time. Angiogenesis is a process of vessel formation, and it is particularly active within the first weeks of life<sup>23</sup>. Therefore an increase in methylation of genes involved in the angiogenesis pathway could be one of the mechanisms to regulate this process later in life. The thyrotropin pathway induces thyrotropin and prolactin release, and prolactin has been shown to both stimulate and inhibit angiogenesis<sup>45</sup>. We also found a decrease in a number of DMGs over time in the histamine and two G-protein signalling pathways. G proteins can act as molecular switches to control chemokines and their receptors; they also affect cell survival, proliferation and migration; and recent evidence suggests non-canonical GPCR signalling in immune cells<sup>46,47</sup>. Histamine signalling is known to regulate immune responses, particularly by regulating TH1 and TH2 cells<sup>48</sup>. Both G-protein and the histamine signalling pathways



could therefore contribute to vaccine-induced immune responses, and this research suggests that these pathways become more active over time.

Other researchers have investigated longitudinal changes in DNA methylation in infants and early childhood. Two studies<sup>49,50</sup> observed dynamic changes in DNA methylation over the first 5 years of life. They report hypermethylation of genes involved in cell development and adhesion, DNA binding, and the formation of cellular components such as dendrite or axon, and hypomethylation of genes involved in the immune system such as regulation of T cell-mediated immunity, cellular response to interferon-gamma or regulation of cell killing. Similarly, we have also observed demethylation of immune genes involved in B cell activation or integrin signalling and observed methylation of development-related genes involved in angiogenesis, axon guidance, or synaptic vesicle trafficking. To our knowledge, no study has compared DNA methylation between a low and high-income country.

Numerous environmental factors including infection, nutrition, radiation and xenobiotics, , have been shown to change the epigenetic profile<sup>51</sup>, and some of these factors could be driving differences in epigenetics between UK and Ugandan infants as well as over time in each country. BCG has been shown to change the epigenetic profile in adults<sup>13</sup>, and similarly, we might expect other vaccines to influence the epigenetic profile of infants. Thus, differences in immunisations could explain some disparate epigenetic profiles between both populations. Bacterial infections have been shown to mediate epigenetic modifications<sup>52</sup>, and Mtb is known to control chromatin at interferon-stimulated gene (ISGs) complexes to inhibit IFN $\gamma$  production<sup>53</sup>. Additionally, there is evidence that maternal diet has an effect on the infant's epigenome in the Gambia<sup>54</sup>. In addition to exposure to environmental factors, infant development over the first year of life has a strong relationship with epigenetic profile<sup>49</sup>. Increased methylation of developmental genes and pathways over time is

associated with tissue morphogenesis and development. Conversely, decreased methylation of immune genes over time is associated with development and activation of the immune system<sup>49</sup>.

Our study has a number of limitations. Firstly, the DNA methylation was measured in whole blood, which is prone to fluctuations in cell composition. Changes in frequencies of cell populations could affect the DNA methylation readout. We argue, however, that in order to truly understand the immunological milieu it is informative to examine the DNA methylation profile of the full complement of venous blood immune cells. This approach has proved fruitful in whole blood gene expression studies<sup>55</sup>. Secondly, in Uganda, infants' mothers were older, and there were more infants that were CMV or EBV infected, or born to LTBI mothers. CMV and EBV infection can affect the immune system by changing immune cell composition and function<sup>56,57</sup>, and maternal LTBI infection is associated with lower infant anti-mycobacterial T-cell responses after BCG immunisation<sup>58</sup>. Due to a small number of samples and no CMV, EBV or maternal LTBI infection detected in the UK, we could not adjust for these factors. Importantly, the prevalence of CMV, EBV and maternal LTBI is high in African populations and should be reflected in the analysis of the DNA methylation. Further studies could be conducted to analyse the role of these pathogens on the infant epigenetic profile.

This is the first study that directly compares DNA methylation profiles of infant whole blood between two distinct geographical locations. We aimed to answer whether differences in DNA methylation, specifically of immune pathways, could explain differences in vaccine immunogenicity observed between high and low-income countries. We report dynamic differences in DNA methylation between the UK and Uganda, and between 10 and 52 weeks of life. Specifically, we found that at 10 weeks, the B cell activation pathway and key immune genes *NFATC1* and *SOCS5*

are more methylated in Uganda than the UK. We also found that at 10 weeks, the integrin pathways, TGF-beta signalling and key interferon and T cell activation signalling genes *JAK1* and *JAK2* are hypermethylated in the UK. Over time, we report a decrease in methylation of B cell activation and integrin pathways in both countries. Future studies should involve *in vitro* models and explore how methylation of the aforementioned genes and pathways affects the immune responses to pathogens and vaccines.

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## **Author Contributions**

Conceptualization M.H.A, S.S, H.M.D.; Methodology M.H.A, J.V.T, J.L., P.M., D.A., J.S., E.W.; Investigation and Formal Analysis M.H.A., Resources D.A., A.E., S.C., H.D., S.S.; Writing-Original Draft M.H.A.; Writing- Review & Editing S.S.; Visualization M.H.A.; Supervision S.S.; Funding Acquisition A.E., H.D.

## **Declaration of Interests**

None

## Figures

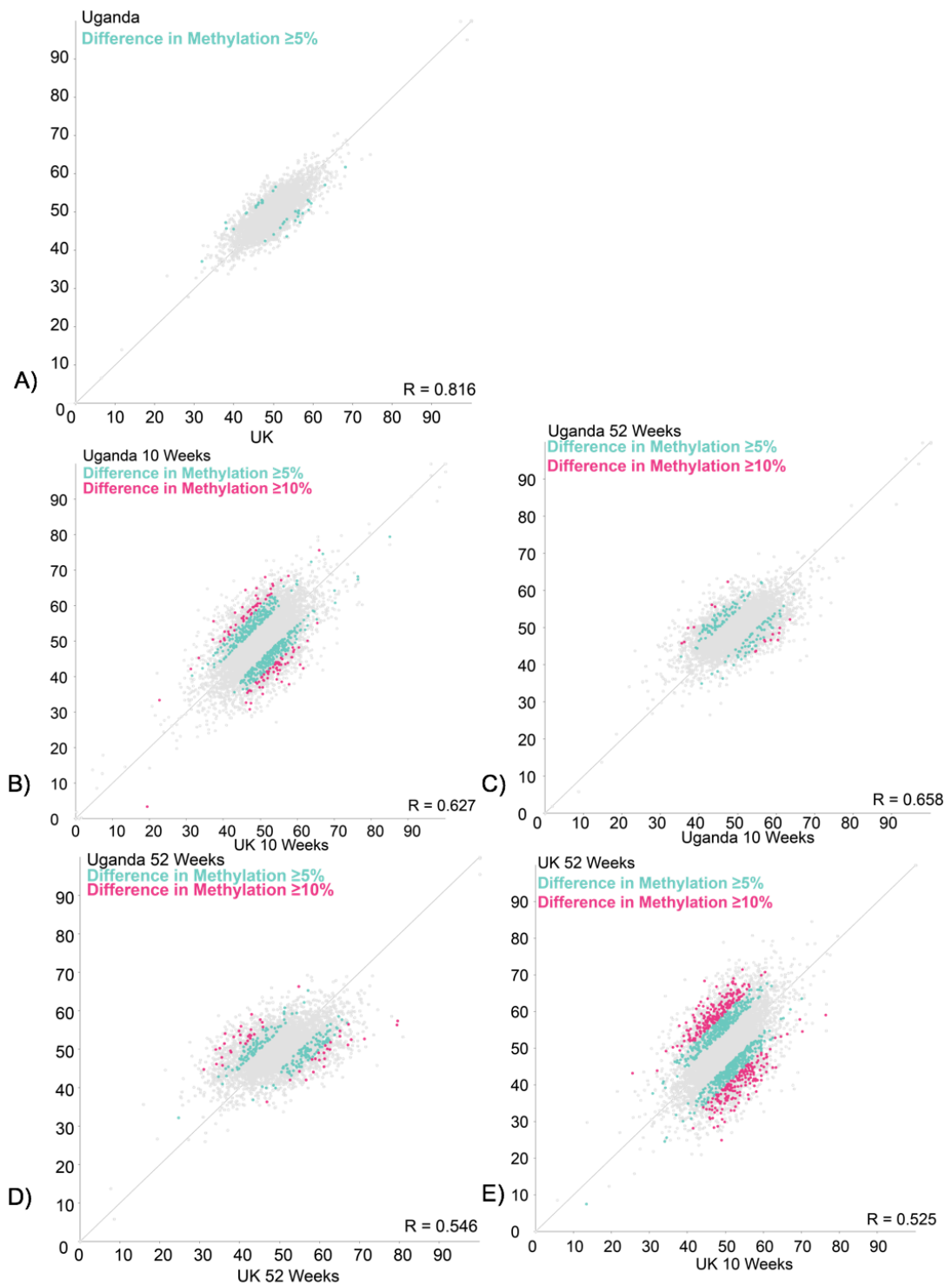


Figure 1. Scatter plots of differentially methylated regions (DMRs). Samples used for analysis included UK infants at 10 weeks of age (n=31), Ugandan infants at 10 weeks of age (n=16), UK infants at 52 weeks of age (n=12), Ugandan infants at 52 weeks of age (n=46). The grey dots represent genomic regions that were not DMRs. The aqua dots represent DMRs of which the minimum difference in methylation between groups was 5%. The pink dots represent DMRs of which the minimum difference in methylation between groups was 10%. The following groups were compared: UK infants vs Ugandan infants (A), UK infants at 10 weeks vs. Ugandan infants at 10 weeks (B), Ugandan infants at 10 weeks vs. Ugandan infants at 52 weeks (C), UK infants at 52 weeks vs. Ugandan infants at 52 weeks (D), UK infants at 10 weeks vs. UK infants at 52 weeks (E). DMRs were analysed using logistic regression with a p value <0.05 considered significant after multiple testing adjustment.

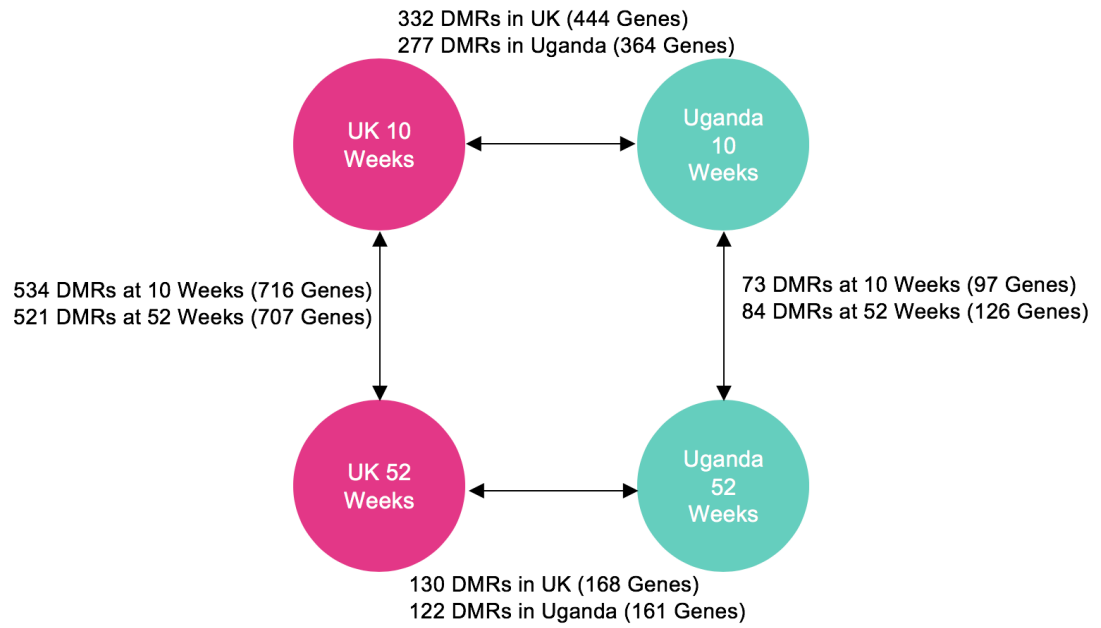
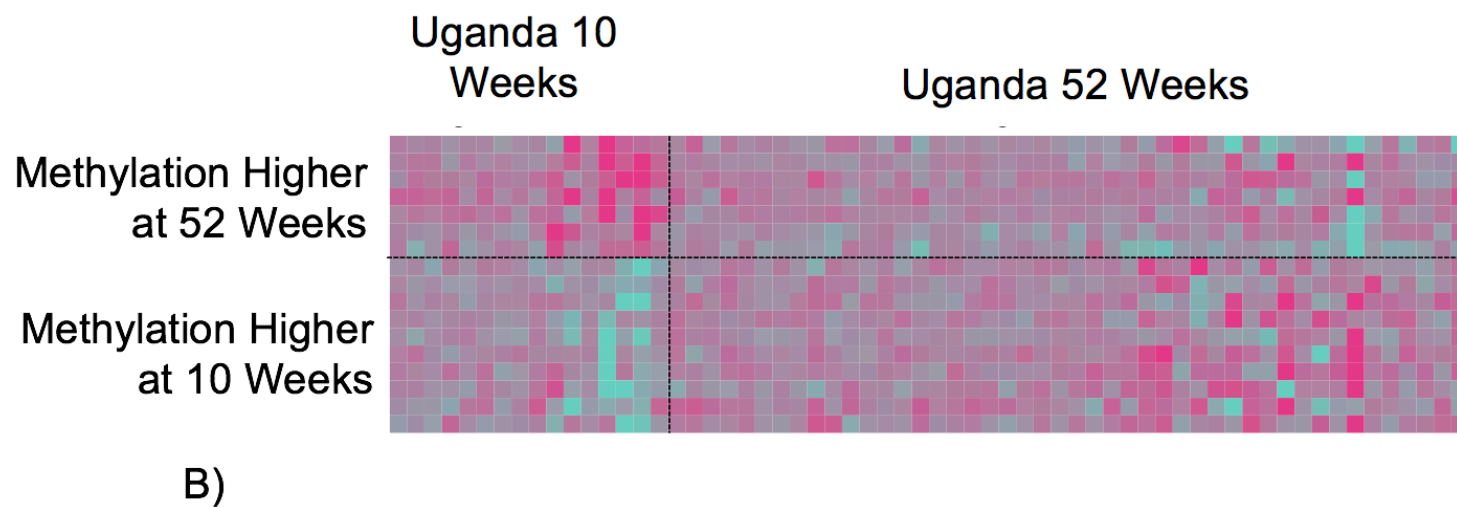
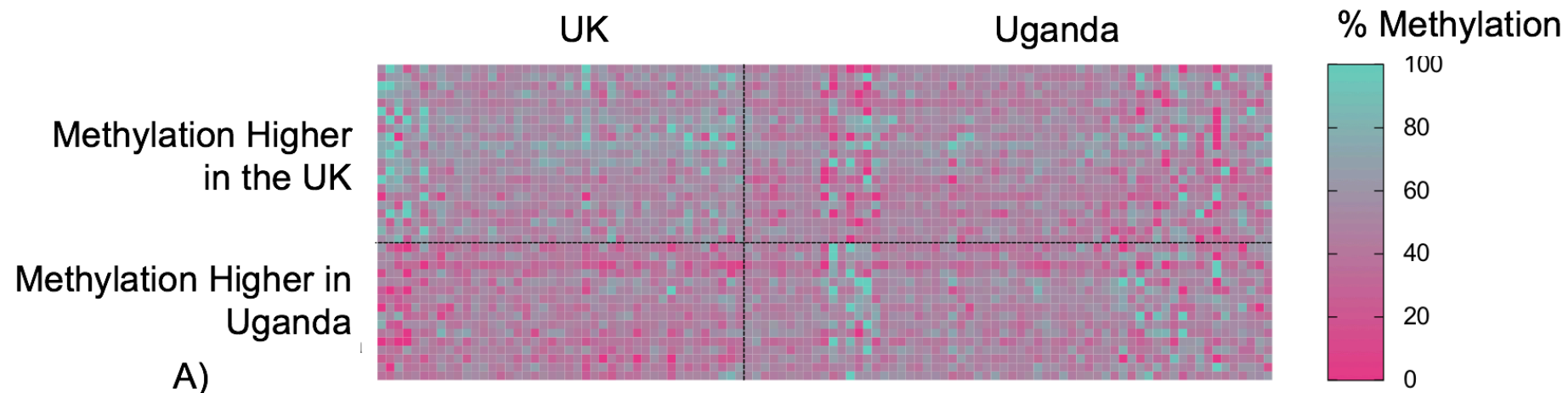


Figure 2. A summary of hypermethylated differentially methylated regions across all comparisons. The number of DMRs informs that there were a number of DMRs hypermethylated in a given condition. DMRs were analysed using logistic regression with a p value  $<0.05$  considered significant after multiple testing adjustment.





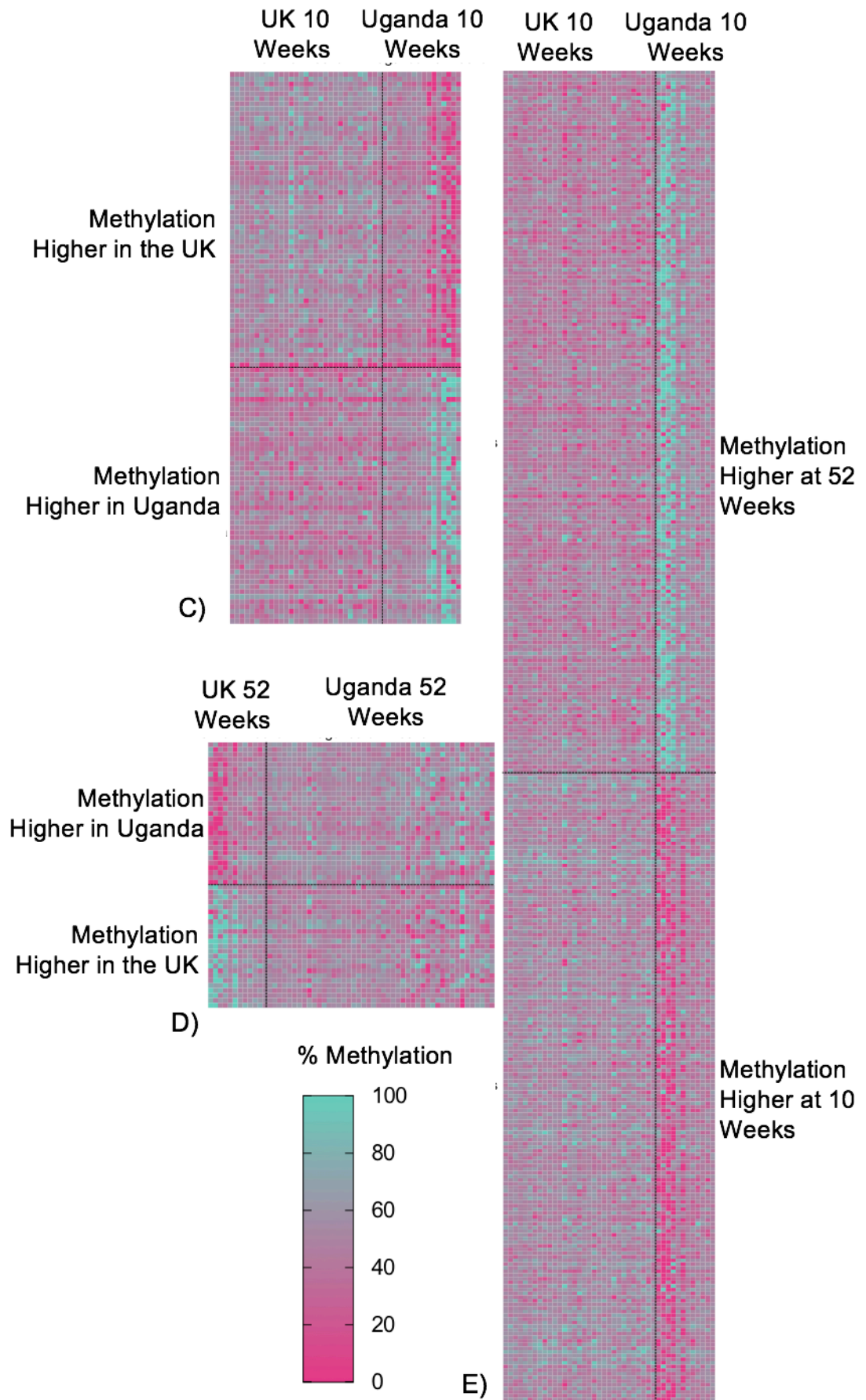


Figure 3. Heatmaps of differentially methylated regions (DMRs) over countries and by infants' age. DMRs between all UK and Ugandan infants (A); Ugandan infants at 10 weeks and 52 weeks of age (B); UK and Ugandan infants at 10 weeks of age (C); UK and Ugandan infants at 52 weeks of age (D); UK infants at 10 and 52 weeks of age (E). The aqua colour represents a 100% methylation whereas the pink colour represents 0% methylation. Each column represents an infant and each row represents 1 DMR. DMRs were analysed using logistic regression with a p value <0.05 considered significant after multiple testing adjustment.

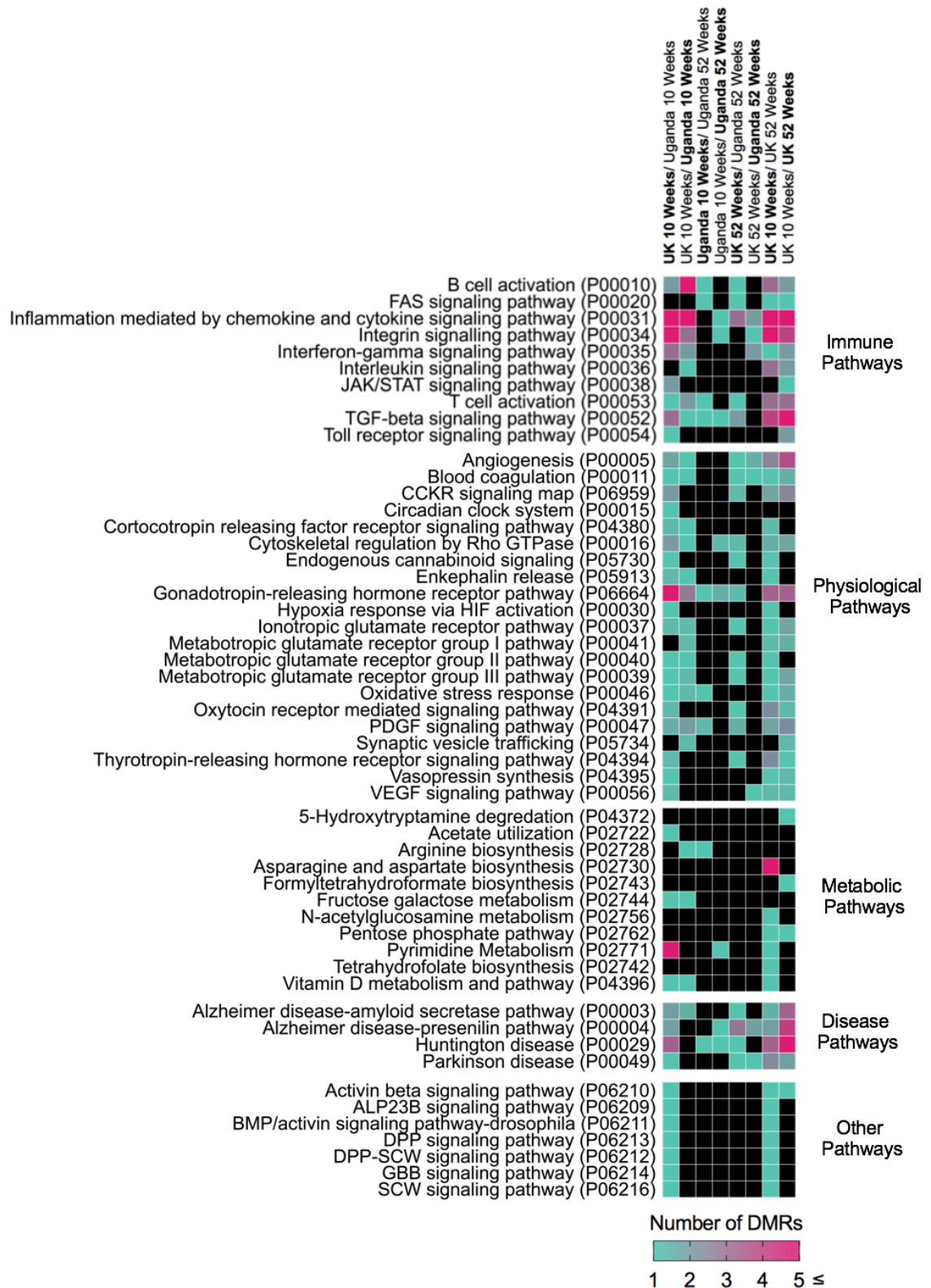


Figure 4. The pathway analysis of differentially methylated regions. Each column represents two groups that were compared. Each row represents a pathway in which at least one DMR was found. The aqua colour indicates one, and pink colour five or more DMRs that are hypermethylated in the group in bold. The black colour indicates that no DMRs were found for a given comparison in the specific pathway. Pathways were classified into immune pathways, physiological pathways, metabolic pathways, disease and other pathways.

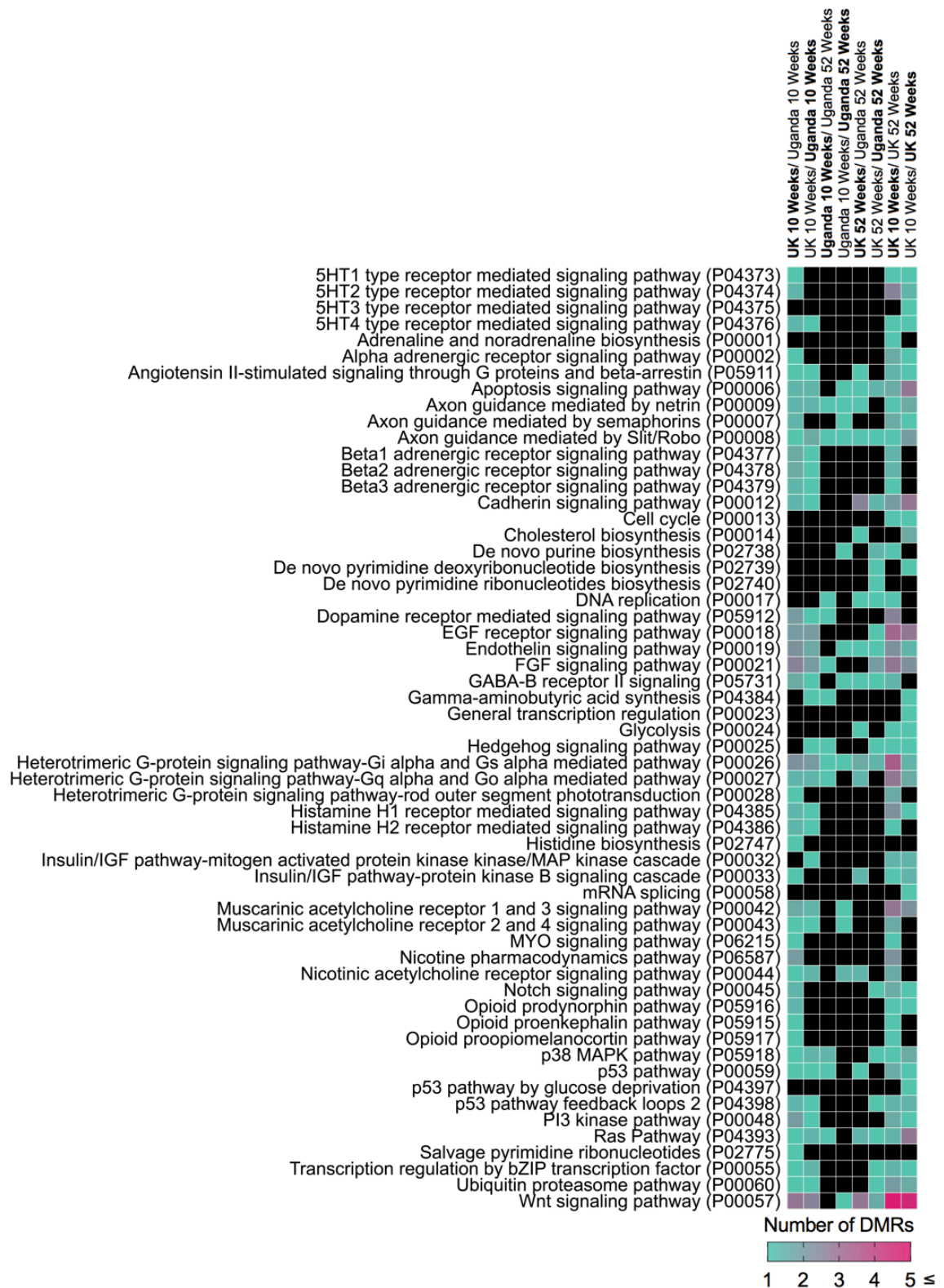


Figure 5. The analysis of cellular processes and signalling pathways of differentially methylated regions. Each column represents the two groups that were compared. Each row represents a pathway in which at least one DMR was found. The aqua colour indicates one, and the pink colour five or more DMRs that are hypermethylated in the group in bold. The black colour indicates that no DMRs were found for a given comparison in the specific pathway.

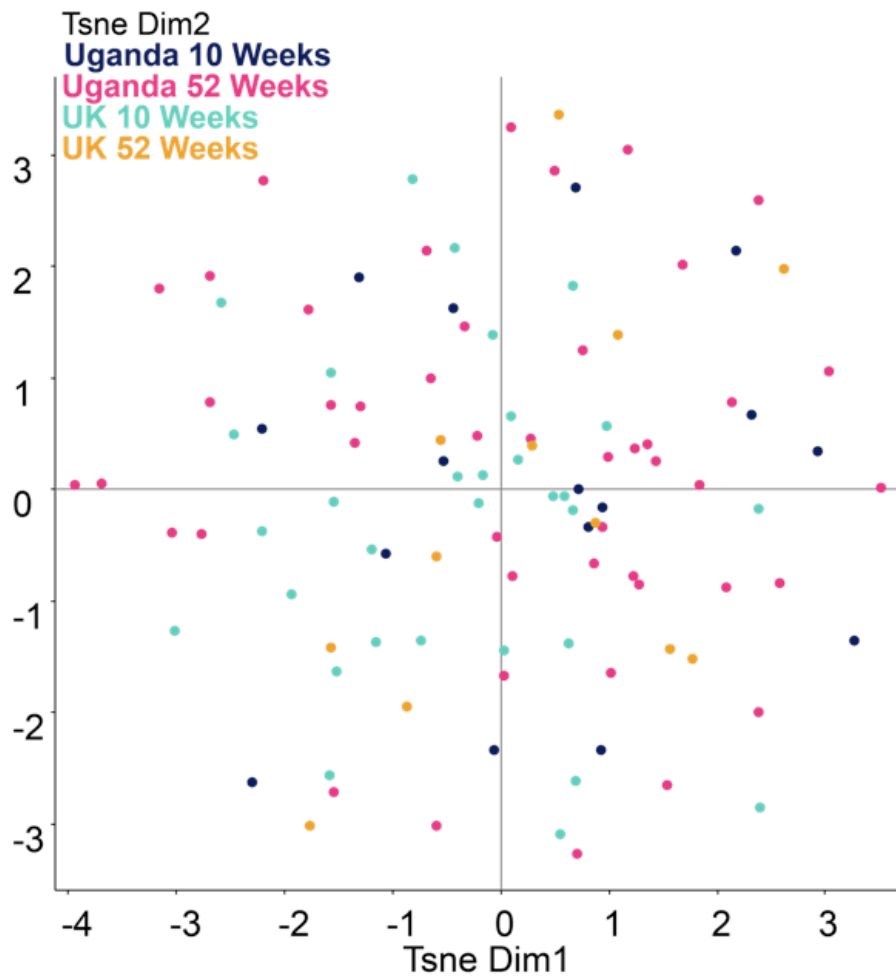


Figure S1. The tSNE analysis of all samples. DNA methylation values for samples in all groups were analysed using the tSNE method. The groups in the analysis included Ugandan infants at 10 weeks (navy), Ugandan infants at 52 weeks (pink), UK infants at 10 weeks (aqua), and UK infants at 52 weeks (orange). tSNE was calculated with perplexity of 21 and 1000 iterations.

## Tables

Characteristics	UK	Uganda	P value
Mother's Age (Median Years)	34	24	<b>&lt;0.001</b>
Birth Weight (kg)	3.31	3.135	0.095
Proportion of Boys	22/43	30/62	0.780
Proportion of Tuberculosis infected Mothers	0/43	7/62	<b>0.023</b>
Proportion of CMV Infected Infants	0/37	14/59	<b>0.001</b>
Proportion of EBV Infected Infants	1/37	16/43	<b>0.002</b>

Table 1.Characteristics of all infant samples included in the DNA methylation analysis in Uganda (n=62) and in the UK (n=43). The p value  $\leq 0.05$  was considered significant and highlighted in bold. The Mann-Whitney test was used for continuous variables (mother's median age and birth weight) and the Chi-square test for categorical variables (the proportion of tuberculosis-infected mothers, the proportion of CMV infected infants, the proportion of EBV infected infants). Due to a limited sample volume, not all infants were tested for CMV and EBV in Uganda and the UK.



Table S1: Primer/Probe sequences used for EBV and CMV detection

Target gene	Forward Primer	Reverse Primer	Probe
EBV- (BALF5)	CGGAAGCCCTCTGGACTTC	CCCTGTTTATCCGATGGAATG	6FAM-TGTACACGCACGAGAAATGCGCC-BHQ1
CMV (UL55)	TGGGCGAGGACAACGAA	TGAGGCTGGGAAGCTGACAT	HEX-TGGGCAACCACCGCACTGAGG-BHQ1

Table S2: A summary of all differentially methylated genes (DMGs) when following comparisons were made: **UK 10 weeks** vs Uganda 10 weeks (A); UK 10 weeks vs Uganda **10 weeks** (B); **Uganda 10 weeks** vs Uganda 52 weeks (C); Uganda 10 weeks vs Uganda **52 weeks** (D); Uganda 52 weeks vs UK **52 weeks** (E); **Uganda 52 weeks** vs UK 52 weeks (F); **UK 10 weeks** vs UK 52 weeks (G); UK 10 weeks vs UK **52 weeks** (H); and the number of hypermethylated DMGs is relevant for the arm of comparison in bold.

Comparison(s)	Total Number of Hypermethylated Genes	Differentially Hypermethylated Gene(s)
E H G C D	1	PAX6
E A H G	1	LHX5
B A H G	1	MSLN
F A G D	2	RP11-934B9.3 ADCY4
F B G C	3	FADS1 IFFO1 FADS2
E B H C	3	FAM219A DNAI1 MLNR
E A H D	4	TNFAIP8L3 NTF3 SMAD3 RP11-108K3.1
F E A	1	TYMP
F E H	1	RP11-89K21.1
F B H	1	HOXA13
F A D	1	PAX7

F H G	1	PTPRN2
E H D	1	HMX1
B A C	1	CCDC106
B A D	1	OSR1
F B G	2	BAIAP2 BAIAP2-AS1
F G D	2	B4GALNT1 CBX8
E B H	2	NFATC1 SLC9B2
E A H	2	BARX2 AUTS2
A H D	2	GPR27 EIF4E3
E H G	3	SPG7 ONECUT3 SKOR1
E H C	3	HTRA1 GDF7 ARL6
B H C	3	B3GNT2 AC007405.4 ERICH2
F A G	4	SLC16A12 CATSPERG NR2E1 SLC35D3
E B C	4	RPSAP52 RP11-572O17.1 SLBP HMGA2
A G D	12	FNDC1 CAMTA1 RP11-268J15.5 PNMA2 SH3D21 FBXO16 DPYSL2 STEAP3 RP11-312B8.1 EVA1B GSX1 RP11-13P5.2
F E	1	KDM2B
F A	1	PLXNA2
F C	1	ZNF398
E C	1	RFC4
G D	1	RCN1
E A	2	TSPAN14 ODF3B

B A	2	GNAS EBF4
H D	2	PDLIM1 GAS7
G C	5	ARHGAP5-AS1 MFSD8 C4orf29 ARHGAP5 FOXO6
F B	6	ZFAND2A KCNIP3 CLDN23 AC091729.9 RASSF2 PARP10
F D	6	CPEB1 PHF21B RP11-752G15.3 YAF2 TRPC6 PPHLN1
A H	11	RP11-111M22.2 NEU1 SPEG TCF7L1 FAM102A OTULIN CCDC78 SLC44A4 FAM173A PRKRIR CTD-2165H16.4
B G	16	STK25 ENTPD2 SPATA2 CDK18 ZCCHC6 SOGA3 PDGFRA BAALC-AS2 BAALC DNAJB14 RP11-229P13.15 FIP1L1 AMN SHISA2 SYT9 PDE12
B C	32	RP11-95G6.1 NAT8L EMP2 ARHGEF7-AS2 FOXL1 TEX2 DUSP10 RP11-126L15.4 FLT1 U2AF2 NUDT8 SLC43A2 ESYT2 PQLC1 EPAS1 KB-1732A1.1 ARHGEF7 TRMT112 FGF20 RP11-93B14.10 SUMO3 SLC12A9 PRDX5 NAGS SLCO4A1 HES6 HTR6 NTN1 FIGNL2 THBS2 E2F6 AC096574.4
A D	44	PXYLP1 KLF3-AS1 RP11-343C2.9 EN1 ZNF395 ZNF747 LINC00682 TGIF2 SLC16A11 COG8 SEC61A1 TBC1D24 IQCJ-SCHIP1 GPRIN1 PPP1R9B VWC2 RP11-343C2.7 ZNF768 IQCJ-SCHIP1-AS1 TUBB4B RNMT AC005256.1 VGF NTN3 ABTB1 KLF3 ST8SIA5 DLGAP4-AS1 DACH1 RP11-158I9.8 PODXL2 TGIF2-C20orf24 EBF3 PLEKHA1 NIP7 DDX6 ELOVL6 FAM210A SOX11 RP11-742D12.2 FAM131B LINC01248 AC002310.14 USP1
B H	55	KCNA6 C20orf27 FEV AC093887.1 KIAA1211L THRB TPT1 PROSER3 AC002398.12 NR3C2 FLJ46906 DEGS1 RAX MAPK11 TPT1-AS1 RP11-386G11.5 FLJ27354 SLC35F2 GALNT8 RAB39A HOXB7 MAPK4 KAZN EIF4EBP2 HSPB6 POU4F2 LRRC8C NTNG2 HOXB3 FBRSL1 ISOC2 SUN1 KCNK17 SYNJ2 HOXB-AS4 CRLF1 TRIM9 THRB-AS1 RP11-413E1.4 DNAJC6 DALRD3 RP11-302M6.4 TMEM158 FOXL2NB SYT3 DDN RP11-290D2.6 CELSR1 PRSS23 ABHD15-AS1 NHSL1 C19orf60 RP1-170O19.14 PDLIM3 HOXB-AS3

A G	59	CREB3L1 NCOR2 FGF17 DMTN PRDM16 AAMDC NGFR UBE2D3 FAM160A2 CRAT ARPC1A PIP5K1C PRRX2 ARHGAP32 PPP2R4 KMT2D TLN2 CSMD2 RP11-10L12.4 AC144831.3 APOLD1 DPP9 ARPC1B NAB1 ZNF512B LINC01006 GNB1 RSF1 MORF4L1 RP11-680F8.1 ADAMTS7 NSUN4 RFX2 TMTC2 RP1-140A9.1 RAI14 EPB41L1 GRHL2 TOB1-AS1 CNRIP1 SHANK1 FGF4 SMAD4 RP11-81K2.2 TCF24 CTB-66B24.1 OSGIN2 B3GAT2 RP11-729L2.2 CTD-2024P10.1 LAMB1 MSX1 SPTB CNGA4 PKN1 ATP12A ZMIZ1 HIVEP3 TOB1
F G	66	RP11-439L18.2 SMO USP6NL RIPK4 RING1 RP11-165N19.2 PXDN F7 AC005013.5 FLJ31306 RP11-545G3.1 TRIL HSPA2 GPRIN2 PRCD RP11-973N13.4 NPAS2 INSM1 SPRY1 C14orf37 MIR219A1 GAS1 RNPS1 ABHD17B ZC3H4 GUK1 ELP2 BACH2 DIO3OS DIO3 C9orf85 CMIP TEK4P2 EFHD1 NACC2 RP11-138I18.1 SLC16A9 AC009065.1 IFNGR2 ZNF217 LINGO1 MEGF9 ITM2C TRIB1 KAT6A RP11-545G3.2 TNFSF12-TNFSF13 ISG15 TBC1D4 PTPRS HIVEP2 CYGB WNT7A CXCL12 FGF18 MIR940 FOXF1 ANK1 MIR1247 ARID4A SMARCA2 SAE1 SP5 HES4 SUSD1 SLC39A6
E H	68	MSL1 REEP6 CHD9 KNDC1 TMEM132E UTF1 GEN1 CIAO1 OXTR SMC6 ECE1 RP5-940J5.3 NEDD4L CAV3 DCLK2 FLJ13224 TOX RAMP3 HUNK ADAMTS15 KCNJ12 CTD-2527I21.7 Six3os1_5 IFNLR1 NABP1 C17orf102 SP3 FAT1 UBALD2 ZNF503-AS2 GAPDH ZBTB25 CH25H ESPNP MCTP1 LBR KIF26B TLX3 CTD-2369P2.8 GRK6 TMEM127 ENAH RASEF RP11-25K19.1 GGPS1 LMO2 PTCHD2 SLC7A10 Six3os1_6 GRAMD1A FAM60A ICAM4 PCSK4 ZBTB1 BCL2L1 MIR1199 NCAPD2 TDRP F12 ARID4B AGAP1 WNK1 NKX3-2 HS3ST4 RP11-546B8.6 ICAM5 FMN2 KLHL24
C	40	CCDC177 LMNB2 LRRC48 TIAM1 ZNF425 SORCS2 CEP131 SVIL SHH VAV3-AS1 ZBTB4 SLC35G6 AP000251.3 BIRC6 POLR2A HNF1B HS3ST2 AJUBA PHF23 LIN54 CIB2 KCNA2 TMEM233 ETS2 TOM1L2 MAP3K10 RN7SL521P RP11-298I3.4 MTMR9 IDH3A RP11-768F21.1 FOXL2 TMPRSS9 PCGF5 FLJ31356 TIMM13 AF131216.1 VAV3 NPTXR RP11-298I3.5
D	46	KCNMA1 PRKRA UFSP1 NRARP FOXA2 FRMD6-AS1 MFSD11 OGFR-AS1 ARPC4-TTLL3 MED18 ATP6V0A2 ANP32A WDR26 ATP6V1E1 FAM19A5 LINC01276 ARPC4 VPS72 PLEKHG1 CPT1C ADM5 DFNB59 LDLRAD4 TADA3 NR5A1 INO80E FRMD6 HIRIP3 BCL2L13 ACHE PVRL2 SPESP1 MIR636 FOXP4-AS1 CTB-33G10.6 RP11-136K14.1 OGFR SRSF2 MEST RP11-318A15.7 GMPS CNIH3 RECQL4 PPP1R3G LRRC14 GATA4

F	62	IRX5 DLX2 LAMP3 MMP15 PP14571 IRX3 EFNA2 LA16c-366D1.3 Six3os1_4 RAB11FIP4 CSRNIP1 SENP3 HOTTIP_1 TNFSF13 SMOG2 MAPKAPK3 RP11-434E6.4 LRP8 ZNRF3 RP11-982M15.5 RP11-47A8.5 TMEM200B RP4-784A16.5 MED12L SCO2 MNT ACOXL DOC2B ASTN1 CAPN15 SOBP CCNA1 PRTG Six3os1_2 DSCAML1 FRAT1 DHRS13 ZNF407 ZNF595 FLOT2 INF2 SMIM19 MRPS26 SENP3-EIF4A1 FERMT2 BCL2L11 LHX3 FGF9 PPP2R1A SLC20A2 PLEC LINC00909 Six3os1_3 FAM84A PRR35 RP11-20B24.4 CDH4 CISH TRIM8 GPC1 HOTTIP TOP1
E	69	LRRC41 MIR3665 POU3F1 FBXO31 WNT3 MT-TP ABHD12 CCDC18 SLAIN1 RP11-615I2.2 PRRX1 RDX CPVL MAP1LC3B CD8A WNT9A CHN2 NFAT5 BAIAP3 PAX2 RP11-1008C21.2 PSMA5 FOXG1 CDH3 PDCC6 WNT1 MAF1 RP5-884C9.2 TIGD4 ARFIP1 TBX3 RP13-582L3.4 RAB34 ENO3 PALD1 CCBE1 RPL23A MKX AC006486.9 SHOX2 UQCRH AP1G1 SNORD42B UAP1L1 MYO1C MGAT1 RP11-2N1.2 PCP4L1 SFSWAP RRP9 MT-CYB STAC2 CCDC134 TMED5 MIR1538 KCNK2 MKX-AS1 CACNA1A PURG CASZ1 AC006486.10 WRN WSB2 PARP3 SHARPIN MT-TT PFN1 SIX1 GSK3A
B	231	ELMO2 SAMD4A LCORL MYO9B IZUMO4 MAP3K3 RP11-298P3.4 NFIA NMNAT1 CDC25B LUZP1 UBE2E2-AS1 LEPREL2 MGAT4B C2CD3 GAS2L1 SYT12 HPCA SEC14L1 PPP2R5A SPTBN1 PHLDA1 DIDO1 RP11-274B21.1 IGDCC3 RP11-290L1.2 TPMT TP53I13 C6orf58 TTC13 PHYKPL ASAP1 RP11-22P6.2 AP4M1 SH2B1 MAP7 SECTM1 RP11-53I6.3 NAV2 MICA SLC7A5P2 C19orf81 NARFL FAM107B NOL4L SNRPG ZBTB7C MAST4 SGSM3 RP11-482M8.3 MAGI1 RP11-84A14.4 RP11-123O10.3 RP11-293M10.6 RPS6KA2 OLFM2 RASSF8-AS1 NDE1 UBASH3B FOXJ3 TSSC4 PPP3CB NAMPT RP1-261D10.2 UGGT2 EFHD2 MCM7 LGALS3 DCBLD1 SMG1P3 SFI1 SHISA4 COMP LPCAT4 TUBA1C EIF4ENIF1 SRGAP1 DNAJC21 RP11-305P22.9 FOXB2 CTC-367J11.1 FBXL17 ZNF160 METTL24 CROCCP2 KIF13A GRINA HERC5 TGIF1 EPS8L2 AC121334.1 UBE2E2 RP11-219B17.3 KCTD8 RP11-209D14.2 KDM1B LZIC RASSF8 SOCS5 RP11-783K16.10 RIMKLA RBM15B AC019181.2 RP11-161H23.5 TSPAN4 TRAPPC3 N4BP2L1 JDP2 RP11-123O10.4 RP11-429P3.3 XKR4 LBH RPRM BHLHE23 LENG8-AS1 MAP7D1 UTP15 ZNF697 RP5-875O13.1 PPP3CB-AS1 COBLL1 OTUD1 ANKRA2 ARV1 HCP5 NDUFAF3 SLC22A4 NDST1 GALK1 IRF2BP2 RP11-410N8.1 GPR162 ST6GALNAC5 SOX21 RXRA ZNF704 TCHH SQSTM1 NMRK1 OSTF1 CTIF AFG3L2 RP11-223A3.1 RP11-977B10.2 RP11-22N19.2 WTIP DSC2 MAPT CLDN7 ABHD15 MSX2 RP11-576D8.4 FAM181B NDUFA4L2 FBXW4 AC131951.1 CHL1 SPON1 CGGBP1 SLC1A2 NGB KIF21A TTBK1 MYH10 SIPA1L1 NRG2 AGFG2 ZSWIM6

		<p>SPECC1 LENG8 C3orf80 GOPC HAUS8 RP5-1042K10.14 DRG1 RNF138 RP11-95O2.5 RP1-4G17.5 CAMK2G KCNK4 MVB12B CREB5</p> <p>RP4-781K5.2 FGFR1 MOB3A MAPT-IT1 P4HA2 NPHP4 MIR3663HG RORA RP11-290L1.3 AC012501.2 MIR1908 AAMP KREMEN1</p> <p>TBC1D30 CDK2AP1 KCNAB2 PPME1 RP3-325F22.5 RP11-410N8.4 KCNQ2 LEMD1 RP11-359E10.1 DPH2 RP4-683L5.1 RP11-212P7.2</p> <p>DSCAS ZNF367 RP1-309F20.3 HOXB6 SOX21-AS1 KCNK13 PRKAR1B RP11-53I6.2 DEAF1 C6orf132 CHL1-AS2 IL12A-AS1 FSTL3</p> <p>TMTC4 RP11-219B17.1 PARD3B C3orf38 JPH3 HEATR3 CELF4 INPP5A chr22-38_28785274-29006793.1 PNKD ITPR3</p>
A	292	<p>MLC1 SLC4A8 RP11-156P1.2 DHRS11 TOPORS-AS1 CA11 CBX3P2 RP4-575N6.4 CTD-2012K14.6 RP11-783K16.5 RP11-656D10.3</p> <p>MAPK12 OTUD3 INTS1 ZNF284 P2RX5-TAX1BP3 EMILIN1 AC009501.4 CDC42EP3 PHF7 LIF CNTFR ITPRIPL2 SLC9A3 FKBP4</p> <p>PTGER1 GFI1 RTN4RL1 SERTAD2 PAQR4 SEMA3F-AS1 DENND5B-AS1 SNTG2 PAQR3 LINC01063 KIT AURKAIP1 RAB20 JAK2</p> <p>NKIRAS1 PEX26 NEGR1 FGF12 RP3-512B11.3 RP11-21J18.1 SMCHD1 RP11-65J3.3 CTD-2545H1.2 TRPC4 DLEU2 NR1D2 TRHDE</p> <p>PANO PARVB CTD-2228K2.7 CHST2 SLC37A1 ZNF223 BCL11B DACT3-AS1 RP11-146B14.1 RP4-591C20.10 EPHB1 MKKS WEE1</p> <p>HES2 COPS6 ADARB1 DBP UCKL1 FAM207A SLC27A6 RP11-685M7.3 UNCX AC073316.1 TUBB2B HECTD1 ZNF717 ATP9A APBB3</p> <p>PPP1R14B NKX2-3 MT-TS2 GALNT6 LRFN1 SIRPA FAM132A EML6 TAX1BP3 PRPF8 CTD-2012K14.7 ARHGEF4 PAOX DLEU2_6</p> <p>TMEM114 PPP1CB MT-TH GLDC AC116614.1 DLGAP3 CHST12 STAU1 TNIP1 DLG5-AS1 TAPT1-AS1 EMC6 CTD-2349B8.1 CRIP1</p> <p>VPS54 FBXL16 ZFAND5 SHANK2 RP5-858L17.1 TMEM41A ZNF496 RP11-776A13.1 CPB2-AS1 ARPC5L PIK3R1 AC083843.2 DLG5</p> <p>PER1 BMI1 GPR63 SEMA3F COMMD3-BMI1 PIDD1 PTPRU TIGD3 MRPL23 TTC9B NCOA7 LRRC37A17P RAPGEF4-AS1 RP11-</p> <p>783K16.13 PYCARD MIR4488 OAT IQGAP2 MIR193B LINC01547 CHRNE RECQL RP5-1029K10.4 DSP RP11-144F15.1 RP11-510C10.2</p> <p>NXPH3 SLC44A2 DLEU1_1 DBNDD2 ZNF581 SLC2A13 RP11-65J21.3 FBN2 SSBP4 ALK DACT3 SYS1-DBNDD2 R3HDM4 RP11-</p> <p>423C15.3 RP11-599B13.6 FLJ42351 RP11-586D19.1 EXO5 PP7080 RP11-65J3.2 PACS2 SKOR2 KHK ELOVL7 THBD ATHL1 SLC25A22</p> <p>C17orf107 MT-TL2 MAP3K8 EGR2 JAKMIP1 DGKZ LINC00092 PPP1R3B C14orf80 RSPO1 RP11-10A14.4 PPP2R5C MT-ND4 FAM168B</p> <p>LHX8 PDS5B RP11-363J20.2 LRFN2 KCNK1 NR6A1 NDUFV2-AS1 WBSR17 PCGF2 DENND5B SSR4P1 CBX3P4 FAM118A ADCY8</p> <p>LRRC10B SOX9-AS1 RAPGEF4 CTCF AADAT DOT1L PBX3 LPAR2 PRLHR ETF1 TOPORS RNF213 CTB-58E17.5 SLC35A4 ZC3H13</p> <p>ARPC2 KLF1 ACSS1 EPB41 CARM1 RP1-102K2.8 TMEM51 ZNF580 WWTR1-AS1 AFAP1L2 TUBA8 BRAT1 S1PR1 CIC RP11-961A15.3</p>

		<p>DAB2IP KDM4C SDK1 TEX22 PABPC4 PTH2R MDK SLC20A1 NR5A2 IQSEC1 PCDH10 AC008079.10 KIAA1456 RP11-326C3.2 RYR2</p> <p>PKMYT1 AP3D1 SLX4IP RP5-1148A21.3 BAP1 SALL4 AC011247.3 TSPAN9 KIAA1614 ANKRD12 RP11-849I19.1 RP11-316O14.1</p> <p>PYCARD-AS1 ZMYND11 WWTR1 COBL RP11-175D17.3 JAK1 QSER1 HNRNPLL EPHB6 ST8SIA2 TAPT1 PTP4A1 RFX4 LRRC16A</p> <p>RP11-510C10.3 ADPRHL2 EIF4G2 MAP4K4 PPP1R18 CHN1 SALL3 ADAMTS5 CAST DLEU1 TRHDE-AS1 GALNT16 AC053503.11</p> <p>GOLT1B DPYSL3 PHF21A RPRML RP11-390F4.6 TP53TG5</p>
G	530	<p>EIF2B5 DPY30 VPS33A RP11-512M8.5 SALL1 RMRP NHLRC3 NTRK1 FRAS1 UQCR11 DCAF5 TRIB3 ZNF131 TRIM33 AK7 GOT1 RP11-201K10.3 CCDC166 UTRN CTC-359M8.1 CORO7-PAM16 RNF103-CHMP3 ZNF529 PLEKHO1 LINC01121 CNGA3 RP11-468E2.2 RP11-347I19.7 IPO4 INSRR SHBG CTD-2265O21.7 OTUD4 GCC2 C1QL1 RP11-106M3.3 TIMM22 AC079790.2 TMEM200A RP5-906A24.1</p> <p>ZSCAN32 ARL16 PRRC2B UPF3A USP46-AS1 ZNF687 SDC2 QKI EZR FUT4 HGH1 ZNF655 HSPA8 PICALM ANKLE2 TLE2 VSIG8</p> <p>ADRA1A KISS1R IL5RA STON1 CDH24 DUSP18 TNFSF12 PLEKHM1P ARID5A LMO4 RP11-571L19.7 GOT2 UIMC1 PPP1R1B CLTCL1</p> <p>RP11-927P21.4 RP11-477I4.4 CASC4 SLC35F1 FHDC1 GPR158 RILPL1 SAMD10 GTF3C5 PARK7 CTC-435M10.3 BAI1 MEMO1 TBC1D8</p> <p>ASCL2 SYDE2 CTD-2089N3.2 NSMF DCAF11 RHPN1-AS1 BARD1 HS3ST6 CTD-2278I10.6 MIR6080 ZNF710 MCM10 H2AFY2 SAFB2</p> <p>AC072062.1 CHTF18 CTD-2562J17.4 RRAS ESR1 AC009236.2 GCH1 PEBP1P1 TNFRSF19 LRRC45 SLC19A1 KIF9 PITPNM2 TGFB1</p> <p>RAVER1 CAHM ADAMTS17 KLHDC2 RP11-353N14.1 B9D2 KRT86 LLNLR-304G9.1 MT-RNR1 PROCA1 NAP1L1 ZDHHC17 MTUS2</p> <p>CCDC107 COL4A2 RP3-510H16.3 RP11-95P2.3 EIF4E FAM200A RSPH14 LSM14B HMHA1 NGEF GFOD1 CTNNBIP1 AGRN PLCB4</p> <p>MRM1 IRX4 ADHFE1 USP46 GNAZ POU4F3 EIF3A YRDC FXR2 NRL PAPOLG SNX9 FAM134A Sep-08 PLCD1 CTD-2369P2.12 DBI</p> <p>LPIN2 CNTN1 LA16c-306E5.2 TENM3 ATP6V0C KCNG3 TMEM91 KIAA1257 LIPG CTD-2313J17.1 C2CD4D NEK8 RP11-347C18.3</p> <p>PPP2R2A C11orf80 JADE2 TRNT1 RP11-411B6.6 TNFRSF21 KLHL18 RP5-1059L7.1 CTD-3184A7.4 CABIN1 IER5 RHOT2 RP11-417L19.4 ARL5C Mar-01 RP11-635N19.1 LINC01089 EZR-AS1 ANKLE1 NMRK2 TP53I11 XRN1 CTD-2186M15.3 CRMP1 GPR158-AS1</p> <p>SYBU AJ003147.8 COL4A1 RP11-755B10.4 LEKR1 VASP SETD1A CTB-180A7.3 LPP-AS2 PTGFRN ZMYM2 GUCD1 CTD-2089N3.3</p> <p>EFCC1 GTF2I SCAF1 SSPN GFOD1-AS1 FAM78B STRA13 DOK5 NOVA1 PNPLA7 MXD1 SOWAHA BOC ATP6C ELMOD3 ARMC4P1</p> <p>NEURL1B SP8 TRIM46 NSUN7 PIP5K1B KIAA1462 AC007040.6 PFKFB4 FUK KCNMB4 SBNO1 PRPF40B HIGD2A CNOT11 RRS1-AS1</p>



		<p>RP6-65G23.3 STON1-GTF2A1L KDSR BHLHE41 PRCC BRAF PBX2 HGS ADARB2 LINC00472 GULP1 SATB1 PPM1D SDHA MAP3K9</p> <p>RHBDD1 STT3B SAFB FAM174B AC005391.2 LPP RGMB MPPED2 RP3-466P17.1 NUFIP2 AXIN1 RP11-1012A1.4 RP11-654A16.3</p> <p>TM9SF1 MIR1470 RNA5SP174 UCN RP5-1024C24.1 FBXL6 FAM20A CHID1 IKZF5 RHOF NRP1 SAMD3 AC010761.9 HRK EPHA2 RP11-436D10.3 SLC18A3 DGKH LINC01424 AC009166.5 C5orf28 MIR8072 IKZF1 TBC1D5 SLC25A1 GAN EVC2 PRKCI FOXC1 WIZ ACBD5</p> <p>NLRP6 CKB MUC1 DYNLT1 AC019131.1 ARID3A GOLPH3 ARF4 CTD-2201E18.3 SEC31B SOS2 GPI AC007040.11 AGPAT3 ABO</p> <p>KCNH2 RP11-441O15.3 CELF6 TEX261 CCM2 ZNF707 ZNF382 NDUFAF6 ECE2 ARF1 POU4F1 CCNG2 ISL2 EVI5 PUS1 RNF103</p> <p>STOX2 RNF219-AS1 PPP3CC TPBGL PROSER1 RP11-97O12.2 FAM149A KAZALD1 RP11-685G9.2 AC010761.6 RP11-159F24.3 TTLL7</p> <p>ARL10 RP11-845M18.7 VEGFC ZFPM1 RGS7 RP11-276H7.2 C1orf122 CHAT RP11-410D17.2 NOP16 ANKRD11 BCAR1 ADO</p> <p>AC005481.5 FRAT2 UACA RRS1 KLF12 AMDHD2 KIAA2026 PPIB FAM155A CCDC127 LINC00982 TPRN TOM1 RP11-347I19.8 C2orf76</p> <p>RCAN3 ERI2 MANEAL LINC01397 PRKAG2 H2AFX TXLNA RAB31 SPATS2 ZNF696 SRSF4 GPR88 MKRN2 RP11-529K1.4 GMEB2 CTC-308K20.1 STAG3 UBE2G2 PHLPP1 RP11-452K12.4 ACE SSSCA1-AS1 PTPRK WDR81 MFSD2B CTD-2369P2.10 ST3GAL2 RHPN1</p> <p>SEMA5B BDH1 LATS2 ZNF174 MKRN2OS LRP6 RBCK1 ZDHHC8 VCPKMT FDX1L MN1 LY75-CD302 CACNB4 SMURF1 FAM105A</p> <p>DNASE2 MT-TF EPN3 MTA3 EPB41L2 PIWIL4 NFIX RP11-402J6.1 LY75 HOXC12 CBL SLC35E4 CYB5R1 RTTN FADD ERBB3 HPCAL1</p> <p>PLD4 AGPAT2 TP53INP1 GNAQ IGDCC4 MMAA AC092675.3 TRAF4 CORO1A PPIL2 TRIM54 EPM2A EXD3 PAPL NEUROG2 PAX8</p> <p>TRRAP AC004381.6 ANPEP TBX20 GOLIM4 RP11-20I23.1 STK35 GS1-259H13.10 GPC2 RP4-753F5.1 MIR4665 TCERG1L TMEM163</p> <p>LHX5-AS1 SSSCA1 ALG3 BEND6 PLEKHH3 CDH23 CDC123 MLEC USHBP1 PPP1R16B AMOTL1 IER3IP1 CHD5 GPD2 RP11-455F5.5</p> <p>CORO7 RGMB-AS1 CDC42BPB CIRBP RP11-106M3.2 SNRPD3 CNP C1orf204 NOVA1-AS1 GATA6-AS1 CNPPD1 SYVN1 NUDT5</p> <p>PPP1R14C GNG13 HDGF PVRL3 CACNA2D4 MIR22HG LINC01482 PMEPA1 MFNG VTI1B RP11-449H3.3 RP11-283G6.3 SRSF5 ETV6</p> <p>AC092675.4 STK38 RETSAT UEVLD CLPP ESPN RNase_MRP MPG FAM132B GPR39 E2F3 RPL26L1 PVRL3-AS1 LRRC37A6P LHX9</p> <p>SLC52A2 C21orf58 AMD1 TRPC3 MBOAT2 CLIC6 VHL ATF7IP2 FAM49B RP11-867G2.8 PCNT CTD-2313J17.6 UBE2J2 PCIF1</p>
H	540	<p>FARP1 RP11-154J22.1 AHDC1 PAK1 GYS1 ERG ANKRD13B G2E3 FAT4 TMCC3 RP11-271C24.2 NR4A2 LRRN2 SNX33 RHOT1 EPT1</p> <p>ARL4A CCDC64 TAOK3 HSD11B2 ZNF48 AC097468.7 RELT ELANE TMEM240 ZNF32-AS3 WHSC1 RTF1 NEFH ALX1 CCNY MSH6</p>

IP6K1 PPP1R3D F2RL1 C1orf216 NTHL1 FAM163A HNRNPR PPM1A NKX6-1 RP11-14D22.2 CHST11 RALY-AS1 CTD-2269F5.1 NCDN  
 PPFIA3 AL356020.1 TRIP12 ERO1L KDM2A SAPCD2 TFAP2B NEBL RP11-758P17.3 LYSMD2 RP11-610P16.1 IPO13 OSR2 Sep-01  
 STAT5A SF3A3 NDNF FBXO36 ZFH4-AS1 ATP10A PHACTR1 KLF4 ARHGEF16 SFRP2 STRIP1 GGN KCNK10 HOXB5 AC090616.2  
 PIEZO2 GRWD1 CYHR1 PDSS1 SMG8 TMEM79 LINC00577 RIMBP2 Sep-05 AC005789.9 UBALD1 TMEM189 ZNF518B TSHZ1  
 LRRC16B LRR1 MIR17HG RPS29 KIAA0319L TALDO1 CTNNB1 EME2 ATP6V1A AC093901.1 TPGS1 DNMT3A INCA1 NCK2 TRPM2  
 CTD-2517M22.16 ZNF565 GLOD4 DVL1 PRMT1 TMOD2 ITGB8 NPNT PCBD2 XYLT2 MFAP3L CEP68 SCARF2 ENOPH1 MFI2 EVX1  
 RNA5SP18 GPR124 MAPK8IP2 RUVBL2 EPN1 ZFAND6 VCP INTS12 RALB POU2F2 GFRA2 TSEN54 NCOA6 PPP4R1 TWSG1 UPF1  
 TRIM47 SLC25A30 PTDSS1 GRIN3A FMNL1 STK24 C9orf116 CTB-147N14.6 HDDC2 RHOQP1 EGR4 MAP3K14-AS1 FOXD2 SOGA1  
 PAM HAS3 EVX1-AS GOLGA7B SLC12A7 RP11-467D6.1 FLJ30679 C1orf229 RP11-647P12.1 CCR6 PLD2 TFAM PPP5D1 ENTPD6  
 SLC31A1 HDAC4 CTNNA1 CTB-158D10.3 PGF RP11-14D22.1 DPM1 GP1BB RP11-54O7.16 PTBP3 RNA5SP492 BLVRA CASC15  
 UNC5C CDK13 CAPN2 CTD-2006C1.2 ITPKB SLC22A31 TMEM50B FEZ1 DDIA5 EIF3J FAM47E-STBD1 PHF2 MCHR2 LRRC26 SEMA6D  
 MTHFD1L GPR113 NCL KCMF1 CTD-2330J20.2 BHLHE40 RNF111 SUS4 TMEM189-UBE2V1 FKBP15 RP11-651L5.2 MAP3K4  
 AC079779.4 LIN9 MAFB CYSRT1 TMEM14B MECOM RP11-428O18.6 RP11-350O14.18 DDHD1 ICA1 CTD-2020K17.4 SLC25A30-AS1  
 SYPL1 ELOVL1 CDCA2 CASKIN2 RP11-1398P2.1 BAG4 ZNF32 MTERF3 ZNF746 SPSB1 JMJD8 RP3-400B16.4 ELAVL1 HRC CFLAR  
 AC013460.1 RP11-81A22.5 WDR37 IMP3 AC062017.1 ZHX1 GDF1 ZNF789 RP11-739B23.1 RP11-348J12.5 SCMH1 IFNAR2 PITPNC1  
 GPX1 DOCK7 LIN28A AP000708.1 KCNJ11 RP11-230B22.1 THUMPD3-AS1 RP11-297D21.2 MGRN1 HAP1 RP11-428C19.4 SETD4  
 MAP2K3 RP11-480I12.10 RHOTB1 CHPF LPCAT1 RP3-400B16.1 LINC00404 GINS2 DCAF17 CCDC24 CERS4 CKAP4 ANKRD37  
 B3GNT5 AC005775.2 E2F8 PRCP RP11-1103G16.1 STK24-AS1 SLTM TRIO NUBP2 KIF7 AC013394.2 RP4-555D20.4 NRM RP11-  
 135A24.4 TMEM9B-AS1 LINC00866 NR2F1 FASN METTL8 EIF3J-AS1 ABCG1 AL391839.1 RP11-324I22.4 RNF208 RALY RP11-662M24.2  
 FAM150B FBLIM1 AC034243.1 SOCS2-AS1 CACHD1 TMEM223 RP11-649E7.5 AKIRIN2 DDAH1 RP11-73M18.2 WDR43 MTHFSD KIF5B  
 RP11-483H20.4 SND1 RP5-942I16.1 AP000688.11 SNRPA PRKD3 LRRC4 TSC2 AC007009.1 PNMAL2 CNN3 JAG1 MCHR2-AS1 SLC1A5  
 GET4 GRIK4 TP53INP2 ALX3 ALDH1A2 RP11-637O19.3 FAM208B CD58 LITAF TRIM37 TMEM165 CTD-2369P2.5 EGFL7 ATP5J2-

PTC1D1 NEK7 ARNTL2 CPA2 MAP4K1 PPM1M PDE9A STUB1 SIM1 NHP2 CDK6 HMX2 ZFH4 MOCS3 ANO1 AKTIP HTT-AS PLEKHG3  
 SENP6 RIF1 FHL2 PCAT6 NCAPG2 NAA50 RP3-428L16.2 CERS1 GPATCH4 PTK7 CSNK1G2 RAD52 B4GALT2 KIAA1147 HELZ2  
 CACUL1 HR TFAP2D ROBO1 TMEM9B RP11-727F15.12 DRAM1 SETD5 CTB-113I20.2 NOC2L CDK5R2 NDFIP2-AS1 NDFIP2 CD9  
 VDAC1 ASRGL1 SCGB3A1 AC007128.1 SEMA3B CXXC5 RP4-639F20.1 TFRC EPC1 CTA-293F17.1 RQCD1 RP11-399E6.1 KCTD9  
 PRNP TRIP13 LINC00237 RPL36AL TAF6L RP11-135A24.2 ECI2 USP37 PRICKLE2 NEBL-AS1 RP11-758P17.2 SPRED3 UBE2D1 MLK4  
 ZNF461 WDR47 PHLDB1 C16orf74 RP11-888D10.3 PRKCE MIR126 AC073343.13 NDUFB10 BRD9 GIT1 MSL2 DPF3 bP-21264C1.2  
 NIPSNAP3A EDIL3 CNM1 TSPAN15 SHISA6 TPM4 PPP4R1-AS1 INPP4B RP11-103J8.1 B3GALNT1 MGAT2 RAI1 C17orf104 RPL21  
 SYT6 ADAMTS1 CYP20A1 HTT BRI3 HNRNPDL RP11-167A14.2 GRIN2D GALNTL6 KIFC2 GRIN1 NAT16 ZKSCAN5 TUBB6 RHOB IDI1  
 WNT10A ASB6 Sep-11 ICAM1 SIX4 LHX6 RBM22 SHISA9 MICAL3 SMG5 DCP2 SRD5A3-AS1 GTDC1 PDZRN4 SH2B3 KIF1C NR2F1-  
 AS1 DUT RP11-522B15.3 MIR3621 FGFR1OP C19orf54 CTD-2510F5.6 SLC12A5 AC099850.1 MRPS2 RP13-16H11.8 SPSB3 TMEM198  
 BAI2 C18orf25 NEUROG1 SOCS2 INSR KLC1 MIR6872 HTRA2 PPFIA1 VSX2 SLC39A11 AUP1 LSM1 QSOX1 CDC34 FAM217B RNMTL1  
 SLC6A9 PTC1D1 CRTAC1 SCN8A PPP1R35 KLHL17 TMEM179B CITED4 PSMD7 EIF3K CPSF4 GPR156 MAK STC2 GAREM RP11-  
 7O11.3 AC009120.4 RP11-12M5.1 LA16c-313D11.9 ZHX1-C8orf76 CTD-3224K15.2 WNT5B AC007391.2 ZNF12 NMD3 MCF2L2 DNER  
 HMGA1 CTD-2231E14.2 KDM3A NRG1 SESN2 RP11-552F3.9 ITPRIP RP11-371M22.1 FAM160A1 RASSF1 BHLHE40-AS1 AC006077.3  
 N4BP3 AMZ1

Table S3: A summary of all pathways and the number of hypermethylated DMGs in each arm of the comparison (highlighted in red). The number in brackets next to the pathway name refers to Panther Pathway Analysis pathway ID.

UK10/ UG10	UK10/ UG10	UG10/ UG52	UG10/ UG52	UK52/ UG52	UK52/ UG52	UK10/ UK52	UK10/ UK52	Pathway	Type
2	5	1		1		3	2	B cell activation (P00010)	Immune Pathways
5	5		1	3	2	8	7	Inflammation mediated by chemokine and cytokine signalling pathway (P00031)	Immune Pathways
8	3		1		1	13	4	Integrin signalling pathway (P00034)	Immune Pathways
3	2				2	1	2	Interferon-gamma signalling pathway (P00035)	Immune Pathways
	1					3	2	Interleukin signalling pathway (P00036)	Immune Pathways
2							1	JAK/STAT signalling pathway (P00038)	Immune Pathways
1	2	1		1		3	3	T cell activation (P00053)	Immune Pathways
3	1	1	1	2		4	5	TGF-beta signalling pathway (P00052)	Immune Pathways
1							2	Toll receptor signalling pathway (P00054)	Immune Pathways
		1		1		1	1	FAS signalling pathway (P00020)	Immune Pathways
1	1			1	1	1	3	Blood coagulation (P00011)	Physiological Pathways
1								Circadian clock system (P00015)	Physiological Pathways
5	1		2	2		3	4	Cytoskeletal regulation by Rho GTPase (P00016)	Physiological Pathways

1					1		Hypoxia response via HIF activation (P00030)	Physiological Pathways
1	2	1			1	3	Oxidative stress response (P00046)	Physiological Pathways
1					1	2	Vasopressin synthesis (P04395)	Physiological Pathways
4	1		1	3	7	12	Angiogenesis (P00005)	Physiological Pathways
5			2		4	7	CCKR signalling map (P06959)	Physiological Pathways
3	5	2	3		4	6	PDGF signalling pathway (P00047)	Physiological Pathways
	2					2	Synaptic vesicle trafficking (P05734)	Physiological Pathways
2			1		2	2	VEGF signalling pathway (P00056)	Physiological Pathways
17	8	1	3	2	10	10	Gonadotropin-releasing hormone receptor pathway (P06664)	Physiological Pathways
2	1				2		Corticotropin releasing factor receptor signalling pathway (P04380)	Physiological Pathways
1			1		1		Endogenous cannabinoid signalling (P05730)	Physiological Pathways
2	1				1		Enkephalin release (P05913)	Physiological Pathways
2	2		1		1	4	Ionotropic glutamate receptor pathway (P00037)	Physiological Pathways
	2				2	3	Metabotropic glutamate receptor group I pathway (P00041)	Physiological Pathways
1	1		1		1		Metabotropic glutamate receptor group II pathway (P00040)	Physiological Pathways

1	2		1		1	4	Metabotropic glutamate receptor group III pathway (P00039)	Physiological Pathways
2			1		6	2	Oxytocin receptor-mediated signalling pathway (P04391)	Physiological Pathways
2			1		6	1	Thyrotropin-releasing hormone receptor signalling pathway (P04394)	Physiological Pathways
3	2		1		2	6	Alzheimer disease-amyloid secretase pathway (P00003)	Disease Pathways
3		1	5	3	3	8	Alzheimer disease-presenilin pathway (P00004)	Disease Pathways
6		1	1	1	6	10	Huntington disease (P00029)	Disease Pathways
1			1	1	4	3	Parkinson disease (P00049)	Disease Pathways
2	2		1	1	2	3	Apoptosis signalling pathway (P00006)	Cellular Pathways and Signalling
2	1		6	2	4	7	Cadherin signalling pathway (P00012)	Cellular Pathways and Signalling
					1	1	Cell cycle (P00013)	Cellular Pathways and Signalling
			1			3	Cholesterol biosynthesis (P00014)	Cellular Pathways and Signalling
		1	1	1	1		DNA replication (P00017)	Cellular Pathways and Signalling
4	1	1			6		Dopamine receptor-mediated signalling pathway (P05912)	Cellular Pathways and Signalling
4	4			1	8	7	EGF receptor signalling pathway (P00018)	Cellular Pathways and Signalling

3	1		1	1	1	2		GABA-B receptor II signalling (P05731)	Cellular Pathways and Signalling
			1			1	1	Glycolysis (P00024)	Cellular Pathways and Signalling
	1	1			1	1	1	Hedgehog signalling pathway (P00025)	Cellular Pathways and Signalling
5	4	1	1	3	2	9	2	Heterotrimeric G-protein signalling pathway-Gi alpha and Gs alpha mediated pathway (P00026)	Cellular Pathways and Signalling
2	2	1		2		7	3	Heterotrimeric G-protein signalling pathway-Gq alpha and Go alpha mediated pathway (P00027)	Cellular Pathways and Signalling
1						3		Heterotrimeric G-protein signalling pathway-rod outer segment phototransduction (P00028)	Cellular Pathways and Signalling
							1	mRNA splicing (P00058)	Cellular Pathways and Signalling
3	2		1			7	5	Muscarinic acetylcholine receptor 1 and 3 signalling pathway (P00042)	Cellular Pathways and Signalling
1	1		1			3		Muscarinic acetylcholine receptor 2 and 4 signalling pathway (P00043)	Cellular Pathways and Signalling
2					1	3	1	Notch signalling pathway (P00045)	Cellular Pathways and Signalling
1	2	2			1	1	3	p38 MAPK pathway (P05918)	Cellular Pathways and Signalling
1	1	1		1		3	1	p53 pathway (P00059)	Cellular Pathways and Signalling

						1	p53 pathway by glucose deprivation (P04397)	Cellular Pathways and Signalling	
2	1			1	2	2	p53 pathway feedback loops 2 (P04398)	Cellular Pathways and Signalling	
4	1				3	1	PI3 kinase pathway (P00048)	Cellular Pathways and Signalling	
1	1			1	4	3	Ubiquitin proteasome pathway (P00060)	Cellular Pathways and Signalling	
7	6		1	7	3	14	13	Wnt signalling pathway (P00057)	Cellular Pathways and Signalling
1					1	1	5HT1 type receptor-mediated signalling pathway (P04373)	Cellular Pathways and Signalling	
2					6	2	5HT2 type receptor-mediated signalling pathway (P04374)	Cellular Pathways and Signalling	
						1	5HT3 type receptor-mediated signalling pathway (P04375)	Cellular Pathways and Signalling	
2	1				1	1	5HT4 type receptor-mediated signalling pathway (P04376)	Cellular Pathways and Signalling	
					1		Adrenaline and noradrenaline biosynthesis (P00001)	Cellular Pathways and Signalling	
1					3	1	Alpha adrenergic receptor signalling pathway (P00002)	Cellular Pathways and Signalling	
1	1			1	2	1	Angiotensin II-stimulated signalling through G proteins and beta-arrestin (P05911)	Cellular Pathways and Signalling	
2	2	1	1	1		1	3	Axon guidance mediated by netrin (P00009)	Cellular Pathways and Signalling
2			1			3	1	Axon guidance mediated by semaphorins (P00007)	Cellular Pathways and Signalling



1	3	1	1	1	1	1	4	Axon guidance mediated by Slit/Robo (P00008)	Cellular Pathways and Signalling
3	1					2		Beta1 adrenergic receptor signalling pathway (P04377)	Cellular Pathways and Signalling
3	1					2		Beta2 adrenergic receptor signalling pathway (P04378)	Cellular Pathways and Signalling
2	1					1		Beta3 adrenergic receptor signalling pathway (P04379)	Cellular Pathways and Signalling
			1		2	1		De novo purine biosynthesis (P02738)	Cellular Pathways and Signalling
					1		1	De novo pyrimidine deoxyribonucleotide biosynthesis (P02739)	Cellular Pathways and Signalling
					1			De novo pyrimidine ribonucleotides biosynthesis (P02740)	Cellular Pathways and Signalling
5	3		1	1	1	5	2	Endothelin signalling pathway (P00019)	Cellular Pathways and Signalling
6	4	1			4	7	5	FGF signalling pathway (P00021)	Cellular Pathways and Signalling
	1	1					1	Gamma-aminobutyric acid synthesis (P04384)	Cellular Pathways and Signalling
							1	General transcription regulation (P00023)	Cellular Pathways and Signalling
2	1					5	1	Histamine H1 receptor-mediated signalling pathway (P04385)	Cellular Pathways and Signalling
2	1					1		Histamine H2 receptor-mediated signalling pathway (P04386)	Cellular Pathways and Signalling

1					2		2		Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade (P00032)	Cellular Pathways and Signalling				
1		2			1		2		Insulin/IGF pathway-protein kinase B signalling cascade (P00033)	Cellular Pathways and Signalling				
4					5				Nicotine pharmacodynamics pathway (P06587)	Cellular Pathways and Signalling				
1		2		2		2		3		Nicotinic acetylcholine receptor signalling pathway (P00044)	Cellular Pathways and Signalling			
2					1		1		Opioid prodynorphin pathway (P05916)	Cellular Pathways and Signalling				
1					1				Opioid proenkephalin pathway (P05915)	Cellular Pathways and Signalling				
1					1				Opioid proopiomelanocortin pathway (P05917)	Cellular Pathways and Signalling				
1		2		1		2		1		2	7	Ras Pathway (P04393)	Cellular Pathways and Signalling	
1		2			1		2		1				Transcription regulation by bZIP transcription factor (P00055)	Cellular Pathways and Signalling
1													Histidine biosynthesis (P02747)	Cellular Pathways and Signalling
1					1								MYO signalling pathway (P06215)	Cellular Pathways and Signalling
1													Salvage pyrimidine ribonucleotides (P02775)	Cellular Pathways and Signalling
							1		5-Hydroxytryptamine degradation (P04372)		Metabolic Pathways			

1		Acetate utilization (P02722)		Metabolic Pathways		
		2	Asparagine and aspartate biosynthesis (P02730)	Metabolic Pathways		
		1	N-acetylglucosamine metabolism (P02756)	Metabolic Pathways		
		1	1	Pentose phosphate pathway (P02762)	Metabolic Pathways	
2	1	1	Pyrimidine Metabolism (P02771)		Metabolic Pathways	
		1	Tetrahydrofolate biosynthesis (P02742)		Metabolic Pathways	
1	1	1	Vitamin D metabolism and pathway (P04396)		Metabolic Pathways	
		1	1	Arginine biosynthesis (P02728)		Metabolic Pathways
			1	Formyltetrahydroformate biosynthesis (P02743)		Metabolic Pathways
1	1			Fructose galactose metabolism (P02744)		Metabolic Pathways
1			1	Activin beta signalling pathway (P06210)		Other Pathways
1			1	ALP23B signalling pathway (P06209)		Other Pathways
1			1	BMP/activin signalling pathway-drosophila (P06211)		Other Pathways
1			1	DPP signalling pathway (P06213)		Other Pathways
1			1	DPP-SCW signalling pathway (P06212)		Other Pathways
1			1	GBB signalling pathway (P06214)		Other Pathways
1			1	SCW signalling pathway (P06216)		Other Pathways

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## 7. Discussion

### 7.1 Final Discussion and Summary

This PhD describes an international and longitudinal study that aimed to achieve a better understanding of the varying efficacy of the BCG vaccine. The immunogenicity of BCG in the UK and Uganda was investigated using multiple assays. I explored immune pathways that could explain immunogenicity profiles in both countries, as well as novel, innovative molecular approaches such as epigenetic profiling, that could drive the immune changes.

In the third chapter, a pilot study was conducted to investigate whether DNA methylation patterns differ between low and high BCG-responding South African infants. The aim was to test the feasibility of combining assays for DNA methylation and for measuring cellular immunity parameters, as well as to establish a DNA methylation data analysis pipeline to be used in future experiments. I found differential methylation of 318 genes between low and high respondents to the BCG vaccine, as measured by BCG-specific cytokine response. I discovered that KCNN4 and ERICH1 genes were differentially methylated between 8/10 cytokine groups. Both genes encode for proteins that mediate calcium influx into the cell and by doing so could directly affect the immune response to the BCG vaccine. After analysing differentially methylated genes, I found 67 pathways in which these genes were involved. Expectedly, I found pathways of the immune system in which genes were differentially methylated between low and high BCG responding infants and could directly affect the BCG-induced immune responses. Unexpectedly, I also discovered the differential methylation of genes that are part of pathways that could indirectly affect the BCG-induced immune responses. These pathways included calcium signalling or WNT pathways, both known to be important in T cell development<sup>1</sup> and proliferation<sup>2</sup>. One might, therefore, conclude that there is scope for DNA methylation to play a role in

the modulation of vaccine immunity. Further studies should investigate the methylation of highlighted pathways and genes and how they affect the overall vaccine-induced immune responses *in vitro*. The differences in DNA methylation of both immunity-related and genes of other pathways were small between low and high responding BCG infants. One could, therefore, conclude that DNA methylation is unlikely to be the only or main molecular mechanism to drive sustained differences of BCG induced immune responses. This study confirmed the robustness of immune and DNA methylation assays and allowed the establishment of a DNA methylation data analysis pipeline. Having observed different immune responses in South African infants, and established immune protocols, I investigated the immune profile of UK and Ugandan infants after BCG vaccination.

In the fourth chapter, I analysed the cytokine immune response to BCG vaccination in UK and Ugandan infants at 10 and 52 weeks post-BCG. I also examined immune parameters such as T cell phenotype to investigate the cellular source of cytokines produced. Surprisingly, no differences were found in TH1 or TH2 cytokines between UK and Uganda when measured by the Luminex assay. Our group has previously reported significant differences in BCG immune responses between Malawian, Gambian and UK infants<sup>3</sup>. We suggest that differences observed in these cohorts might have been accentuated by different timing of vaccination, different vaccine strains, and rural vs urban cohorts. In contrast, UK and Ugandan infants were both from urban settings, had the same time of BCG administration, and both groups received the BCG Danish strain.

Interestingly, I observed an inflammatory profile in Ugandan infant samples in response to the BCG vaccine, but also without stimulation, and when stimulated with the PHA antigen. Ugandan infants produced higher amounts of IL1 $\beta$  and TNF $\alpha$  at 10 and 52 weeks post BCG vaccination when measured by the Luminex assay. The

increased inflammatory profile could indicate underlying inflammation or higher responsiveness of the immune system to pathogens in Ugandan infants. Increased inflammatory cytokine production by macrophages and monocytes have been observed before in people of African descent<sup>4,5</sup>. Interestingly, whereas the direct recognition of PAMPS activates DCs into fully immunogenic cells, the recognition of inflammatory signals from epithelial, hematopoietic, and non-hematopoietic tissue activates DCs to non-immunogenic phenotype<sup>6</sup>. This non-immunogenic but mature DC phenotype has been shown to induce the clonal expansion of CD4 T cells but failed to produce IL12 and subsequently resulted in impaired TH differentiation<sup>6,7</sup>. This has important implications for BCG immunogenicity and efficacy as the fully functional TH1 response was shown to be important to elicit a sufficient BCG response and subsequent TB protection<sup>8</sup>. The analysis of dendritic cell phenotypes did not directly compare UK and Ugandan infants and did not measure the capacity of DC-mediated TH differentiation. However, a small increase was found in a frequency of IFN $\gamma$  or IL2 producing CD4 or CD8 T cells at 52 weeks in the UK. The difference at 10 weeks was not significant, although the sample size was much smaller. A slight increase in the frequency of IL4/5/13 producing CD8 T cells at 52 weeks in Uganda was also reported. The observed differences in frequencies of cytokine-producing T cells somehow reflect the immune profile of Malawian and Gambian infants as they also produced lower levels of IFN $\gamma$ , IL2 and higher levels of IL4, IL5, and IL13 cytokines. However, these differences were very small and diminished when the overall whole blood immune response was measured with the Luminex assay. It is therefore plausible that high inflammatory signals such as TNF $\alpha$  and IL1 $\beta$  derived from epithelial, hematopoietic (including monocytes and macrophages) cells, and non-hematopoietic tissue could activate DCs into a mature but non-immunogenic phenotype that induces clonal T cell expansion but impairs TH1 and favours TH2 differentiation. Interestingly, the increased levels of TNF $\alpha$  and IL1 $\beta$  could be indirectly

mediated by DNA methylation as observed in chapter six. I reported a higher number of hypermethylated genes in the UK than Uganda that regulate integrin-mediated processes. Integrins play a strategic role in the recruitment of leukocytes to sites of inflammation<sup>9</sup>. Thus, higher DNA methylation could lead to reduced integrin-mediated leukocyte recruitment and lower levels of proinflammatory cytokines as observed in the UK. To complement this, I examined the differential DNA methylation between BCG-specific low and high TNF $\alpha$  producers in South African infants in chapter three. I have found 146 differentially methylated regions and 128 genes between low and high TNF $\alpha$  producers. One of the genes, ITGAX, had lower DNA methylation in high TNF $\alpha$  producers. ITGAX encodes the X chain of the integrin protein (CD11b)<sup>10</sup>, which regulates leukocyte adhesion and subsequently affects leukocyte-mediated inflammatory responses<sup>11</sup>. Thus, low methylation of ITGAX could result in higher levels of CD11b, increased leukocyte recruitment and elevated levels of proinflammatory cytokines, including TNF $\alpha$ , as observed in South African and Ugandan infants. Taken together, I propose that low DNA methylation of genes that are associated with TNF $\alpha$  production, such as ITGAX, could increase levels of CD11b. The increased levels of CD11b would drive leukocyte recruitment and increase the production of proinflammatory cytokines as observed in Uganda. Inflammatory signals could induce mature but non-immunogenic profile of DCs, leading to impaired TH1 differentiation, with important implications for BCG immunogenicity. This cascade of events should be further explored with *in vitro* models as well by using novel technologies that allow for a precise edition of DNA methylation such as CRISPR and Cas9<sup>12</sup>.

Lastly, I found that at 52 weeks there is a higher frequency of naïve CD4 or CD8 T cells in the UK, whereas, in Uganda, CD4 and CD8 T cells have an increased terminally differentiated phenotype. Naïve T cells differentiate into their mature

phenotype after antigen exposure in the periphery<sup>13</sup>, and this process is mediated by epigenetic mechanisms<sup>13–15</sup>. One would expect for Ugandan infants to have a higher exposure to environmental mycobacterial and increased antigen exposure, and these factors could drive the differentiation of T cells from their naïve to mature and terminally differentiated phenotypes. Interestingly, when I compared DNA methylation between UK and Ugandan infants in chapter six, I found that UK infants had higher methylation of JAK1 and JAK2 genes. JAK proteins play a central role in cell growth, survival, development and differentiation of immune cells. There is growing evidence that JAK1 and JAK2 are essential for an effective function of CD4 T cells, as JAK1/2 inhibition impairs T cell functions in vitro<sup>16,17</sup>, and JAK proteins are vital for the expansion of CD4 T cells<sup>18</sup> and their differentiation from the naïve phenotype<sup>19</sup>. Thus, it could be plausible that higher methylation of JAK1 and JAK2 proteins in the UK results in their lower levels and delayed or impaired differentiation of naïve T cells to their memory and terminally differentiated phenotypes, as observed in the UK. However, this novel pathway of T cell differentiation should be investigated further.

I was not able to directly compare immune responses between UK, Ugandan and the South African infant cohort described in chapter three. South African samples were stimulated with BCG for 12 hours (including 5 hours with Brefeldin A), whereas UK and Ugandan samples were stimulated with PPD for 24 hours (including 24 hours with Brefeldin A). I conclude that the immune response to the BCG vaccine between UK and Uganda is similar, and perhaps this can be explained by the fact that both cohorts of infants live in urban areas, although this needs further investigation. I highlight the need to conduct an international trial to examine the immunogenicity of the BCG vaccine, spanning more than two countries, and including infants from both rural and urban backgrounds while controlling for BCG timeliness and strain administered.

Having investigated the immune response to the BCG vaccine in Uganda and the UK, I examined immune pathways that may contribute to BCG immunogenicity. In chapter four I explored T cell phenotypes, and in chapter five I investigated the phenotypes of dendritic cells in Uganda. The Ugandan study collected samples at multiple time points throughout the first year of life, including cord blood. This gave us an excellent opportunity to investigate the dynamics of dendritic cell phenotype in the first year of life. I employed state-of-the-art techniques for flow cytometry data analysis such as viSNE and SPADE. Surprisingly, I found that observed dendritic cell phenotypes are absent in blood at birth but appear within the first month of life. Previous studies have observed decreased frequencies of DCs in blood at birth but our study is the first longitudinal and comprehensive analysis to observe dynamic changes in DC phenotypes in the first year of life of African infants. The appearance of DCs in the first month of life could be due to a natural development of the immune system and differentiation of hematopoietic stem cells to dendritic cell progenitors and dendritic cells. However, this appearance could also be accentuated by the exposure to multiple environmental pathogens in the first month of life; or repeated vaccinations, including BCG. It would be beneficial to conduct a similar study that compares the phenotype of dendritic cells in infants with the standard vaccination schedule and with a delayed vaccination scheduled within the first month of life. This would inform us of the contribution that vaccines have on the development of DCs phenotypes. The finding that known DCs are mostly absent at birth has significant implications for vaccine efficacy. Although except for polysaccharide vaccines<sup>20</sup>, there is no evidence that vaccines given at birth have a limited efficacy<sup>21</sup>. For logistical reasons, infants' samples were not collected in the early weeks of life in the UK, therefore, a comparison between countries cannot be made.

Interestingly, I found that maternal latent TB infection is associated with an increase in the frequency of cDC1, cDC2 and MDDC populations in infants. The increased

number of DC populations may be detrimental to BCG efficacy as it has been shown that elevated DC numbers impair protective immunity to intracellular bacteria<sup>22</sup>. The exposure to TB antigens *in utero* could also mask the subsequent BCG mediated immune response and result in a lower production of IFN $\gamma$  and TNF $\alpha$  as observed previously<sup>23</sup>. Besides, I have found that infant EBV infection decreases numbers of known DC phenotypes, including plasmacytoid DCs, which mediate viral induced immunity by producing type I IFNs. Thus, an EBV driven decrease in DC populations, specifically pDCs, might be one of the mechanisms to regulate host-mediated immune responses against EBV. Surprisingly, I found limited evidence of the effect of infant CMV infection on the phenotype of dendritic cells. I have observed a moderate delay in the development of crucial DC populations but no changes in marker expression in CMV infected infants. I conclude that maternal LTBI, infant EBV and to a lesser extent CMV affect the phenotype of dendritic cells. I highlight the importance of the development of vaccines or new treatments for these pathogens and also urge to include these pathogens and infections in clinical efficacy trials of new vaccines. I was not able to analyse the dendritic cell profile in South Africa, as I was limited by sample availability. I examined the dendritic cell phenotype in a limited subset of UK infants, however, when I compared findings from both countries I discovered technical issues that prevented me from a direct comparison of UK and Ugandan DC phenotypes. I also realised that the isolated analysis of UK infants was not sufficient to complement an understanding of the dendritic cell story.

Lastly, in chapter six I continued the investigation of DNA methylation between UK and Ugandan infants at 10 and 52 weeks of life. I observed dynamic differences in DNA methylation between the UK and Uganda. Interestingly, at 10 weeks, genes from the B cell activation pathway as well as vital immune genes *NFATC1* and *SOCS5* have higher methylation in Uganda than in the UK. In contrast, in the UK, integrin



pathways, *JAK1* and *JAK2*, as well as TGF $\beta$  signalling pathways were more methylated at 10 weeks of age. These findings suggest that B cell activation might be impaired in Ugandan infants when compared to the UK. *NFATC1* is a ubiquitous immune modulator, and its increased methylation might indirectly affect numerous immune pathways including T cell activation. Lastly, increased methylation of TGF $\beta$  signalling pathways could indicate that TGF $\beta$  regulation of immune responses in the UK is limited and other regulatory mechanisms such as IL10 of Tregs might be more important. Interestingly, I did not find any differences in DNA methylation patterns for cytokine genes, reflecting the Luminex findings for most cytokines, as observed in chapter four. However, I report that DNA methylation patterns do not mirror the proinflammatory profile of the BCG vaccine in Ugandan infants. As found in chapter four, Ugandan infants produced more IL1 $\beta$  and TNF $\alpha$  at 10 and 52 weeks post-BCG vaccination, however, the DNA methylation of these genes was not decreased in Uganda, nor increased in the UK (an increase in DNA methylation of a gene would limit the protein transcription of that gene). These findings indicate that DNA methylation plays a moderate role in the regulation of immune responses, although well-controlled, *in vitro* studies are needed to confirm this. Highlighted genes and pathways and what effect they have on the overall vaccine response should be examined. Alternatively and similarly to chapter three, I could stratify immune responses to low and high vaccine responders and compare the DNA methylation between two groups and whether these findings support findings from chapter three. I decided however, not to proceed with this comparison as the racial background of samples differs; the origin of samples is different (PBMCs in South Africa and whole blood in the UK and Uganda); and samples were stimulated according to different protocols. This analysis could be conducted in future.

## 7.2 Conclusions

This study was conducted to investigate the spectrum of immune responses to the BCG vaccine in the UK and Uganda and examine the molecular mechanisms that drive them. To address that I analysed BCG immune responses and DNA methylation of samples from South Africa, Uganda and the UK. I hypothesised that that DNA methylation patterns or dendritic cells phenotypes and composition are underlying mechanisms that can explain the spectrum of BCG immunogenicity and in this study, are associated with the immune response to the BCG vaccine. I found that the contribution of DNA methylation to the overall BCG response is modest, as limited number of immune pathways was associated with the immune response to the BCG vaccine. In addition I found that phenotypes of dendritic cells change change over time. Specifically, known DC phenotypes are missing at birth and appear within the first month of life, and the relationship between marker expression and age is dynamic. In summary, DNA methylation is an unlikely mechanisms to largely influence the immunogenicity of BCG and further studies need to be conducted to measure the impact of DC development dynamics on BCG immune responses.

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## 8. Appendices

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LONDON  
SCHOOL of  
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#### Observational / Interventions Research Ethics Committee

LSHTM

11 May 2015

Dear

**Study Title:** The impact of maternal infection with *Mycobacterium tuberculosis* on the infant response to BCG immunisation

**LSHTM Ethics Ref:** 8720

Thank you for responding to the Observational Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

#### Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

#### Conditions of the favourable opinion

Approval is dependent on local ethical approval having been received, where relevant.

#### Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document Type	File Name	Date	Version
Covering Letter	response letter ethics	17/04/2015	1
Protocol / Proposal	UgUK BCG study Protocol ver2	17/04/2015	2
Information Sheet	UgUK BCG study Consent form ver3_150415_1	17/04/2015	3

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**Observational / Interventions Research Ethics Committee**

Mr Mateusz Agopsowicz  
LSHTM

**18 February 2016**

Dear Mateusz,

**Study Title:** The impact of maternal infection with *Mycobacterium tuberculosis* on the infant response to BCG immunisation

**LSHTM Ethics Ref:** '8720 - 1'

Thank you for your letter responding to the Observational Committee's request for further information on the above amendment to research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

**Confirmation of ethical opinion**

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above amendment to research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

**Conditions of the favourable opinion**

Approval is dependent on local ethical approval for the amendment having been received, where relevant.

**Approved documents**

The final list of documents reviewed and approved by the Committee is as follows:

Document Type	File Name	Date	Version
Other	S088-04 LSR II LSR Fortessa flowcytometers Intracellular cytokine staining and assay[1]	12/10/2015	1
Other	S093-05 Generic 12hr Whole blood Intracellular Cytokine AssayUNC[1]	12/10/2015	1
Other	CC & nested study approvals	12/10/2015	1
Other	CCS uctrec initial appr Mar01	12/10/2015	1
Covering Letter	Answer letter to ethical committee_2	27/11/2015	1
Covering Letter	Answer letter to ethical committee_3	16/02/2016	3
Other	UgUK BCG study Protocol ver3	16/02/2016	3
Other	SKMBT_28316020309470 (1)	16/02/2016	1
Other	UCT LSHTM MTA Draft v3 8 February 2016	16/02/2016	1

**After ethical review**

The Chief Investigator (CI) or delegate is responsible for informing the ethics committee of any subsequent changes to the application. These must be submitted to the Committee for review using an Amendment form. Amendments must not be initiated before receipt of written favourable opinion from the committee.

The CI or delegate is also required to notify the ethics committee of any protocol violations and/or Suspected Unexpected Serious Adverse Reactions (SUSARs) which occur during the project by submitting a SUSAR/protocol violation form.

At the end of the study, the CI or delegate must notify the committee using an End of Study form.

All aforementioned forms are available on the ethics online applications website and can only be submitted to the committee via the website at: <http://leo.lshtm.ac.uk>

Additional information is available at: [www.lshtm.ac.uk/ethics](http://www.lshtm.ac.uk/ethics)

Yours sincerely,



27 February 2015

Professor Hazel M Dockrell  
Professor of Immunology  
London School of Hygiene and Tropical Medicine  
Keppel Street, London  
WC1E 7HT

Dear Professor Dockrell

<b>Study title:</b>	<b>The impact of maternal infection with <i>Mycobacterium tuberculosis</i> on the infant response to BCG immunisation</b>
<b>REC reference:</b>	<b>15/LO/0048</b>
<b>IRAS project ID:</b>	<b>162186</b>

Thank you for your letter of 25 February 2015, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact the REC Manager, Hayley Henderson, [nrescommittee.london-camdenandislington@nhs.net](mailto:nrescommittee.london-camdenandislington@nhs.net). Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

**Confirmation of ethical opinion – Favourable Opinion**

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation

A Research Ethics Committee established by the Health Research Authority





## Uganda National Council for Science and Technology

(Established by Act of Parliament of the Republic of Uganda)

17/02/2014

Our Ref: IIS 1526

Dr. Stephen Cose  
MRC/Uganda Virus Research Institute on AIDS  
Uganda Virus Research Institute  
Entebbe

**Re: Research Approval:** The impact of maternal infection with *Mycobacterium tuberculosis* on the infant response to BCG Immunization

I am pleased to inform you that 20/01/2014, the Uganda National Council for Science and Technology (UNCST) approved the above referenced research project. The Approval of the research project is for the period of 20/01/2014 to 20/01/2017.

Your research registration number with the UNCST is HS 1526. Please, cite this number in all your future correspondences with UNCST in respect of the above research project.

As Principal Investigator of the research project, you are responsible for fulfilling the following requirements of approval:

1. All co-investigators must be kept informed of the status of the research.
2. Changes, amendments, and addenda to the research protocol or the consent form (where applicable) must be submitted to the designated local Institutional Review Committee (IRC) or Lead Agency for re-review and approval prior to the activation of the changes. UNCST must be notified of the approved changes within five working days.
3. For clinical trials, all serious adverse events must be reported promptly to the designated local IRC for review with copies to the National Drug Authority.
4. Unanticipated problems involving risks to research subjects/participants or other must be reported promptly to the UNCST. New information that becomes available which could change the risk/benefit ratio must be submitted promptly for UNCST review.
5. Only approved study procedures are to be implemented. The UNCST may conduct impromptu audits of all study records.
6. A progress report must be submitted electronically to UNCST within four weeks after every 12 months. Failure to do so may result in termination of the research project.

Below is a list of documents approved with this application:

	Document Title	Language	Version	Version Date
1	Research proposal	English	1.1	31 July 2013
2	Consent Form	English, Luganda	1.1	6 Jan 2014
3	Consent for Storage	English, Luganda	1.1	6 Jan 2014

Yours sincerely,

Leah Nawegulo Omongo  
for: Executive Secretary  
UGANDA NATIONAL COUNCIL FOR SCIENCE AND TECHNOLOGY

cc Chair, Uganda Virus Research Institute SEC, Entebbe

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Our Ref: GC/127/15/02/432

Your Ref:

09<sup>th</sup> March 2015

Drs. Steven Cose & Sarah Prentice,

RE: UVRI REC review of protocol amendment titled **"The impact of maternal infection with Mycobacterium on the infant response to BCG immunization"**

Thank you for submitting the above study amendment dated 06<sup>th</sup> March 2015 to the UVRI Research Ethics Committee (REC).

This is to inform you that the amendment to the above named protocol was reviewed and met the requirements of the UVRI Research Ethics Committee. UVRI REC approval has been given for you to continue with the proposed amendment.

The reviewed and approved amendments are;

1. Use of gene and gene expression profiling to determined gene and gene expression profiles in freshly collected placental samples.
2. Increasing recruitment area by deleting the requirement for mothers to reside in Entebbe Municipality or Katabi sub-county.

You can continue with your study and remember to notify Uganda National Council for Science and Technology (UNCST).

Yours sincerely,



Mr. Tom Lutalo

**Chair, UVRI REC**

C.C. Secretary, UVRI REC