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Determinants of Innate Immune Responses to Mycobacteria

Thesis Presented for the Degree of

Doctor of Philosophy

by

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Abstract

Innate cells such as macrophages, monocytes, myeloid dendritic cells and granulocytes recognise mycobacteria and initiate immune responses such as phagocytosis, cytokine production and expression of maturation markers. The type and magnitude of innate responses to mycobacteria may determine the subsequent adaptive responses generated. Our aims were to determine maturational changes in innate immune responses to mycobacteria over the first 9 months of life, and to assess effects of genetic variations in toll-like receptors on host responses to mycobacteria. This knowledge is important for designing rational strategies for vaccination against tuberculosis.

We collected blood from newborns, 10- and 36-week old infants, and adults. We stimulated blood with mycobacteria for 6 hours and determined expression of intracellular cytokines, or for 18-20 hours and determined expression of maturation markers and secretion of cytokines in supernatants. Different innate cell subsets were identified through expression of specific surface markers expressed on these cells. The toll-like receptor 6 coding region was sequenced to identify polymorphisms occurring in this gene in our local population, and associate these with host responses to mycobacteria.

We showed that pro-inflammatory cytokine production and co-stimulatory molecule expression increased over the first 9 months of life, while anti-inflammatory cytokine production did not change. We described a novel toll-like receptor 6 polymorphism, and demonstrated an association between toll-like receptor 6 variations and responsiveness to mycobacteria.

These findings suggest that immaturity of innate immune cells may result in sub-optimal immune responses to vaccination against tuberculosis at birth. These results also suggest that genetic variations between individuals should be considered when designing vaccination strategies to control tuberculosis.

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Publications

The work directly related to this thesis has been published in the following manuscripts and attached as appendices.

1. **Shey MS**, Randhawa AK, Bowmaker M, Smith E, Scriba TJ, de Kock M, Mahomed H, Hussey G, Hawn TR, and Hanekom WA. Single nucleotide polymorphisms in toll-like receptor 6 are associated with altered lipopeptide-induced interleukin (IL) 6 secretion. *Genes and Immunity* 2010; 11(7): 561-72.
2. **Shey MS**, Hughes EJ, de Kock M, Barnard C, Stone L, Kollmann TR, Hanekom, Scriba TJ. Optimization of a whole blood intracellular cytokine assay for measuring innate cell responses to mycobacteria. *Journal of Immunological Methods* 2011 Dec 1. Doi:10.1016/j.jim.2011.11.011.

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List of abbreviations

%	Percent
°C	Degrees centigrades
APCs	Antigen presenting cells
APC	Allophycocyanin
BCG	Bacille Calmete-Guerin
BSA	Bovine serum albumin
CD	Cluster of differentiation
CCL	Chemokine ligand
CCR	C-C chemokine receptor
CFP-10	Culture filtrate protein-10
CFUs	Colony forming units
CO ₂	Carbon dioxide
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
CXCL	CXC chemokine ligand
DCs	Dendritic cells
DC-SIGN	Dendritic Cells-Specific Intracellular adhesion molecule-3-Grabbing Non-integrin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribunucleic acid
DOTS	Directly observed treatment strategy
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EPI	Expanded programme on immunisation
ESAT-6	Early secretory antigenic target 6
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FMO	Fluorescence-minus-one
FSC-A	Forward scatter area
FSC-H	Forward scatter height
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
ICS	Intracellular staining
IFN- γ	Interferon gamma
IL	Interleukin
IGRA	Interferon gamma release assay
kDa	Kilodalton
LTBI	Latent tuberculosis infection
log	logarithm
LPS	Lipopolysaccharide
<i>M. bovis</i>	<i>Mycobacterium bovis</i>
MDR	Multidrug resistant

MHC I	Major histocompatibility complex class I
MHC II	Major histocompatibility complex class II
mL	Milliliter
mRNA	Messenger ribonucleic acid
<i>M.tb</i>	<i>Mycobacterium tuberculosis</i>
MTCT	Mother to child transmission
NK	Natural killer
NO	Nitric oxide
NTM	Non-tuberculosis mycobacteria
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programme death-1
PE	Phycoerythrin
PE-Cy7	Cyanine-7-phycoerythrin
PerCP-Cy5.5	Cyanine-5.5-peridin-chlorophyll
PFC	Polychromatic flow cytometry
PHA	Phytohaemagglutinin
PRR	Pattern recognition receptor
Qdot	Quantum dot
RNI	Reactive nitrogen intermediate
ROI	Reactive oxygen intermediate
SATVI	South African Tuberculosis Vaccine Initiative
SN	Signal to noise
SNP	Single nucleotide polymorphism
SSC	Side scatter
TAP	Transporter associate with antigen processing
TB	Tuberculosis
TCR	T cell receptor
Th	T helper
TNF- α	Tumour necrosis factor alpha
Treg	Regulatory T cell
TST	Tuberculin skin test
UNS	unstimulated
WHO	World health organisation
$\gamma\delta$	Gamma delta
μ L	microliter

Objectives of this thesis

1. To optimise stimulation and cell processing conditions to reliably measure monocyte, dendritic cell (DC) and granulocyte responses in whole blood by flow cytometry (**Chapter 2**).
2. To develop flow cytometry antibody panels to identify monocyte, dendritic cell and granulocyte subsets and measure: (i) expression of intracellular cytokines, and (ii) maturation markers (**Chapter 2**).
3. To investigate maturational changes (intracellular cytokine and maturation marker expression) in monocyte, DC and granulocyte responses to mycobacteria over the first 9 months of life (**Chapter 3**).
4. To identify toll-like receptor 6 (TLR6) single nucleotide polymorphisms (SNPs) occurring in a Cape Town population (**Chapter 4**), and to determine the role of TLR6 SNPs in innate immune responses to mycobacteria (**Chapter 4**).

CHAPTER 1: General introduction and literature review

In this chapter, we will discuss the general components of the immune system including the innate and adaptive arms and how these arms independently or in combination, help in immune control of mycobacterial infection.

1 The immune system

The immune system is comprised of innate and adaptive immune arms (1). The innate arm acts as the first line of defense against infectious agents, and has anatomical features that function as barriers. The adaptive arm acts as a second line of defense and may afford protection following re-exposure to the same antigen. Although each arm of the immune system has distinct functions, both arms are integrated into a complex network that together provides optimal immunity to infection (2).

Different components of the immune system have developed to control invading pathogens that replicate intracellularly or extracellularly. If not controlled, infection with a pathogen may lead to disease. Disease occurs either when the immune system is overwhelmed by the pathogen dose or when the interplay between the innate and the adaptive immunity is compromised. Infection also leads to inflammation. Inflammation is the (patho)physiologic condition that is induced by various infectious and non-infectious agents, which entails recognition of pathogen and recruitment of immune cells (2). The outcomes of inflammation can be resolution without damage, tissue remodeling or immunopathology (2). In addition, in some cases the immune response may be directed toward self-tissues resulting in autoimmune disease.

In this thesis, we focus on the innate immune responses. Other projects in our laboratory have already been investigating adaptive immune responses to mycobacteria.

1.1 Innate immunity

The innate immune arm is made of several cell subsets that can provide immediate protection against infection (3). These cell subsets include monocytes, macrophages, DCs, granulocytes and natural killer (NK) cells. Each cell type is equipped with different functions that allow the cell to recognise and respond to pathogens in the body. These cells can eradicate the pathogen before establishment of infection and the development of an adaptive immune response (1, 4). Innate cells recognise pathogens through a variety of different receptors expressed either on their surface or intracellularly. These receptors, known as pathogen recognition receptors (PRRs, discussed in detail under receptors of the innate immune arm in **section 1.3**) bind to pathogen-associated molecular patterns (PAMPs), found on pathogens (4, 5) (discussed in detail below). These receptors can also bind to damage-associated molecular patterns (DAMPs). DAMPs, also known as alarmins, are danger signals that result from necrotic, stressed or sometime apoptotic cells (6). Examples of DAMPs include heat shock proteins (Hsp), uric acid, galectins, adenosines, high mobility group box protein 1 (HMGB1), interleukin 1 alpha (IL-1 α) and IL-33 (7). The binding of PRRs to DAMPs or PAMPs can enable the innate immune system to recognise these as non-self antigens and generate an immune response (5, 6, 8). The innate immune arm is not antigen-specific as innate cells can bind to a variety of pathogens. Innate myeloid cells typically do not demonstrate immunological memory in vertebrates (9)

In this thesis, we focus on identification of innate cell subsets, and the roles of these cells in recognition of mycobacteria, cytokine production and expression of maturation markers.

1.2 Innate cell subsets

In this section we describe three major innate cell subsets found in blood: monocytes, DCs and granulocytes. We also discuss, in less detail, other subsets that may not be found in blood but have major innate function, such as macrophages and NK cells. In the subsequent chapters, we describe the role of monocytes, DCs and granulocytes as these cells are more frequent in blood and can be detected in small volumes of blood.

1.2.1 Monocytes

Monocytes perform multiple functions including phagocytosis of pathogens and production of effector molecules. Monocytes in human blood have been subdivided into three subsets: the classical monocytes characterised by high level expression of the CD14 and low expression of CD16 ($CD14^{++}CD16^{-}$), non-classical monocytes with low level expression of CD14 and CD16 ($CD14^{+}CD16^{+}$), and intermediate monocytes, characterised by low expression of CD14 and high expression of CD16 ($CD14^{+}CD16^{++}$) (10, 11). Other studies describe two types of monocytes only: $CD14^{hi}CD16^{-}$ and $CD14^{lo}CD16^{+}$ (12, 13).

$CD14^{hi}CD16^{-}$ monocytes predominate in the steady state, and make up 80-90% of peripheral blood monocytes while the $CD14^{lo}CD16^{+}$ monocytes increase during inflammation and disease (12, 13). These subsets differentially express cell surface receptors for recognition and uptake of pathogen as well as migration to the sites of infection (12). Upon stimulation, both $CD14^{+}CD16^{+}$ and $CD14^{+}CD16^{lo}$ monocytes produce high amounts of cytokines such as $TNF-\alpha$, IL-12, IL-6 and IL-10 (12, 13). In this thesis we focus on $CD14^{hi}$ monocytes that are the most frequent in blood.

1.2.2 Dendritic cells

DCs are relatively rare cell subsets in blood, comprising less than 1% of peripheral blood leukocytes. The major function of DCs is to process pathogens and present antigenic material to T cells, thus functioning as professional antigen presenting cells (APCs) (14). DCs therefore act as a link between innate and adaptive immunity (discussed under antigen presentation in **section 1.8**). DC function may result in immunity or in tolerance (15-17), as demonstrated in **Figure 1**.

Immature DCs (iDCs) are efficient phagocytes of pathogens (18). Once DCs recognise a pathogen there is upregulation of cell-surface markers involved in migration to lymph nodes and co-stimulation of T and B cells such as CCR7, CD80, CD86, and CD40. Previous studies have shown that once activated, mature DCs shut down their antigen capture mechanisms, through reorganisation of the endocytic compartments, and become more adapted to antigen presentation (19-21). However, recent data from mice suggests that mature DCs may continue to capture and present antigen (22). DCs can

present antigens on both MHC II and I and through non-MHC mechanisms (see **section 1.8**).

DCs are a heterogenous population of cells that cannot be identified with a single lineage marker (16). There are two major subsets of dendritic cells in human blood: myeloid DCs and plasmacytoid DCs. The other major types of DCs include epidermal Langerhans cells and dermal DCs (not described in this thesis).

1.2.2.1 Myeloid dendritic cells

Myeloid DCs (mDC) are also referred to as conventional or classical DCs. These cells are usually identified by high expression of CD11c and low expression of CD123. mDC express a wide range of PRRs for recognition and phagocytosis of pathogens. Upon infection, mDC produce a series of cytokines and chemokines including IL-12, TNF- α , IL-6, IL-1 β and IL-10.

Two major subsets of mDC have been identified. These include mDC1, which express CD11c and CD1c (BDCA-1), and mDC2 that express CD11c and CD141 (BDCA-3). The CD11c⁺CD141⁺ mDC2 are known as the human functional equivalent of mouse CD8 α DCs and are more efficient at capturing exogenous antigens for cross presentation on MHC class I (18). Regardless, CD11⁺CD1⁺ mDC1 can also cross present antigen and secrete cytokine such as IL-12 (18).

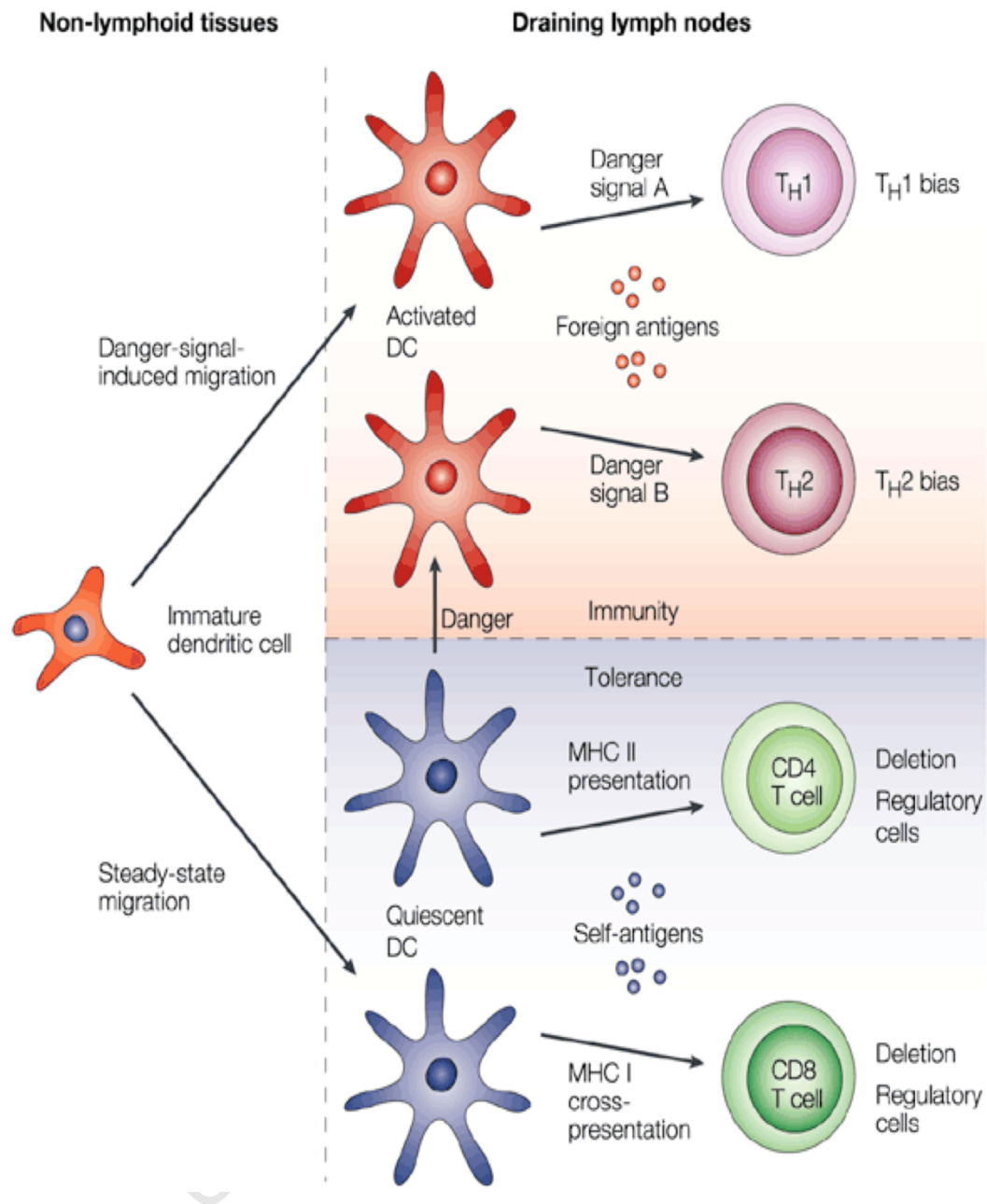


Figure 1: Dendritic cell activation and role in immunity and tolerance. DCs are activated in non-lymphoid tissues and after maturation, and these cells migrate into draining lymph nodes and present antigens to naïve T cells and shape the developing adaptive immune response. In the absence of microbial infections and or 'danger' signals, there is a low-level, steady-state entry of DCs into lymphoid tissues, in which quiescent DCs help to maintain a state of peripheral T-cell tolerance to self-antigens. Microbial infection, inflammation and tissue damage all activate the DCs and increase their rate of migration into lymphoid tissue, where these cells signal to T cells that are specific for the foreign antigens that are presented by DC to initiate immune responses. MHC, major histocompatibility complex; T_H1, T helper 1 cell; T_H2, T helper 2 cells (adapted from Shortman and Liu, 2002)(17).

1.2.2.2 *Plasmacytoid dendritic cells*

Plasmacytoid DCs (pDC) express high levels of CD123 and low levels of CD11c. These can also be identified by expression of CD303 or BDCA-2 (23). pDC play a major role in immunity to viral infections and are also known as interferon-producing cells (IPC). Key features of pDC that make these cells efficient antiviral agents include: the presence of an early endosomal compartment containing MHC class I molecules for antigen presentation and activation of CD8⁺ T cells; late endosomal compartment that contains MHC class II molecules for viral antigen presentation to CD4⁺ T cells; and an extensive ER compartment that facilitates high-capacity secretion of antiviral factors, including type I interferons (18, 24). The secretion of type I interferons and IL-6, and the expression of CD70 upon stimulation with CpG make pDC efficient activators of plasma B cells and antibody responses (25, 26)

In this thesis, we focus on mDC, as pDC did not respond to mycobacterial stimulation as described in **Chapter 2**.

1.2.3 *Granulocytes*

Granulocytes comprise three major cell subsets: neutrophils, basophils and eosinophils. Neutrophils are professional phagocytes and are the most frequent white blood cells in blood, and are rapidly recruited to sites of infection as part of the inflammatory response (27). Neutrophils are able to recognise, ingest and destroy pathogens without help from the adaptive immune system. Upon infection, neutrophils produce cytokines and chemokines including TNF- α , IL-12, IL-6 and IL-1 β as well as chemokines such as IL-8 (27, 28). Neutrophils undergo spontaneous apoptosis after interaction with pathogen releasing apoptotic blebs that may be engulfed by immature dendritic cells and macrophages (28-31). This facilitates processing and presentation of antigen contained in the apoptotic particles (**see section 1.8**), and leads to the induction of an adaptive immune response (32). When activated, neutrophils upregulate the expression of CD83 and MHC II (33, 34).

1.2.4 Macrophages

Although macrophages are not found in human peripheral blood, these cells play a major role in innate immunity. A major role of macrophages is phagocytosis. In the absence of infection or activation, macrophages are involved in elimination of apoptotic bodies and cell debris as part of normal housekeeping process (35) (**Fig. 2A**). Macrophages can also phagocytose pathogens. Upon recognition of pathogen or PAMPs through PRRs (**Fig. 2B**), macrophages express cytokines, costimulatory molecules and other immune proteins that regulate the immune system and can lead to the development of inflammation. These cells can also act as APCs presenting exogenously derived antigens to T cells and other cells of the immune system, although not as efficiently as DCs. The role of macrophages in anti-mycobacterial immunity is discussed further under immune responses to *Mycobacterium tuberculosis* (*M.tb*) in **section 2.4.4**. Furthermore, macrophages also play an essential role in wound healing or resolution of inflammation following tissue injury (36). Wound healing is divided into three phases: inflammation, new tissue formation and remodeling. The inflammation phase involves immune response to prevent pathogen invasion, and coagulation cascade to prevent fluid or blood loss. The phase involving new tissue formation is characterized by cellular proliferation and migration of different cell types from neighboring tissues. The remodeling phase is characterized by down-regulation of molecular processes initiated after tissue damage. Because of the ability of macrophages to produce cytokines (both pro- and anti-inflammatory), phagocytose pathogens and migrate in response to an inflammatory gradient, these cells are centrally involved in the different phases of wound healing (36).

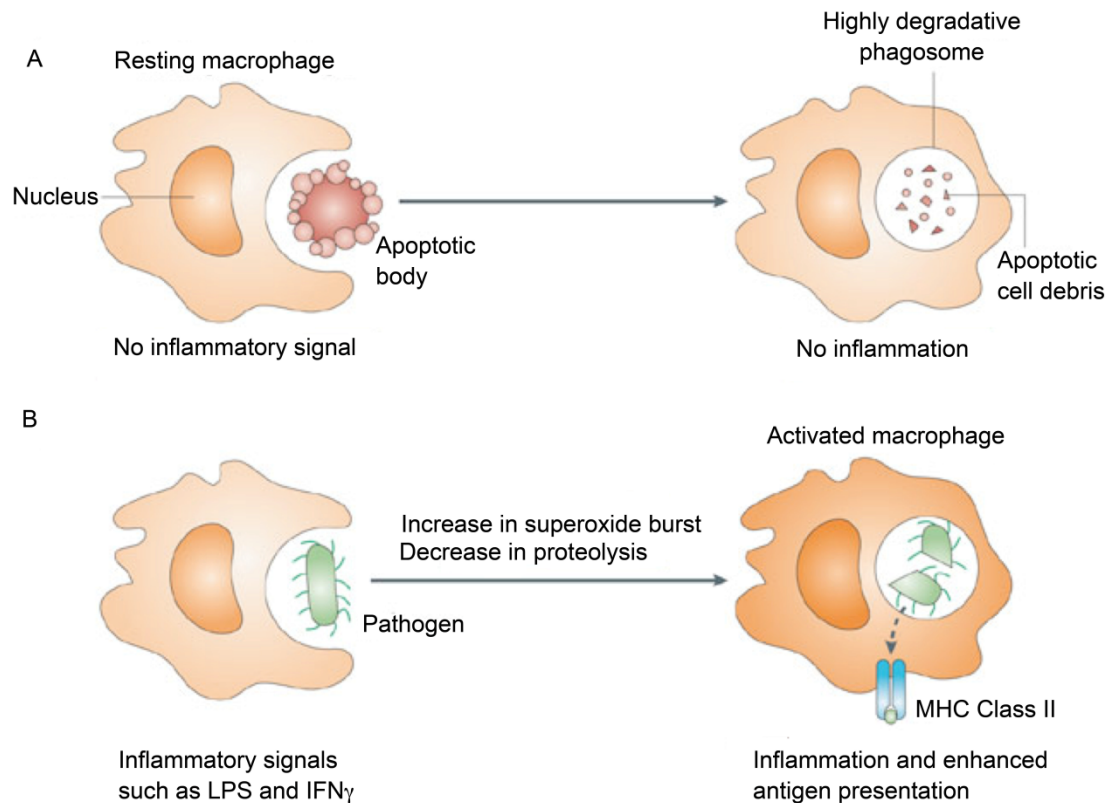


Figure 2: Macrophages and their functions. (A). Uptake of apoptotic bodies and breakdown in the absence of an inflammatory signal as part of the homeostatic maintenance function (B). Phagocytosis of pathogen, activation and antigen processing in the presence of an inflammatory signal such as LPS (TLR4 ligand) or IFN- γ (adapted from Russell *et al.*, 2009)(35).

1.2.5 Natural killer and gamma-delta T cells

These cells are also found in blood and have important roles in anti-mycobacterial immunity. NK cells play an important role in responses to pathogen. These cells produce IFN- γ and cytotoxic molecules such as perforin, granzyme B, and granulysin (37, 38). NK cell activation has been described in individuals with TB/HIV co-infections who developed unmasking TB-associated immune reconstitution inflammatory syndrome (uTB-IRIS) (39).

Gamma-delta ($\gamma\delta$) T cells represent a small subset of lymphocytes that express the γ - and δ -chain T cell receptor (TCR) (40). Activation of these cells does not require antigen presentation on MHC molecules, and these cells are involved in the recognition of phospho-antigens (40). These cells also develop a memory-like response to, and may

be important in rapid recall responses during infection with mycobacteria (41, 42). $\gamma\delta$ cells are known as effector lymphocytes with innate functions (**Fig. 3**).

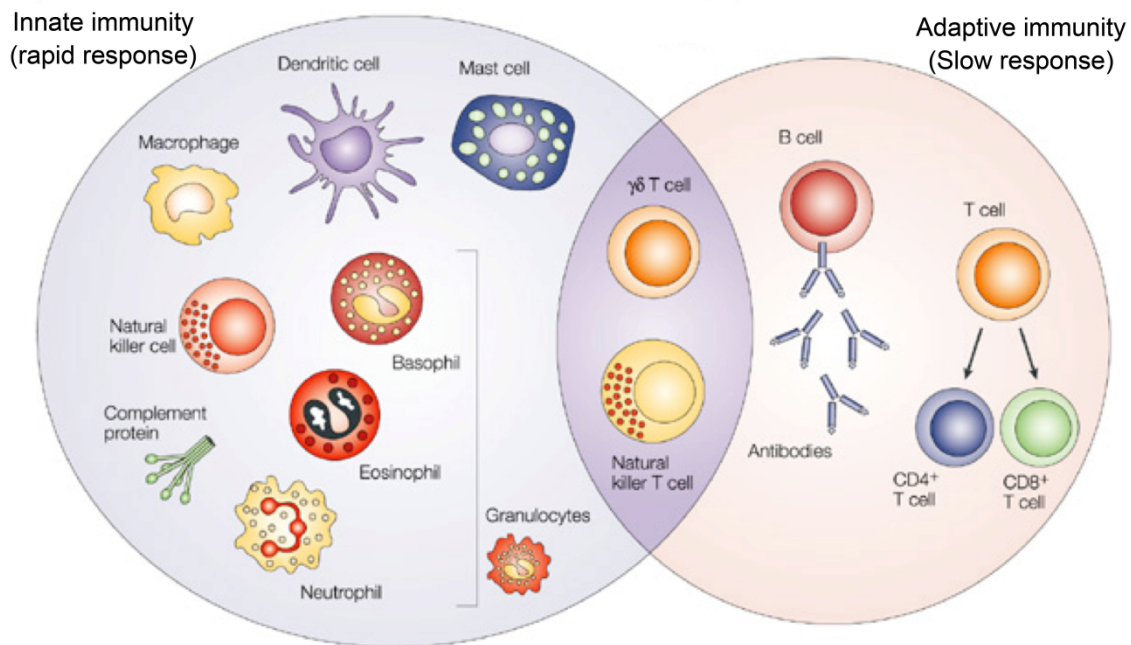


Figure 3: Schematic representation of the major innate and adaptive immune cells. NK T and $\gamma\delta$ T cells exhibit both innate and adaptive immune functions. (Adapted from <http://people.eku.edu/ritchisong/301notes4b.html>, accessed 13 July 2011).

1.3 Pattern recognition receptors and other binding receptors in innate immunity

Pattern recognition receptors are expressed mostly on innate cells and are centrally involved in recognition of invading pathogens and induction of immune responses (5, 43). These receptors include: Toll-like receptors (TLR), nucleotide-binding oligomerisation domain (Nod)-like receptors (NLR) (**Fig. 4**), and C-type lectins such as mincle, dectin-1 and mannose receptor (MR), retinoic-acid-inducible protein RIG-1-like receptor (RLR), complement receptors (CR) and scavenger receptors. Innate cells also express Fc receptors (FcRs) and soluble PRRs, such as collectins or pentraxins, that contribute to recognition and immune responses to pathogens (44). The type of PRR triggered may determine the outcome of an innate immune response, as triggering different receptors result in different responses.

The focus of this thesis is on TLRs and will be discussed in details below and in subsequent chapters. Other PRRs will also be discussed below in less detail.

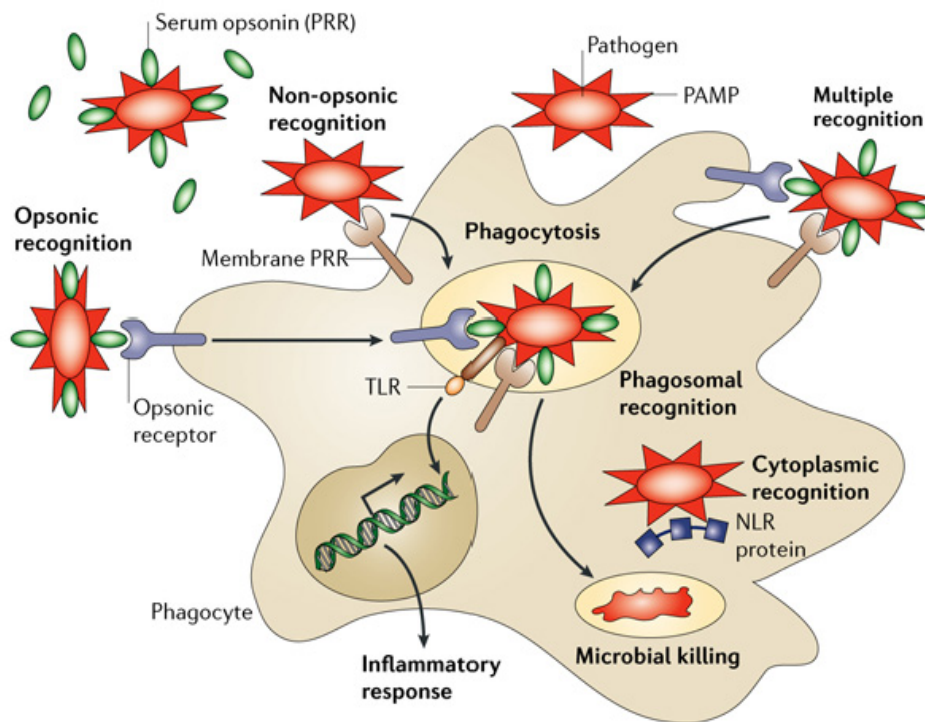


Figure 4: Recognition of microbes and PAMPs by PRRs (Adapted from Brown, 2006)(45).

1.3.1 Toll-like receptors

Toll-like receptors (TLRs) were the first class of PRRs to be recognised and are also the most widely characterised (46). These receptors were discovered due to the homology to the toll receptor, which mediates embryonic development and anti-fungal immunity in *Drosophila* fruit flies (47). TLRs play a central role in the initiation of immune responses to pathogens. The TLR family consists of 10 functional members (TLR1-10) (**Fig. 5**), while there are 12 functional TLRs in mice (46). These receptors recognise PAMPs present in a wide variety of microbes. The TLR consists of three regions (48): the extracellular leucine-rich repeat (LRR) motif, the transmembrane regions and the cytoplasmic toll interleukin 1 receptor (TIR) region. PAMPs recognised by TLRs include lipids, lipoproteins, proteins and nucleic acids derived from a wide range of microbes such as bacteria, viruses, parasites and fungi (**Fig. 5**).

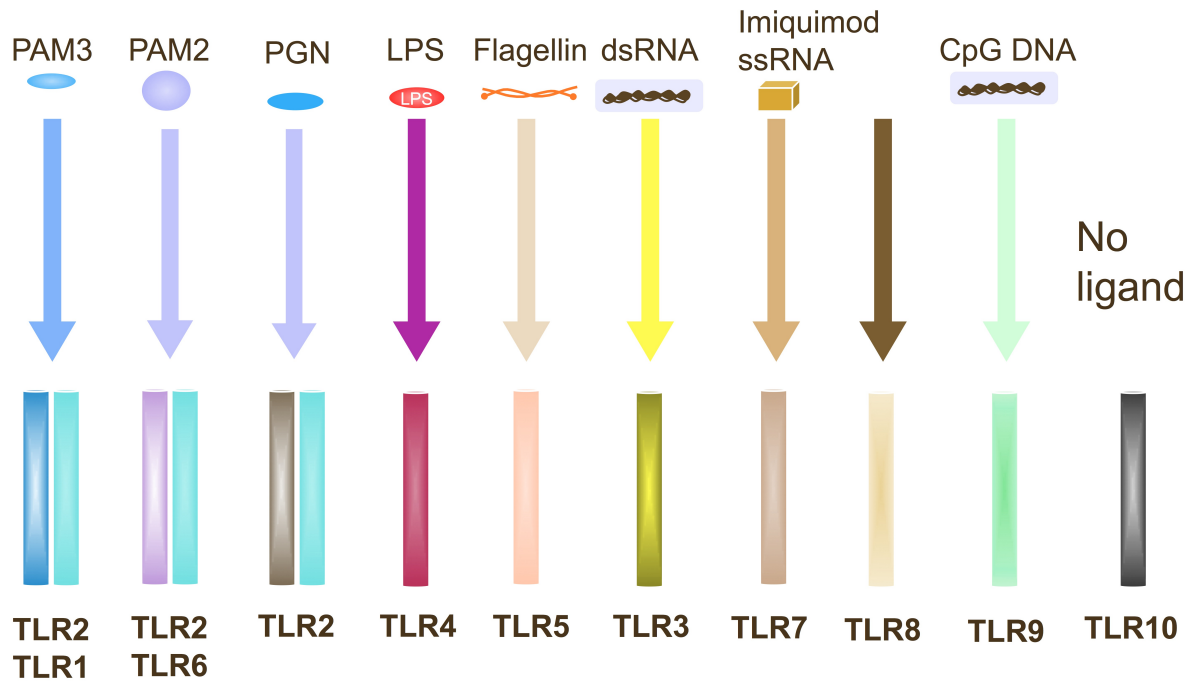


Figure 5: Summary of human TLRs and their ligands (Courtesy: Dr Thomas Hawn). PAM3, Pam3CSKKKK, *N*-palmitoyl-*S*-[2,3-bis-(palmitoyloxy)-propyl]-(*R*)-cysteiny-(lysyl)3-lysine; PAM2, PAM2CSKKKK, *S*-[2,3-bis(palmitoyloxy)-propyl]-(*R*)-cysteiny-(lysyl)3-lysine. PGN, Peptidoglycan; LPS, Lipopolysaccharide; CpG, 2'-deoxyribo(cytidine-phosphateguanosine).

1.3.1.1 Localisation of TLRs

The cellular localisation of TLRs is thought to be important for ligand accessibility, the maintenance of tolerance to self-molecules such as nucleic acids, and downstream signal transduction (49). TLRs are largely divided into two subgroups, depending on their cellular localisation and respective ligands. One group is expressed on cell surfaces and recognises mainly microbial membrane components such as lipids, lipoproteins and proteins. The other group is expressed mainly in intracellular vesicles such as the endoplasmic reticulum (ER), endosomes, lysosomes and endolysosomes, where these receptors recognise microbial nucleic acids (49).

Cell surface TLRs include TLRs 1, 2, 4, 5, 6, and 10. These receptors are expressed on many cell types, which include mDC, monocytes and macrophages. TLRs may form heterodimers with other receptors to enhance their signaling effects. For example, a heterodimer of TLR2 and TLR1 (TLR2/1) recognises triacylated lipopeptides, while

TLR2/6 recognises diacylated lipopeptides. In the presence of CD36, TLR2/6 can recognise other TLR2 agonists. TLR2 recognises peptidoglycans. TLR4 forms a complex with MD2 and CD14 to recognise LPS (49, 50) (**Fig. 6**). In addition to recognition of their microbial ligands, TLR2 and TLR4 also recognise endogenously derived ligands such as heat-shock proteins, extracellular matrix degradation products, β -defensin, and surfactant protein A, so-called DAMPs (50). These endogenous ligands are referred to as danger signals because these are released during inflammatory responses. TLR4 and TLR2 have been reported to bind respiratory syncytial virus (RSV) (51, 52) as well as mycobacteria (2, 53, 54). TLR5 recognises mostly bacterial flagellin (55), while the ligand for TLR10 has not yet been identified.

Intracellularly expressed TLRs include TLR3, TLR7, TLR8 and TLR9 (49, 56). These receptors recognise DNA and RNA motifs after phagocytosis and intracellular degradation of pathogens. TLR3 recognises double-stranded RNA motifs of viral origin and polyinosinic-polycytidylic acid (poly I:C), which usually mimics viral infection and induces antiviral immune responses. TLR7 and TLR8, either as homodimers or heterodimers, can recognise single stranded RNA derived from bacteria or viruses (55). TLR8 has also been implicated in mycobacteria recognition or immunity (57). TLR9 recognises unmethylated 2'-deoxyribo(cytidine-phosphateguanosine) (CpG) DNA motifs that are frequently present in bacteria and viruses (58). TLR9 also directly recognises the insoluble crystal hemozoin generated as a byproduct of the detoxification process after digestion of host hemoglobin by *Plasmodium falciparum* (59). These TLRs with intracellular localisation are mostly expressed in plasmacytoid DCs (60).

Although TLR8 and 9 are mostly expressed intracellularly, some studies have suggested that these receptors are also expressed on the cell surface (61-63).

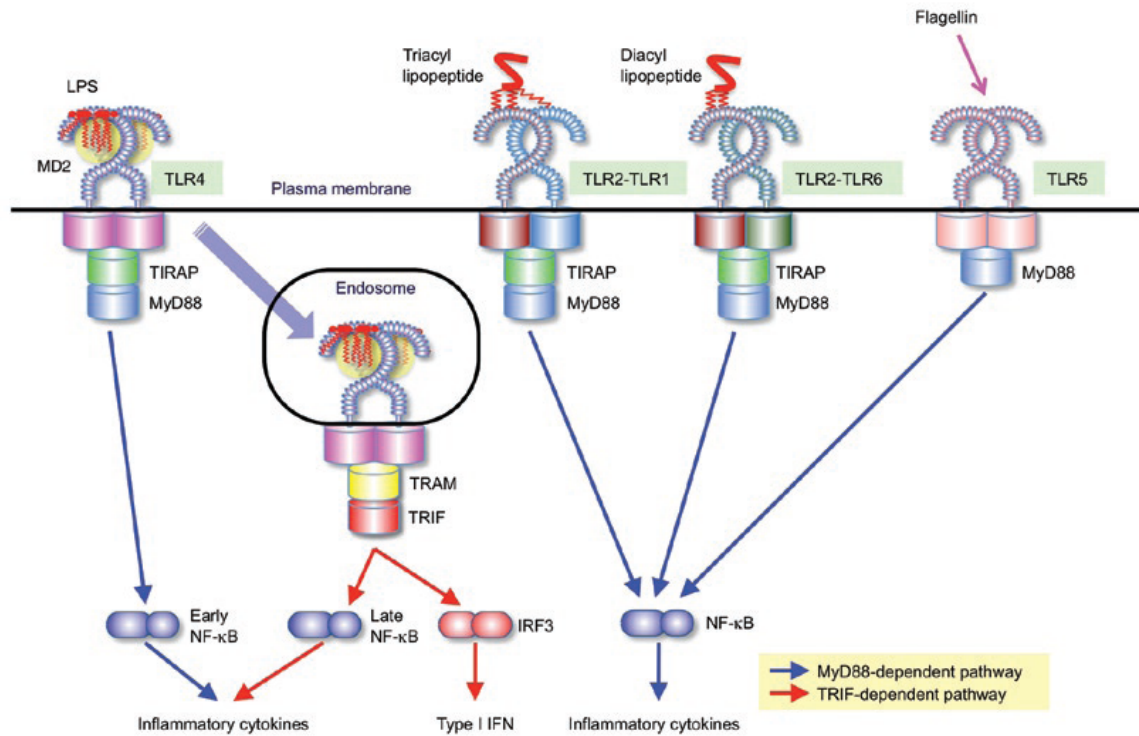


Figure 6: A simplified TLR signaling pathway showing MyD88-dependent (blue) and MyD88-independent (red) signaling pathways (Adapted from Kawai and Akira, 2010).

1.3.2 Nod-like receptors

Nod-like receptors (NLRs) are cytosolic receptors that detect PAMPs in the cytosol. These receptors are expressed mostly in innate cells (macrophages and DCs) but also in lymphocytes and non-immune cells like epithelial cells (64). These receptors consist of three principal domains: the N-terminal protein interaction domain, the central nucleotide-binding domain and the C-terminal leucine rich repeat (LRR) domain. Twenty-three human and 34 murine NLR genes have been identified but the physiological function of most of these receptors is poorly understood (65). NLRs are classified into 5 subfamilies: NLRA, NLRB, NLRC, NLRP and NLRX which are distinguished by their N-terminal structures (5). The well-characterised members of the NLR family are NOD1 and NOD2, which recognise distinct structural motifs derived from peptidoglycans (PGN). NOD2 recognises muramyl dipeptide (MDP) (**Fig. 7**) derived from *Streptococcus pneumoniae*, gram-positive and gram-negative bacteria (5, 66). Signaling via these receptors triggers the induction of several inflammatory cytokines and other antimicrobial factors that contribute to host defense, as well as cell death and

survival mechanisms (67). NOD1 recognises *meso*-diaminopimelic acid (*meso*DAP) or γ -D-glutamyl-*meso*-diaminopimelic acid (*iE*-DAP)-containing peptidoglycan, found predominantly in gram-negative bacteria (66).

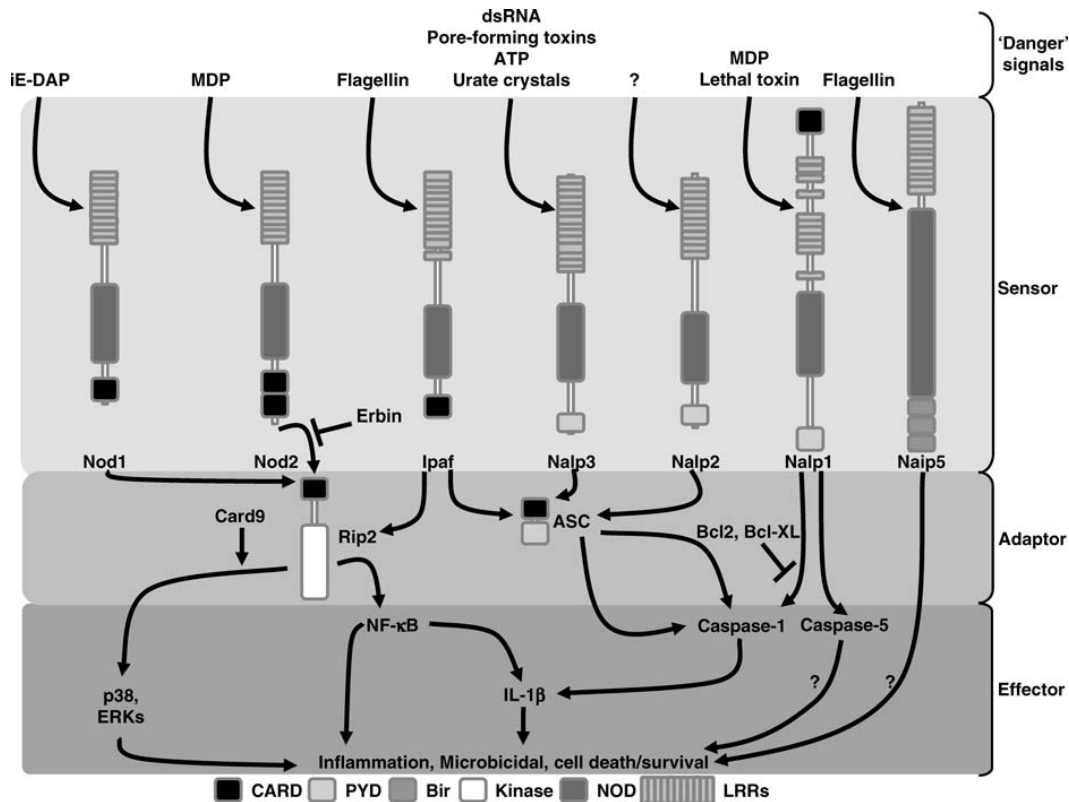


Figure 7: Schematic representation of the NLR signaling pathway. (Adapted from Sirard *et al.*, 2007)(67).

1.3.3 C-type lectin receptors

Mannose receptors (MRs), dendritic cell-specific intracellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN), mincle and dectin-1 form a class of C-type lectin receptors that are also important in mediating innate responses to pathogens (43). MRs mediate phagocytosis through interactions with mannose-capped lipoarabinomans (ManLAM), components of the cell wall of mycobacteria. This interaction also limits the phagosome-lysosome fusion and allows immune evasion by intracellular mycobacteria (68). DC-SIGN and MR interact with phosphatidyl-myoinositol mannosides (PIMs), LAM and trehalose-6, 6-dimycolate (TDM) in cell walls of mycobacteria to alter the phagosome lysosome fusion and di-acyl lipoarabinomans (69). This interaction may lead to inhibitory effect on macrophage and DC maturation (2, 70).

Dectin-1 is expressed on DC, macrophages, monocytes, neutrophils, T cells, B cells, mast cells, and eosinophils (45, 71, 72). Dectin-1 recognises β -glucan found in cell walls of fungi. Engagement of different C-type lectins by mycobacteria results in differential innate responses, which in turn induces differential adaptive responses. For example, binding of mycobacteria by dectin-1 promotes induction of Th1/Th17 responses, while binding by MR and DC-SIGN inhibits the induction of Th17 and promotes Th1 responses (73).

Macrophage inducible C-type lectin (mincle) is a recently identified PRR that is expressed on macrophages subjected to several types of stress. This receptor possesses a carbohydrate recognition domain (CRD) within the extracellular region (74). Mincle recognises TDM, also known as cord factor, which is found in the cell wall of mycobacteria, and is also a derivative of some pathogenic fungi (69, 75-77). Mincle can also recognise small nuclear ribonucleoproteins that are released from damaged cells (78).

1.3.4 RIG-like receptors

RIG-like receptors consist of three members: RIG-1, MDA5 and LGP2 (79). These are cytoplasmic receptors that recognise RNA derived from RNA viruses in the cytoplasm of infected cells. These receptors can also bind polyinosinic-polycytidylic acid (poly (I:C)). Binding of RLR to ligands leads to the induction of inflammatory responses including the expression of type I interferons (5, 79).

1.3.5 Complement and scavenger receptors

Complement receptors (CRs) are also important in induction and regulation of immunity. CRs form part of the complement system of the body, which consists of a series of plasma proteins (complement proteins) that opsonize pathogenic components and mark the pathogen through these components for phagocytosis and destruction. CRs 1, 3 and 4 are involved in binding and internalization of mycobacteria (80, 81). B-glucan can also be recognised by complement receptor 3 (CR3) (71).

Class A scavenger receptor (SR) comprises receptor family such as macrophage receptor with collagenous structure (MARCO), which can recognise TDM, with TLR2 and CD14 as coreceptors (82).

1.4 Innate receptor signaling and generation of an immune response

Upon recognition of the ligand by a PRR, a signaling cascade is initiated, which involves adapter molecules and transcription factors. This signaling cascade is necessary for the generation of an immune response such as cytokine production or maturation of innate cells (2).

Adapter molecules associated with TLR signaling include MyD88, TIR-domain-containing adapter-inducing IFN- β translocating chain-associating membrane protein (TRIF), and TIR domain-containing adapter proteins (TIRAP). All TLRs except TLR3 signal through MyD88. TLR3 signals through TRIF while TLR4 signals through either MyD88 or TRIF (2, 49).

The signaling transmitted through the above adaptor molecules culminates in activation of transcription factor, nuclear factor kappa B (NF- κ B), which translocates into the nucleus for induction of pro-inflammatory molecules (55, 83, 84)(**Fig. 6**). The resulting inflammation is a critical early step in immune responses aimed at controlling the invading microbe. Although TLRs are essential for protective immunity against infection, inappropriate TLR responses may contribute to acute and chronic inflammation, as well as to systemic autoimmune diseases (5). Assays determining NF- κ B signaling are described in **Chapter 4**.

NLRs and CLR s typically signal through caspase recruitment domain family, member 9 (CARD9) (85). NLRs can also signal via interacting protein 2 and TANK-binding kinase 1. CLR s also signal via Raf1, and FcR-gamma converging to CARD9 for signaling (2, 85).

Other transcription factors involved in PRR signaling pathways include activator protein-1 (AP-1), mitogen-activated protein kinase (MAPK) and IFN regulatory factors (IRF). Activated transcription factors translocate into the nucleus and initiate transcription of immune response genes, such as cytokines and chemokines (2). The production of

cytokines and chemokines as well as other molecules involved in the immune response then shape and determine the type of subsequent adaptive immune response generated.

1.5 Cytokines and chemokines produced by innate cells

Cytokines are important mediators of immunity to pathogens. Several cytokines mediate inflammation and innate immune responses. These cytokines include IL-12, IL-10, IL-6, IL-1 β , TNF- α , and IFN- α amongst others. These cytokines have important functions in the development of inflammation and the polarization of naïve T cells into Th1, Th2, Th17 or regulatory T cells (2) as suggested in **Figure 8**. The type of PRR triggered determines the nature of cytokine produced by innate cells and the outcomes.

Below, we discuss the function of major innate cytokines which are the focus of this thesis, such as IL-12, IL-6, IL-10 and TNF- α . We also discuss, other cytokines and chemokines that are important in innate immunity.

IL-12 is produced by most innate cells including monocytes, dendritic cells and monocytes. IL-12 exists as IL-12p40 or IL-12p35 subunits, which can combine to form IL-12p70. IL-12 plays an essential role in immunity as this cytokine promotes Th1 responses characterised by the production of IFN- γ . DCs in IL-12p40-deficient mice failed to migrate from lungs or respond to chemokines after *M.tb* infection suggesting an essential role of this cytokine in immunity to *M.tb* (86). Polymorphisms in the IL-12 receptor (IL-12R β 1) were also shown to predispose individuals to tuberculosis (TB) (87).

TNF- α is a pro-inflammatory cytokine produced in high amounts by innate cells, which is essential for the control of pathogenic infections. TNF- α mediates and maintains inflammation and granuloma formation after mycobacterial infections (88). TNF- α is also a mediator of apoptosis, which is important in the control of mycobacterial infection by macrophages (89). Individuals on anti-TNF therapy for psoriasis, an autoimmune disorder, were shown to be highly susceptible to TB, suggesting a role for TNF- α in immunity to *M.tb* infection and TB disease (90). TNF- α can also mediate tissue damage, which may lead to dissemination of mycobacterial infection (91).

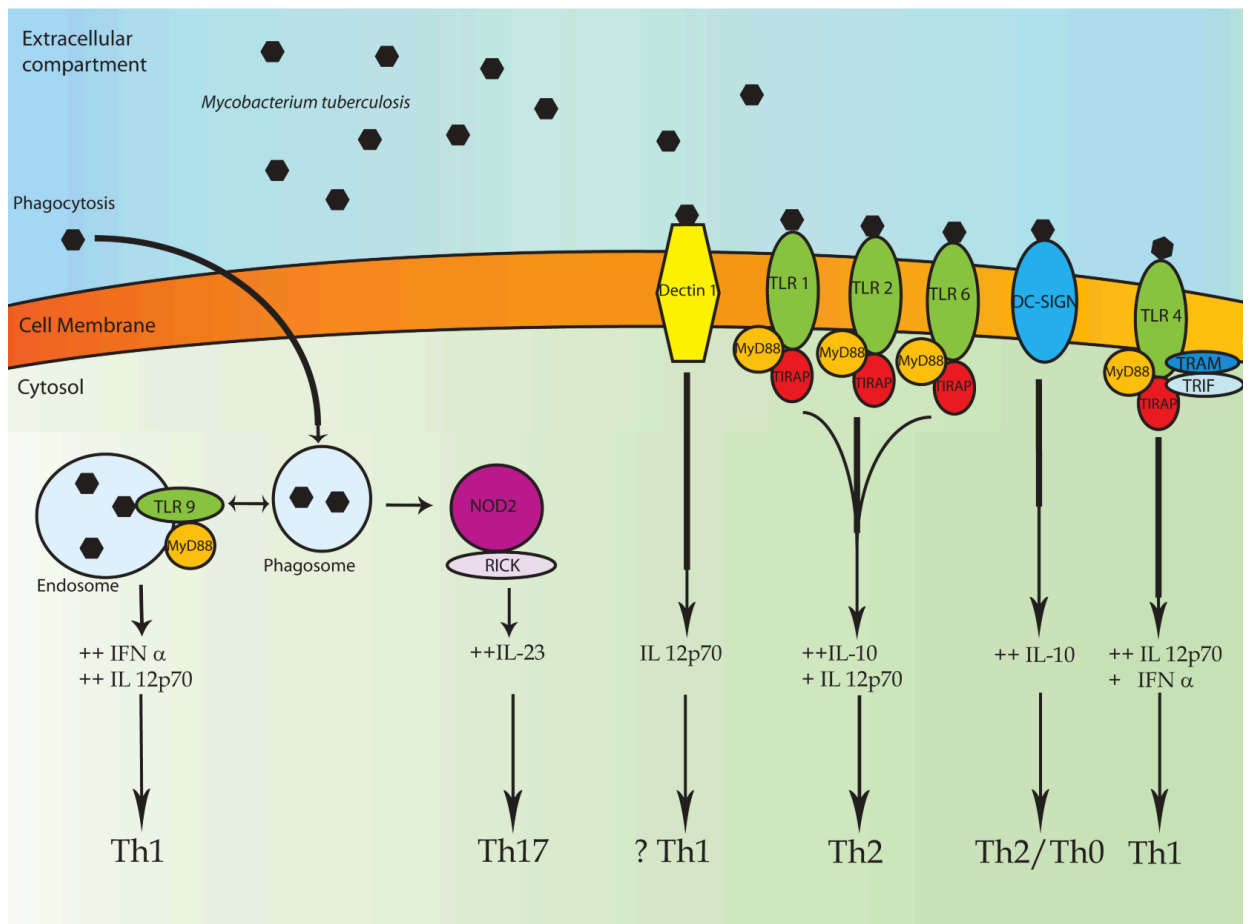


Figure 8: Cytokines produced by innate cells in response to mycobacteria and their functions (Adapted from Arentz and Hawn, 2007)(92).

IL-6 is produced in high amounts by innate cells and acts both as an anti-inflammatory and pro-inflammatory cytokine. The production of IL-6 in the presence of other cytokines including IL-1 β , IL-23 and TNF- α may lead to the development of Th1 or Th17 immune responses (73, 93).

IL-10 is an anti-inflammatory cytokine with inhibitory effect on T cell responses (94). In the presence of low IL-12 and high IL-10 production, the subsequent adaptive immune response is skewed towards Th2 immunity (**Fig. 8**). IL-10 is also known to inhibit migration of DC to the lymph nodes and local IL-12 production after pathogenic infection (95, 96). IL-10 can also downregulate pro-inflammatory cytokine responses, T cells proliferation and can balance the response between bacterial eradication and host survival (96, 97).

IFN- α is an important inflammatory cytokine in antiviral immunity. IFN- α is expressed by pDC upon stimulation with ligands for TLR3, TLR7 and TLR9 (60). Upon viral infection, IFN- α and other type I interferons, which inhibit viral replication, are secreted and activate NK cells, cytotoxic T cells, and macrophages to eliminate infected cells (98, 99). IFN- α and IFN- β are type I interferons. IFN- $\alpha\beta$ -inducible gene transcripts have been shown to be upregulated in blood neutrophils of individuals with active TB, compared with individuals with latent TB infection, suggesting a role for these cytokines in pathogenesis of TB (100).

IL-1 β is known as the central mediator of inflammation (101). IL-1 β is produced in abundant quantities after infection with mycobacteria. In mice, deletion of IL-1 β results in fatal consequences after infections (102).

Chemokines (chemoattracting cytokines) are also important in innate immune responses to pathogens. Chemokines facilitate leukocyte migration and positioning as well as other processes such as leukocyte degranulation (103). Chemokines are also involved in processes that do not involve leukocyte migration such as angiogenesis and metastasis (104). There are many chemokines with diverse functions in immunity (inflammatory chemokines) and homeostasis (homeostatic chemokines). Inflammatory chemokines are secreted in response to infection and are often stimulated by pro-inflammatory cytokines such as IL-1 β , and serve to attract other immune cells to the site of infection (104, 105). Inflammatory chemokines include CCL3 (MIP-1 α) and CCL2 (MCP-1) that bind to CCR1 and CCR2, respectively, to induce innate and adaptive immune responses. Homeostatic chemokines navigate leukocytes during homeostasis in the bone marrow and thymus, and initiate adaptive immune responses and immune surveillance of healthy peripheral tissues (104). An example of a homeostatic chemokine is CCL19, which serves to attract mature DC expressing chemokine receptor CCR7 to the lymph nodes where antigen presentation occurs (32). Chemokines such as CCL20 and CXCL9, which bind to CCR6 and CXCR3, respectively, are both inflammatory and homeostatic (104).

1.6 Maturation markers in innate immunity

Maturation comprises morphological and functional changes associated with activation of innate cells by microbial stimuli such as TLR agonists (18). Maturation markers are important in innate immunity and indicate changes in the maturational status of innate cells. These markers include CD86, CD80, CD83, CD40 and CCR7. CD80 and CD86 belong to the B7 family of co-stimulatory molecules and provide co-stimulation during antigen presentation (discussed in details **section 1.8** under antigen presentation). In this thesis, we focus on CD86, CD83 and CD40.

CD86 is the most abundant costimulator and is highly expressed on DC relative to other leukocytes. CD86 or CD80 binds to two molecules on T cells: CD28 for activation, and CTLA-4 for inhibition of T cell responses (106).

The role of CD83 is a maturation marker that is expressed on DCs and upregulated upon activation. Expression of CD83 on DCs is also essential in the activation of T cells responses (107-109). Neutrophils have also been shown to upregulate the expression of CD83 upon activation (33, 34).

CD40 is expressed by innate cells such as DCs and monocytes, and also expressed on B-lymphocytes. Upon ligation with the CD40 ligand (CD154) expressed on T cells, T cells become activated (110). The binding of CD40 on B-lymphocytes also leads to activation of B cells and this promotes antibody class switching and memory B cell development (111).

1.7 Phagocytosis by innate cells

Phagocytosis is a critical function of innate cells that is required for control or killing of invading pathogens and for antigen processing and induction of adaptive immune responses. Innate cells become activated upon phagocytosis of pathogens and may express cytokines, chemokines and maturation markers. Alternatively, activation and cytokine and/or maturation marker expression may occur in the absence of phagocytosis.

Innate cells can perform phagocytosis using intermediary proteins such as antibody or complement that coat the pathogen, as well as by binding to the microbe directly via

PRRs or phagocytic receptors (**Fig. 4**). Phagocytosis of pathogen is mediated by several receptors including DC-SIGN, mannose receptors, complement receptors, and Fc receptors (80, 112-114). Of the mechanisms involved in phagocytosis, Fc-receptor-mediated phagocytosis is the most extensively studied (112). Antigens coated with antibodies (opsonised antigens) are recognised by Fc receptors and initiate the process of internalisation. This process leads to clustering of several Fc receptors leading to phosphorylation and recruitment of actins and actin-associated proteins to form a phagocytic cup (112). The phagosome is formed containing the pathogen, which eventually fuses with lysosomes to form the phagolysosome. This phagolysosome degrades the pathogen for eventual antigen presentation and induction of adaptive immune response.

In this thesis, we describe the acquisition (binding and/or internalisation) of mycobacteria by innate cell subsets.

1.8 Antigen presentation

A critical part of immunity against pathogens is antigen presentation to and the induction of memory T cells. DCs, macrophages, and B cells are the three major APCs. Antigen presentation is made possible by the expression of molecules such as major histocompatibility complex (MHC) on APC. Two major MHC classes of molecules are involved in antigen presentation: MHC class I (MHC I) and MHC class II (MHC II). Antigen loaded onto MHC I and II are presented to CD8⁺ and CD4⁺ T cells, respectively.

MHC II is synthesised in APC as alpha and beta chains. These chains associate with an invariant chain (Ii) in the ER. This complex then transits through the Golgi apparatus into the endosomal/lysosomal pathway (115). In this pathway, the Ii is proteolytically degraded and a small fragment, known as class II associated Ii chain peptide (CLIP), is left with the MHC II alpha-beta dimer. HLA-DM, a class II-related dimer, bearing antigen from the endosomal/lysosomal compartment then replaces CLIP on the MHC II dimer with the antigen that is derived from the breakdown of phagocytosed pathogens. The antigens loaded onto MHC II are generated from extracellular proteins that have been endocytosed and processed by proteases that reside in the secretory system (116, 117). The peptide-MHC II complex (pMHCII) then traffics from the endosomal/lysosomal

compartment to the surface of the cells where the antigen is presented to CD4⁺ T cells (Fig. 9A).

Antigens are loaded onto MHC I in either the endoplasmic reticulum (ER) or the endosomal/lysosomal compartment. Endogenous peptides loaded onto MHC I in the ER are generated from cytosolic proteins. These proteins arise from defective forms of nascent proteins or intracellular pathogens that have been degraded by proteasomes (118). These peptides are then transported to ER by the transporter associated with antigen processing (TAP) protein for binding to MHC I. When the peptide binds to MHC I, the peptide-MHC I complex (pMHC I) leaves the ER and is transported to the surface via the Golgi apparatus where the antigen is presented to CD8⁺ T cells (Fig. 9B).

Cross-presentation most often occurs via endosomal/lysosomal antigen processing, when exogenous antigens gain access to the cytosol and are degraded by the proteasome. The resulting peptides are then transported to the ER by TAP and are loaded on MHC I (119, 120).

Antigen presentation is needed to successfully prime a cognate T cell response. Three signals are required during antigen presentation.

Signal 1 - This involves the MHC-peptide complex that binds with the T cell receptor (TCR), as described above.

Signal 2- Co-stimulation: DCs express many co-stimulators including CD80, CD86, CD40, ICOS-ligand, and PD-L1/2 (18). At the DC surface, CD86 and MHC-peptides complexes (pMHC) are often clustered together to prepare DCs for interaction with T cells when CD86 and pMHC bind to CD28 and TCR, respectively, on T cells. This interaction forms a supramolecular immunologic synapse (14, 121, 122).

Signal 3- Soluble cytokines: The types and relative amounts of cytokines produced determine which type of T cell response is induced. For example, high levels of IL-12 polarise differentiation of Th1 responses; IL-23 polarises Th17 responses and IL-4 polarises Th2 responses (123-127).

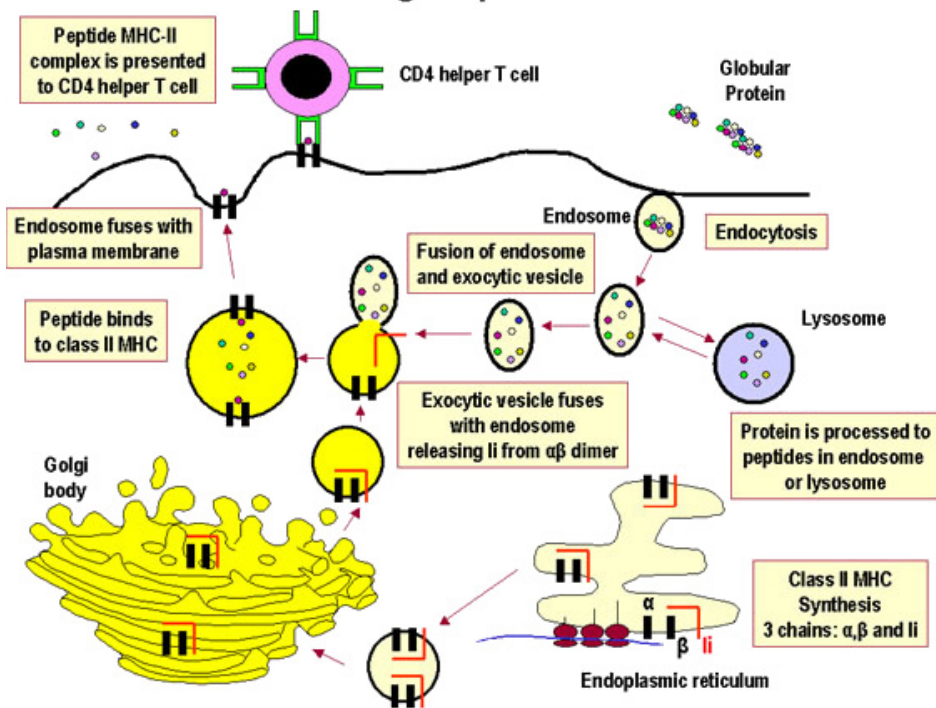
Antigen presentation can also occur through non-classical pathways. This occurs when antigen is presented on CD1, which is an MHC-like molecule expressed on DCs and monocytes (128). CD1 family consists of five members classified into three groups with

distinct antigen-presenting functions. Group 1 CD1 molecules consisting of CD1a, CD1b and CD1c present antigens to alpha beta ($\alpha\beta$) T cells, while Group 2 CD1 molecule, CD1d, presents antigens to NK T cells (129). Group 3 CD1 molecule, CD1e, is involved in intracellular CD1b antigen loading (130, 131). CD1 molecules bind and present mycobacteria-derived lipid and glycolipid antigens to T cells (132).

Below and in subsequent chapters, we describe some of processes involved in antigen presentation such as acquisition of mycobacteria, cytokine production and expression of maturation (co-stimulatory) markers. Detailed intracellular processes of antigen presentation are beyond the scope of this thesis.

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A. MHC Class II antigen presentation



B. MHC Class I antigen presentation

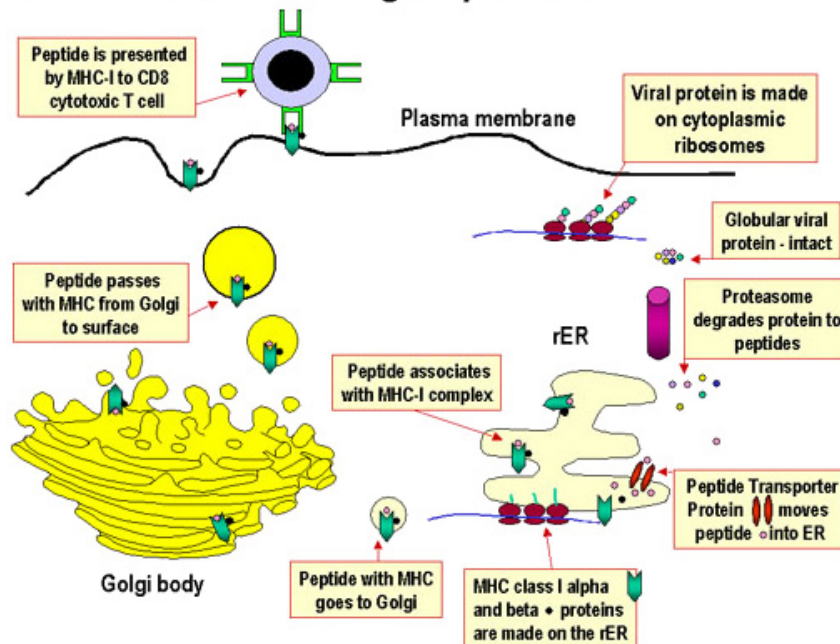


Figure 9: The MHC class I and MHC class II antigen presentation pathways. (A) Pathway of class II MHC-restricted presentation of an exogenous antigen. (B) Pathway of class I MHC restricted presentation of an endogenously synthesised antigen. (Adapted from <http://pathmicro.med.sc.edu/bowers/ant-pres.htm>, accessed 15 April 2011).

2 Tuberculosis

Tuberculosis (TB) is a disease primarily of the lungs caused by *M.tb*. However, *M.tb* can also cause disease in most organs in the body (133). When a person with TB disease coughs, *M.tb* bacilli spread into the air allowing airborne transmission to uninfected individuals who inhale the bacilli. A third of the world's population is estimated to be infected with *M.tb*. People who are already infected may be re-infected. About 5-10% of people infected with *M.tb* will develop TB disease in their life-time (133). Symptoms and signs of TB include prolonged coughing, fever, night sweats and weight loss. These symptoms and signs may be absent in early stages of TB disease as 1 in 4 people with cultured confirmed TB may be asymptomatic (134).

2.1 Epidemiology of tuberculosis

The World Health Organization (WHO) estimates that there were 8.8 million incidence cases of TB in 2010, 1.1 million deaths among HIV-uninfected people and 0.35 million HIV-associated TB deaths (135). Since 2006, the absolute numbers of TB cases have been declining (135). Despite this decrease, Africa and Asia still have the highest case burdens of TB. Out of 22 high burden countries with 81% of global TB burden, South Africa ranks 3rd (0.4-0.59 million cases) after India (2-2.5 million cases) and China (0.9-1.2 million cases), respectively (135). In Cape Town, South Africa, the highest incident rates of TB were reported in infants less than 2 years of age while the lowest incident rates were in adolescents (**Fig. 10**) (136). With the high incidence of TB in early life, we therefore need to understand the development of immune responses to mycobacteria in early life, which is the focus of **Chapter 3**.

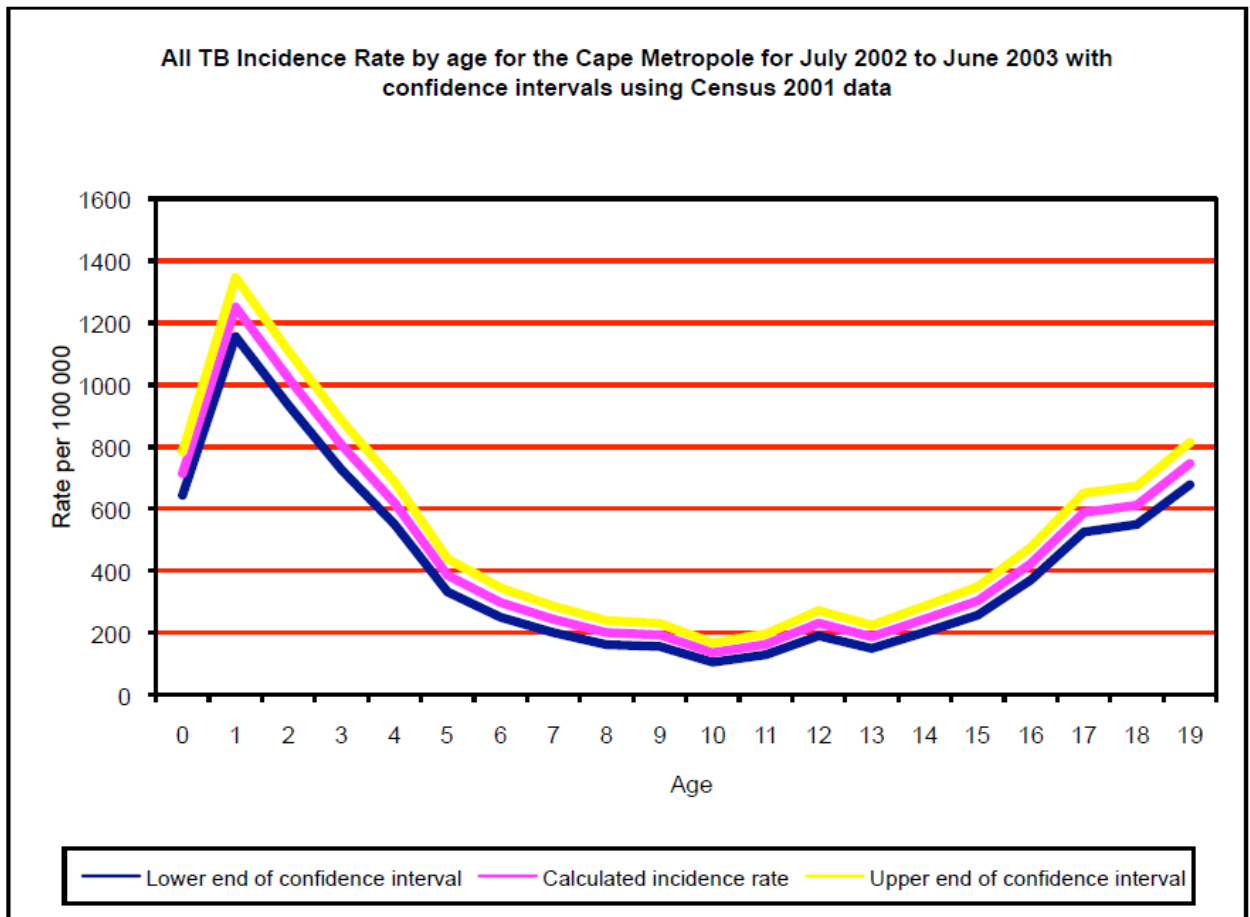


Figure 10: All tuberculosis incident rates in Cape Town by age, in years (adapted from Mahomed *et al.*, 2011) (136).

2.2 Tuberculosis control

Several interventions are employed for the fight against TB. These include:

- i. Vaccination: Bacille Calmette-Guerin (BCG) vaccine derived from virulent *Mycobacterium bovis* by serial passages is the only licensed vaccine against TB. BCG was first tested in human in 1921 (137). BCG is effective in preventing disseminated forms of TB especially in children but highly variable in preventing pulmonary TB (138). Up to 80% protection against severe forms of TB has been reported in children and adults, with the lowest efficacy in countries closer to the equator (138-140). Several factors are hypothesised or known to be responsible for the poor efficacy of BCG. These include host genetic factors, host immune deficiency, exposure to environmental

mycobacteria, different vaccine strains used, route of immunization, and age of individuals vaccinated (141-146). Development of more effective TB vaccines is thus urgent. Pre-exposure vaccines to reduce initial bacterial burden, and post-exposure vaccines to prevent reactivation of latent TB and reduce risk of re-infection in high prevalence areas are also needed (147). At SATVI, we are currently testing multiple novel vaccines, which are at different phases of clinical trials. Some of the vaccines are designed to boost the immunity induced by BCG at birth. These vaccines include MVA85A (148), M72, HyVac4/AERAS-404, Hybrid-I+IC-31, Hybrid-I+CAF01 and Crucell Ad35/AERAS-402 (149). M72, Hybrid-I+IC-31, Hybrid-I+CAF01 and HyVac4/AERAS-404 contain ligands of PRRs especially TLRs as adjuvants to enhance the immune response generated. For example, IC-31 used in Hybrid-I+IC-31 vaccine signals through TLR9 and induces Th1, cytotoxic and antibody responses (150, 151). Cationic adjuvant formulation (CAF01), in Hybrid-I+CAF01 vaccine, signals through multiple TLRs and also induces both cell-mediated and antibody responses (152). AS01 used in M72 vaccine, is an adjuvant containing monophosphoryl lipid A (MPL) and QS21, which signal through TLR4 and induce Th1 responses (153). All participants in our studies described in subsequent chapters were vaccinated with BCG at birth.

- ii. Diagnosis. Effective and early diagnosis of TB or *M.tb* infection is essential for its treatment and control. Some of the methods used to diagnose *M.tb* infection include the tuberculin skin test (TST) and IFN- γ release assay (IGRA). Methods used to diagnose TB disease include sputum microscopy, culture, X-rays, and nucleic acid amplification technology (NAAT) method (gene Xpert MTB/RIF assay) (147, 154). Diagnosis of childhood TB and sputum smear-negative pulmonary and extrapulmonary TB in adults remains a challenge (147).

Even though these are used to diagnose TB or *M.tb* infection, there are disadvantages associated with these diagnostic methods. For example, TST that assesses delayed host reaction to purified protein derivative (PPD) derived from *M.tb* has been extensively used in under-resourced settings for diagnosis, but may also be positive in individuals previously vaccinated with BCG or exposed to environmental mycobacteria, and sensitivity can be

reduced by HIV infection (155, 156). IGRA tests, which measure IFN- γ responses to *M.tb*-specific antigens, early-secreted antigenic target 6 (ESAT6) and culture filtrate protein 10 (CFP10), are more sensitive compared with TST, but may also be influenced by HIV infection (156, 157).

Sputum microscopy or automated liquid culture systems (such as the mycobacterial growth indicator tube (MGIT)) are most often used for TB diagnosis but presents difficulties in infants as sputum samples are difficult to collect coupled with the fact that very few bacilli are often found in sputum samples obtained from infants (158). Also, the MGIT technique requires highly trained operators that may not be available at point-of-care in high incidence and under-resourced settings (159, 160). Chest radiography or X-rays are most often used to diagnose TB especially in infants, in conjunction with other assays, but misdiagnoses are common where specialist radiology support is lacking (154). Also, in individuals co-infected with HIV, other opportunistic infections may be present with overlapping clinical and radiological findings (160). The gene Xpert MTB/RIF assay, which involves the use of sputum samples to detect *M.tb* DNA or RNA and produces results in less than 2 hours, may provide the best diagnostic method in high incidence areas but the current use of this method, especially in under-resourced areas of the world, is limited by high cost, the need for specialised molecular laboratories and highly experienced staff (147, 159). This test was endorsed by WHO at the end of 2010 (135).

Other diagnostic assays that are currently being developed and tested include the use of polymerase chain reactions (PCR) or real-time (RT)-PCR to detect *M.tb* DNA or drug resistance in both sputum and urine samples (161-163). These assays equally require highly trained operators and specialised equipment.

We used the IGRA assays to screen participants in our studies for *M.tb* infection.

- iii. Treatment: Treatment of TB may involve treating LTBI to prevent progression to active disease or treatment of active TB (164). Successful treatment as targeted by WHO is a cure rate of 85% of the 70% TB cases detected (165). Drugs used to treat TB are classified into first-line, second-line and third-line depending on drug sensitivity (166). The first line treatment often includes

isoniazid and rifampicin. Treatment of LTBI in children can take 3 months with isoniazid and rifampicin, or up to 6 months with Isoniazid (167). The length of active TB treatment may be at least 6 months (164). In the case of resistance to the first line regimens, referred to as multidrug resistance (MDR), which is often due to non-adherence, second-line regimens are used. These include drugs like capreomycin and neomycin (166). Third line regimens, which include drugs like rifabutin and linezolid, are used to treat extensively drug resistant TB (XDR) (166). The Directly Observed Therapy, Short-course (DOTS) strategy implemented by WHO has been very successful in identification and treatment of TB (168). STOP TB Strategy and the Global Plan to Stop TB (2006-2015) also aim to address issues surrounding HIV associated TB, MDR, and weak health systems among others (147). Participants in our studies were all healthy and not on treatment.

- iv. Public health interventions: Effective national TB programs are necessary to ensure rapid diagnosis, adherence to treatment and high vaccination coverage for those at risk (169). Effective health sector reforms have been shown to improve management of TB in South Africa (170).

2.3 Clinical manifestations of tuberculosis

When *M.tb* is inhaled by a healthy individual, one of several outcomes may result depending on the individuals' immune system (171).

- i. The immune system may eliminate the infection.
- ii. The infection may establish itself but does not develop into active TB. This is known as latent TB infection (LTBI). In LTBI, the host maintains control over the replication of *M.tb* and remains free of tissue damage and symptoms.
- iii. The infection may establish as LTBI and later develop into active TB.

About 5-10% of those infected may develop active TB in their lifetime but in the presence of immunosuppressive conditions such as HIV, the risk of developing active TB increases to approximately 10% a year (97, 172). Several factors may determine the progression of an individual from latent to active TB. These include:

- i. Host genetics: Genetic variations in several PRRs and other immune response genes have been linked with susceptibility to tuberculosis (See **section 2.5** below, and **Chapters 4 and 5**). Genes that affect susceptibility include TLRs (173, 174), DC-SIGN (175), TNF- α (176), and nitric oxide synthase (177), among others.
- ii. Age of individuals: Previous studies have shown that the newborn and infant immune system is generally less responsive to TLR ligand and mycobacterial antigen stimulation compared with that in older children or adults (see **Chapter 3**) (3, 178, 179). Consequently infants may be more susceptible to *M.tb* and other infections compared with adults. Our studies reported in **Chapter 3** also suggest that the innate immune responses at birth are different and generally less mature compared with those in infants and adults and this may translate to suboptimal control of *M.tb* infection or progression to disease.
- iii. Immune compromise: HIV and other immuno compromising conditions, such as diabetes make individuals with latent TB more susceptible to developing active TB (180, 181). HIV infection leads to selective depletion of CD4⁺ T cells that have been shown to play an important role in immunity against *M.tb* infection (182, 183). In South Africa, A previous report showed that infants with HIV are highly susceptible to progress to active TB, compared with HIV-uninfected (180). Also, in people who are co-infected with HIV and *M.tb*, HIV treatment may lead to conditions such as immune reconstitution inflammatory syndrome that may have detrimental effects for the host (184, 185). This thesis is focused on innate responses in HIV-uninfected individuals.
- iv. Socio-economic factors: Socioeconomic factors that have been associated with susceptibility to TB in low and high TB incidence settings include, poor nutrition, co-infections with helminths and malaria, homelessness and/or poverty (186, 187). We recruited our participants from the same geographical area with similar socio-economic status.

2.4 Immune responses to *M.tb* infection

The observations that a proportion of exposed people do not become infected, and others who are already infected control progression to active TB, point to the fact that interaction between the host and the pathogen determines outcome of *M.tb* infection. Epidemiological evidence suggests that a proportion of persons who are exposed to *M.tb* bacillus possess innate immune mechanisms that eliminate the pathogen before sensitisation of an antigen-specific T cell response (188, 189). However, some exposed individuals would develop a detectable *M.tb*-specific T cell response, suggesting successful *M.tb* infection. In household contact studies in a TB endemic setting in South Africa (190, 191), the maximal annual TB incidence rate in pre-school children was reported to be 3%, which was mostly determined by the number of resident adult TB cases in the household (190). Among children aged 3 months to 6 years with household exposure, the odds of being *M.tb*-infected increased by 74% (191). The establishment of infection and progression to active disease is evidence that *M.tb* can overcome the host responses. Some of the evidence that the host immune response is essential in controlling *M.tb* infection includes

- i. Genetic polymorphisms in immune gene pathways that influence susceptibility to TB.
- ii. Development of TB in people who take anti-TNF treatment for rheumatologic disorders.
- iii. Immunocompromised individuals.

Both the innate and the adaptive immune arms are important in immune responses to *M.tb*. These immune arms interact with each other for effective control of *M.tb*. In the sections below we discuss the role of the innate arm, which is the focus of this thesis. The role of the adaptive arm will be discussed in less detail.

2.4.1 Innate immune responses to *M.tb*

M.tb bacilli, when inhaled, are recognised by innate cells that are resident or recruited to the lungs, such as macrophages, DCs and neutrophils, through PRRs expressed on

their cell membranes. This recognition leads to immune responses such as phagocytosis and pathogen killing, cytokine production and inflammation, antigen presentation and granuloma formation.

2.4.2 Recognition of *M.tb*

TLRs and NLRs have been identified as the major PRRs for *M.tb* and other mycobacteria (43). Increased susceptibility to *M.tb* in MyD88-deficient mice suggested TLRs were important in the initial host response (192, 193), although the exact TLRs involved were not known. *In vitro* and *in vivo* studies in mice have implicated TLR2, TLR4, and TLR9 (192, 194-196), although other studies did not support these findings (197, 198). Human TLR6 and TLR1 as heterodimers with TLR2 recognise whole *M.tb* lysates, cell wall and membrane extracts from *M.tb* and induce NF- κ B signaling (see **Chapter 4**) (199). C-lectin receptors such as mincle, DC-SIGN and MRs can also recognise *M.tb* and initiate pathogen internalisation. Recognition through some of these receptors leads to activation of macrophages while others allow quiescent entry of *M.tb* into the cells (2).

2.4.3 Inflammatory responses to *M.tb*

After phagocytosis of *M.tb*, intracellular processes may occur, such as the formation of inflammasomes. Inflammasomes are protein complexes containing various members of the NLR family of intracellular proteins including NLRP3, NLRC4, AIM2 and NLRP6 (200). Recognition of a diverse range of microbial, stress and damage signals by inflammasomes results in direct activation of caspase-1. This eventually leads to production and activation of the classical inflammatory mediators such as IL-1 β , IL-6, TNF- α , as well as chemokines, which mediate the recruitment of other immune cells, including neutrophils, T cells, and B cells, to the site of infection (201). This recruitment of immune cells to the site of infection is the hallmark of an inflammatory response and is required for containment or elimination of infection. The inflammatory response to *M.tb* can be beneficial as well as detrimental if not well controlled. The presence of regulatory T cells at the site of inflammation, and the expression of inhibitory molecules

such as PD-1 and IL-10, regulate the inflammatory response to *M.tb*. An excessive inflammatory response may lead to tissue damage and is thought to contribute to progression to TB and lung cavitation (202). As reported in **Chapters 2 and 3**, we determined the production of pro-inflammatory cytokines (IL-12, IL-6 and TNF- α) and anti-inflammatory cytokine (IL-10) in response to mycobacteria.

2.4.4 Innate mechanisms of pathogen control

Neutrophils and macrophages are the predominant cell population infected by *M.tb* and are found in high numbers in airways of people with TB (203). Neutrophils and other mononuclear cells express antimicrobial peptides such as cathelicidin LL-37 and human neutrophil peptides (HNP) 1-3 that can kill mycobacteria (204, 205). Neutrophils infected with *M.tb* rapidly undergo apoptosis and are taken up by DCs and macrophages, through DC-SIGN, that mature and present antigens to T cells (29, 206). Macrophages can be classically or alternatively activated. Both classically and alternatively activated macrophages are important in immunity to *M.tb* (97). Macrophages are classically activated to kill *M.tb* by cytokines such as IFN- γ and TNF- α , which are produced in response to infection and antigen presentation or through vitamin D pathway that increases IFN- γ production by T cells (207, 208). Classically activated macrophages produce bactericidal molecules such as nitric oxide catalysed by inducible nitric oxide synthase (iNOS). Alveolar macrophages are alternatively activated by cytokines such as IL-4 and IL-13, and are anti-inflammatory in nature producing IL-10 and TGF- β that limit excessive inflammation and contribute to clearing infections in the airways (97). Other control strategies for *M.tb* include granuloma formation (discussed in **section 2.4.5**) and apoptosis by cells that have internalised the bacilli. The apoptotic cells are then taken up by APCs such as DCs that present antigens to initiate an adaptive response. DCs and monocytes may also be involved in uptake and killing of *M.tb*, but the major function of DCs is antigen processing and presentation, while that of monocytes is cytokine production and replenishing DCs and macrophages (18, 80, 209).

M.tb has evolved several strategies to evade killing by activated macrophages including inhibition of phagosome maturation and killing (210). *M.tb* is also known to inhibit inflammatory responses through release of bacterial zinc metalloproteases (211). The

persistence of *M.tb* in endosomal compartment and eventual escape to the cytosol also suggests *M.tb* interferes with inflammatory processes (212). In chronic *M.tb* infection models, contrary to acute infection, monocyte-derived inflammatory DCs have been shown to downregulate expression of MHC II, CD40, CD80, and CD86 accompanied by decreased IFN- γ production by *M.tb*-specific CD4⁺ T cells and increased expression of PD-1 ligands, PD-L1 and PD-L2 (213, 214). *M.tb* may also cause necrosis, which leads to tissue damage and dissemination of the bacilli (88).

2.4.5 Granuloma formation and immunopathology

Granuloma formation is regarded as a hallmark of *M.tb* infection. A granuloma is a well-organized structured collection of innate and adaptive immune cells that is formed to contain the pathogen (202). This is often made up of infected innate cells such as macrophages, DCs, neutrophils and giant foamy cells at the centre, which are typically surrounded by a cuff of T and B cells (97, 202, 215) (**Fig. 11**). Granuloma types include caseous granulomas, non-necrotising granulomas, necrotic neutrophilic granulomas and completely fibrotic granulomas (97). The caseous granuloma is characterised by the formation of a cheese-like substance, which indicates liquefaction of the tissue. The caseous granuloma consists of epithelioid macrophages, surrounded by lymphocytic cuff of T and B cells. Neutrophils can also be found in a caseous granuloma. The non-necrotising granulomas are characterised by macrophages surrounded by few lymphocytes (2, 97).

The granuloma can be a hypoxic environment and may be characterized by low nutrient content. *M.tb* can persist in these conditions by altering gene transcription patterns and adopting a dormant lifestyle (216, 217). T cells activated through antigen presentation by DCs secrete IFN- γ that activates infected macrophages to kill *M.tb* through the production of NO. TNF- α is important in activating macrophages and maintaining the granuloma structure. *M.tb* may continue to replicate or remain dormant in the granuloma and may eventually resume replication if host immunity wanes. Studies using isoniazid therapy, which acts on actively replicating bacilli, to prevent reactivation showed that *M.tb* continue to replicate during clinical latency (reviewed in (218)). Macrophages containing *M.tb* may undergo apoptosis or necrosis releasing viable *M.tb* (88). When *M.tb* is not effectively killed or controlled within the granuloma, *M.tb* can eventually be

transmitted. Transmission of *M.tb* occurs when the caseous granuloma breaks and the caseum with viable bacilli is deposited into the airways, which are then coughed up into the air and are inhaled by another host either uninfected or with an existing infection (2). However, the formation of caseum and eventual calcification of the granuloma may also be an indication of a successful containment of the bacilli.

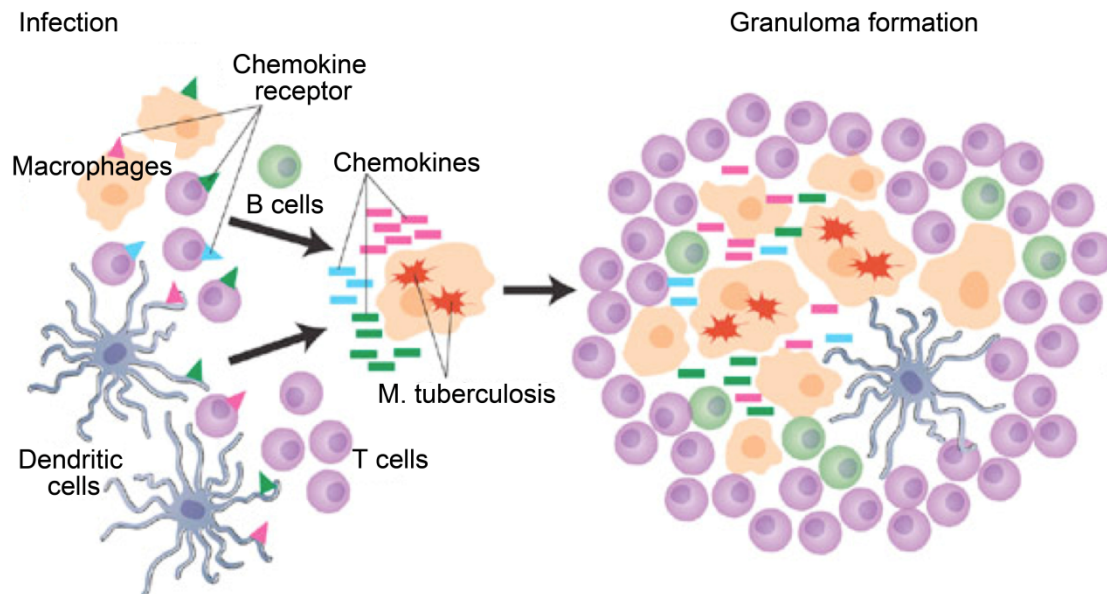


Figure 11: Granuloma formation after *M.tb* infection. Infected macrophages secrete chemokines that attract DCs, B cells and T cells bearing chemokine receptors to the site of infection to form an organised structure with infected macrophages and DC in the center surrounded by T and B lymphocytes (adapted from Flynn J, 2004) (215).

2.4.6 Adaptive immune responses to *M.tb*

Recruitment of T cells that are specific to mycobacteria and activation of macrophages is essential for effective control of *M.tb* within the granulomas (219). The generation of a T cell response is delayed until about 8-21 days post infection (97, 220). T cells involved in immune responses against *M.tb* include cytotoxic T lymphocytes ($CD8^+$) and $CD4^+$ T cells. $CD4^+$ T cells include Th1, Th17, Th22, Th2, and regulatory T cells (Tregs) (126, 133, 221-224). BCG is known to activate $CD4^+$ and $CD8^+$ T cells to produce various cytokines or cytotoxic molecules, which may contribute to immunity to *M.tb* (143, 225, 226).

CD4⁺ T cells are the primary producers of TNF- α , IFN- γ and IL-2 and also express cytotoxic molecules (126). CD4⁺ T cells expressing cytotoxic molecules have been detected in healthy tuberculin skin test (TST) positive individuals and patients with active TB (227, 228). The importance of CD4⁺ T cells in immune responses to mycobacteria is seen in HIV/TB co-infected individuals who have a 10% annual risk of developing active TB as well as a progressive loss in CD4⁺ T cells (229, 230). Although the role of CD4⁺ Th1 T cells and Th1 cytokines such as IFN- γ and TNF- α is well established, other types of T cell responses are also important (221, 231).

Mycobacteria-specific CD8⁺ T cells priming mostly occurs through process of cross-presentation where exogenously derived antigens are loaded onto MHC I molecules (232). CD8⁺ cytotoxic T cells may also mediate direct killing of pathogens through the expression of cytotoxic molecules such as granzyme B, granulysin and perforin (222). These cells can also produce cytokines such as TNF- α , IFN- γ and IL-2 (126).

Th17 cells that produce IL-17 have been described with pro-inflammatory functions and may also be essential in immune responses to mycobacteria (233). BCG induces low production of IL-17 in children but high IL-17 production in adults (234, 235). Another pro-inflammatory cytokine IL-22, which is produced by Th22 and Th17 T cells have also been described and implicated in immunity to *M.tb* (236, 237). BCG also induces the production of IL-22 in adults (235). CD4⁺ T cells producing IL-17 and IL-22 are decreased in peripheral blood of people with active TB compared with healthy individuals. The same was observed for CD4⁺ T cells producing IFN- γ (235). However, Scriba *et al.* (235) showed that there was increased IL-22 secretion in bronchoalveolar lavage (BAL) from individuals with pulmonary TB compared with healthy individuals. IL-22 producing cells have been shown to restrict *M.tb* growth in human macrophages through enhanced phagolysosomal fusion (238).

Tregs mostly produce IL-10 and TGF- β , which can inhibit proliferation and cytokine production by other T cells and are also involved in a homeostatic control of immune responses (224, 239). Inactivation of murine Tregs before BCG vaccination has been shown to enhance T cell responses 14 days after vaccination, but was not associated with protection against TB (240). Another study recently showed that a decrease in BCG-mediated protection against murine TB was associated with an emergence of Tregs (241). In humans, elevated frequencies of Tregs have been reported at the site of

infection and peripheral blood of TB patients compared with healthy TST+ controls (242, 243).

The role of Th2 T cells in immunity to *M.tb* is less well understood but may have a detrimental role, since production of IL-4, IL-13 and IL-10 may inhibit autophagy, which is required for killing of *M.tb*, or induce alternative activation of macrophages (244). Also, individuals with helminth infections have been reported to have high Th2 responses and a down-regulation of Th1 responses to mycobacteria-specific antigens (245).

The role of B cells in immunity to *M.tb* is not as well established as that of T cells, but their presence in lungs and granulomas indicates that these cells may contribute to immunity against *M.tb* (97, 202). B cells probably play a role through production of cytokines that mediate the recruitment of other cells to the lungs to form the granuloma, as well as polarisation of T cells towards Th1 or Th2 (97, 246). Infiltration of B cells in pleural fluid has been shown to increase IL-17 and IL-22 expression in lungs of patients with TB, suggesting a role of B cells in Th17 and Th22 polarisation (247).

2.4.7 Innate and adaptive immune responses in children

Several studies have reported that immune responses may differ with age from newborns through infancy to adulthood. Innate responses have been shown to increase with age in response to TLR ligands (178, 248) (discussed in details in **Chapter 3**). Mycobacteria-specific T cell responses have been shown to increase in early childhood following vaccination with BCG (249). Data from our lab also show that BCG-specific T cell proliferation, cytokine production and cytotoxicity increase with age and peak at 6 weeks of age following BCG vaccination at birth (A Soares, K Cheong, T Scriba, W Hanekom., unpublished data). The high incidence of TB in young infants (Fig. 10) may suggest that the immune responses that limit progression from infection to disease are suboptimal in children.

2.5 Genetic variations in PRRs and immune responses to mycobacteria

Many studies have suggested that host genetics can influence susceptibility to mycobacterial diseases. In addition to environmental factors, there are also genetic factors that influence susceptibility to tuberculosis (250). Genetic variations in NLRs have been associated with altered immune responses or susceptibility to disease including TB and leprosy (251, 252).

Genetic variations of human TLRs affect signaling and inflammatory responses and are associated with susceptibility to human disease (174, 253, 254). Single nucleotide polymorphisms (SNPs) and mutations in the TLR pathway have also been described to regulate cellular function and are associated with susceptibility to TB. These include hypofunctional polymorphisms in TLR1 (T1805G), TLR2 (C597T & G2258A), TLR4 (A896G & C1196T), and TIRAP (C539T, G558T) (173, 174). TLR2 and TLR9 genetic variants have been shown to influence susceptibility to pulmonary TB in different ethnic populations (146). Our studies reported in **Chapter 4** and discussed in **Chapter 5** show that polymorphisms in TLR6 alter innate immune responses to *M.tb* and adaptive immune responses to BCG (255).

2.5.1 Principles and techniques in human genetic studies

Genetic studies are important in understanding genetic factors that contribute to human disease prevention or progression. Several genetic principles and techniques are employed (256). Some of these principles include linkage analyses to identify gene loci, SNP analyses and copy number variants (CNV) for mapping probable causal variants, and genome-wide association. The techniques generally used in these studies include polymerase chain reactions (PCR), quantitative real-time PCR (RT PCR) and microarrays for gene expression. The major applications of these techniques include gene expression profiling, genotyping and DNA sequencing (256). PCR is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand with the use of primers to add nucleotides onto a preexisting 3'-OH group. This requirement makes it possible to delineate a specific region of template sequence to be amplified. This leads to accumulation of billions of copies (amplicons). Unlike PCR, RT PCR enables the detection of PCR amplification during

the early phases of the reaction. Microarray techniques exploit the ability of a given mRNA molecule to bind specifically to, or hybridize to, original DNA template. The expression levels of genes within a cell can be determined by measuring the amount of mRNA bound to each site on the array. A computer is then used to precisely measure the amount of mRNA bound to the spots on the microarray, generating a profile of gene expression in the cell.

In the following chapters, we focus on the identification of innate cell subsets in infants and characterisation of their functional profiles, including expression of cytokines and maturation markers. Changes in these functional profiles were evaluated over the first 9 months of life. We show associations of polymorphisms in TLR6 with production of IL-6 by innate cells. We also discuss the importance of the findings and consequence of our findings in development of novel strategies to control TB and other infectious diseases.

University of Cape Town

Chapter 2: Optimisation of whole blood assay and flow cytometry methods to measure innate cell functions

Results reported in this chapter have been accepted for publication in the Journal of Immunological Methods, and additional data that were left out in the publication have been included.

Title: Optimization of a whole blood intracellular cytokine assay for measuring innate cell responses to mycobacteria

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2.1 Introduction

Monocytes, DCs and granulocytes are important components of the immune responses to microbial infection. There is a need to characterize and optimise assays to measure the function of these cells in human whole blood. In our setting, most of our research involves infants. Obtaining blood samples from this group of individuals is challenging because only small volumes of blood can be obtained. We aimed to optimise assays to measure innate cell function and maturation in small volumes of blood.

Flow cytometry is a technique used to analyse fluorescently labelled cells in fluidics channels, which entails combination of multiparameter measurements and high-speed analysis (257). This allows measurement of the phenotype and functional profile on a single cell level (257, 258). Multiparameter flow cytometry antibody panels can be difficult to optimise especially when larger antibody panels are involved (258). Typically, steps that are addressed in the design and development of flow cytometric antibody panels include; (i) selection of phenotypic and functional markers, (ii) configuration of the flow cytometer, (iii) selection of optimal antibody-fluorochrome combinations, (iv) antibody concentrations (v) determining the optimal voltages, (vi) assessing spectral overlap by fluorescence-minus-one (FMO) controls, (vii) development of optimal gating strategy (259, 260).

Flow cytometry assays may measure intracellular cell processes such as cytokine expression, or cell surface markers. In this chapter, we aimed to develop and optimise flow cytometric assays that measure intracellular expression of key pro- and anti-inflammatory cytokines and maturation markers by peripheral blood innate cells in response to live mycobacteria. We describe multiple important factors for assay success and apply these intracellular cytokine and surface marker staining assays to characterize the innate cell response to the live mycobacterium, *M. bovis* BCG, using 200 μ L of whole blood per condition.

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2.2 Objectives

1. To establish stimulation and cell processing conditions to reliably measure monocyte, DC and granulocyte responses in whole blood by flow cytometry.
2. To develop flow cytometry antibody panels to identify monocytes, DCs and granulocytes and reliably measure: (i) expression of intracellular cytokines, and (ii) maturation markers.

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2.3 Materials and methods

2.3.1 Participant recruitment and enrollment

The study protocol was approved by the Research Ethics Committee of the University of Cape Town, and all participants provided written informed consent. Healthy adults, aged 18-50 years, were recruited at the South African Tuberculosis Vaccine Initiative Field Site in the Cape Town region of South Africa. Exclusion criteria included pregnancy, HIV-1, *M.tb* and any other acute or chronic infections (**See 2.3.2** below). Eligible participants were then enrolled.

2.3.2 Testing for HIV and *M.tb* infection

Participants were consented by trained staff and tested for HIV infection using a finger prick rapid HIV antibody test. This tests for the presence of antibodies against HIV 1/2 and the presence of HIV-1 p24 antigen. If the test was positive blood was taken and sent for a confirmatory test by enzyme-linked immunosorbent assay (ELISA) or Western blot or viral amplification tests by PCR. If confirmed, the individual was referred to appropriate health care services for further management, which included antiretroviral therapy, according to national guidelines.

M.tb infection status was determined by measuring an IFN- γ response to early-secreted antigenic target 6 (ESAT6) and culture filtrate protein 10 (CFP10) peptide pool (2 μ g/mL per peptide, final concentration), which are specific to *M.tb*. Plates were pre-prepared with peptide pools at 2X final concentration and RPMI 1640 as unstimulated control. The plates were then stored at -80°C until required for use, when 100 μ L of blood diluted 1:5 in RPMI 1640 was added. A mitogen, phytohaemagglutinin (PHA, 5 μ g/mL final concentration), was used as a positive control and was added for the last three days of incubation. This is because PHA is a powerful mitogen, which may kill cells if culture exceeds 3 days. IFN- γ secreted into the supernatants after 6 days was determined by ELISA.

ELISA: Ninety-six well flat bottom plate was first coated with capture antibody for IFN- γ and incubated for 2 hours at 37°C. The plate was then washed with PBS containing 0.05% Tween-20 and blocked with PBS containing 1% BSA (bovine serum albumin) for

1 hour at 37°C. The plate was again washed and supernatants and IFN- γ standards (with known values) added to appropriate wells and incubated overnight at 4°C. During this incubation period, IFN- γ in the supernatant or standards binds to the capture antibody. The plate was washed and the detection antibody, which binds to the capture antibody-IFN- γ complex, was added and incubated for 45 minutes at room temperature. The plate was again washed and avidin peroxidase (enzyme), which binds to the detection antibody, added and incubated for 30 minutes at room temperature. The plate was again washed and o-phenylenediamine dihydrochloride (OPD) tablets (Sigma Aldrich), substrates for the enzyme, added and incubated in the dark for 30 minutes. Sulphuric acid was added to stop the conversion reaction. The intensity of the colour formed gives an indication of the amount of IFN- γ present in the supernatant. The plate was read with an ELISA reader and the levels of IFN- γ calculated from standards. Individuals who had IFN- γ values to the peptide pool above the mean + 3x the standard deviation (STDEV) of the unstimulated condition, i.e. [peptide pool value – (mean + 3xSTDEV of unstimulated condition) > unstimulated value] were considered positive and excluded from further analyses.

2.3.3 Toll-like receptor ligands and bacteria

Ultrapure lipopolysaccharide (LPS, TLR4 ligand, 100ng/mL), isolated from *Salmonella minnesota*, was obtained from Invivogen. Viable BCG expressing green fluorescent protein (BCG-GFP, Pasteur strain, donated by Dr Muazzam Jacobs, University of Cape Town) was cultured in our laboratory. Lyophilised live BCG (1.6×10^6 CFU/mL) was obtained from Statens Serum Institute.

2.3.4 Blood collection and processing

6-hour blood stimulation: Heparinised blood was collected from each participant and 180 μ L added to wells of a 96-well plate containing 20 μ L LPS, bacteria or RPMI 1640 (media). The cultures were incubated at 37°C, 5% CO₂ in humidified conditions for 6 hours. For blood stimulated for 6 hours, a reagent to block export of cytokine (Brefeldin A (BFA) or Monensin or both) was added to each well after 3 hours and the plate was incubated for 3 additional hours. After a total incubation of 6 hours, EDTA was added

(2mM final concentration, Sigma-Aldrich), to detach adherent cells, and the plates were incubated for 10 minutes at room temperature. We also tested the effect of no EDTA treatment. Red blood cells were lysed at room temperature for 10 minutes with lysis buffer (**See 2.3.4.1**). This lysing step was repeated to ensure complete red cell lysis. Fixed white cells were cryopreserved in 10% DMSO in heat inactivated fetal calf serum (10% DMSO/FCS) or in FACS Lysing Solution. The cells were then thawed and stained with antibodies for lineage markers and cytokines.

18-hour blood stimulation. Whole blood was stimulated as above without addition of reagent to block cytokine export. White cells from the 18-hour stimulation were harvested in EDTA and cryopreserved as described above. The cells were thawed and stained with antibodies for lineage and maturation markers.

2.3.5 Selection of buffers for cell lysis and staining

We evaluated whether the type of lysis buffer used could affect the frequencies of cells detected in whole blood after staining. Fresh whole blood was collected and immediately stimulated with BCG, LPS or left unstimulated for 6 hours. Red blood cells were either lysed with FACS Lysing Solution (BD Biosciences) or alternative lysing solution made in-house. The alternative lysis buffer solution is made up of ammonium chloride (150mM), potassium bicarbonate (10mM) and EDTA (1mM). Unlike BD FACS Lysing solution, the alternative lysis buffer does not contain fixatives. After stimulation, whole blood was incubated with either of the lysis buffers for 10 minutes, and then spun down and the process repeated once. White blood cells were frozen and later thawed for staining. For alternative lysis buffer, the cells were fixed with the BD FACS Lysing solution after thawing. Cells treated with either of the lysis buffers were then stained and acquired on the flow cytometer.

2.3.6 Titration of antibodies

Antibodies were titrated by serial dilution to determine optimal titer volume. The starting volume was usually twice the volume recommended by the manufacturer to ensure saturation. At least 6 serial dilutions were completed for each antibody. We used whole

blood from adult participants for the titration experiments. The signal to noise (S/N) ratio or frequency of positive cells was determined for all the titration volumes used. The lowest saturating antibody titer volume that yielded the highest S/N ratio or had highest frequency of positive cells was chosen as optimal concentration.

The S/N ratio, a measure of signal intensity relative to background fluorescence, uses the median fluorescence intensities of the cells that stain positive for the antibody (signal) divided by the median fluorescence intensities of the cells that do not stain for the antibody used (noise). This may provide the level of saturation of the antibody binding to the cells expressing the marker of interest. The S/N is highest at saturating titer of the antibody.

2.3.7 Effect of cryopreservation on innate cell function

Because whole blood samples may be cryopreserved before staining and acquisition and also to allow batch staining of samples, we evaluated the effect of cryopreservation on frequencies of cells detected and the function. After 6-hour culture with BCG, we either immediately stained cells without cryopreservation, or stained after cryopreservation. We tested cryopreservation of cells in either FACS Lysing Solution or 10% DMSO in fetal calf serum (FCS). We compared the frequencies of monocytes and mDC or the frequencies of these cells expressing IL-6, when cells were stained immediately after culture or stained after culture and cryopreservation.

2.3.8 Antibody conjugation

The CD66 antibody (Biolegend) is conjugated in-house to QDot 565 (Invitrogen) according to the manufacturer's protocol. Briefly, the QDot nanocrystals are first activated with activating agent for 1 hour. The unconjugated antibody is also reduced with a reducing agent for 30 minutes in parallel with the QDot activation. The Qdot and antibody are both desalted and mixed together for 1 hour for the conjugation reaction to take place. The conjugation reaction is then quenched. The sample is concentrated through ultrafiltration and the conjugated antibody is then separated from the unconjugated antibody by a separation column.

2.3.9 Staining and flow cytometry acquisition

Cryopreserved cells were thawed in batch, and washed twice with either phosphate buffered saline (PBS, without calcium and magnesium) for surface marker staining or BD Perm/Wash buffer for intracellular cytokine staining. Cells were stained with fluorescent antibody cocktails for specific markers (depending on what outcome was being evaluated) in either PBS or BD Perm/Wash, at 4°C for 1 hour. Stained cells were washed and 1 million or all cells acquired on a BD LSR II flow cytometer. Only samples with a total leukocyte number between 500,000 – 1 000 000 were included in the analysis.

2.3.10 Effect of activation on expression of lineage markers

Pathogen recognition may alter expression of lineage markers used to identify cell subsets. We evaluated whether activation of cells with BCG influences expression of these markers. We left whole blood unstimulated or stimulated with live BCG or LPS. We harvested the cells and cryopreserved. Later, we thawed the cells and stained for lineage markers.

2.3.11 Fluorescence-minus-one (FMO)

FMO experiments are necessary to assess spectral overlap for antibody-fluorochrome combinations that may result in false positive signals. In an FMO experiment, all the antibodies in the panel except one are used to stain cells (261). Absence of a signal from an FMO control indicates zero spectral overlap, while detection of a signal in an FMO control indicates spectral overlap from other fluorochromes in the panel into the detector. For the intracellular cytokine panel, we stimulated whole blood samples with LPS in 9 tubes. We also included an unstimulated condition. One of the 9 tubes and the unstimulated condition were stained with the full 8-colour antibody-fluorochrome combination (FP). The other 8 aliquots were stained with 7 of 8 antibody fluorochromes omitting one for each aliquot. The signal for each marker was determined by measuring the frequencies of the stained markers.

For maturation markers, we incubated whole blood with BCG-GFP. A full 7-colour antibody panel with all maturation markers for BCG-stimulated and unstimulated samples was included and used for gating strategy. FMO controls were also included for the 7 colours.

2.3.12 Data analysis

Flow cytometry data were analysed using FlowJo v9.2. Results from single-stained and unstained mouse κ beads were used to calculate compensations, for each run. Cytokine co-expression by innate cell subsets was assessed by boolean gating. Subtraction of background cytokine expression (unstimulated samples) was done using Pestle V1.6.2, while data sorting and analysis were done with Spice V5.1 (262) (<http://exon.niaid.nih.gov/spice>, accessed February 25th, 2011). Background was not subtracted when evaluating the amount of cytokine expressed per cell (MFI). GraphPad Prism v5 was used for data presentation and statistical analysis. The Mann-Whitney or Wilcoxon signed rank tests were used to compare data sets. P values <0.05 were considered significant.

2.4 Results

2.4.1 Selection of phenotypic and functional markers

We were interested in markers that are expressed by innate cells and are relevant to mycobacterial immunity. We selected markers to: (i) identify innate cell subsets (lineage markers); (ii) measure innate cell function (cytokines); and (iii) measure maturation of innate cells (maturation markers). Markers selected to address the study have been introduced in **Chapter 1** (See sections **1.2, 1.5 and 1.6**) and included in **Tables 1 and 2 below**.

Fluorochrome conjugates were then assigned to these markers. The criteria used to assign these fluorochromes included instrument configuration, commercial availability of antibodies, expression levels, fluorochrome staining index and amount of spectral overlap contributed by each fluorochrome. Markers with low expression were assigned fluorochromes with high staining index while markers with high expression were assigned fluorochrome with low staining index. For example, IL-10 was expressed at low levels and was assigned to PE, which has a high staining index. Final fluorochromes assigned to each marker are listed in **Tables 3 and 4**.

2.4.2 Antibody titrations

Titration of new antibody-fluorochrome combinations is important to obtain an optimal signal when compared with background noise. This occurs when the antibody concentrations have reached a saturation point (258). Antibody concentrations that are too low may result in suboptimal signals while antibody concentrations that are too high may result in non-specific binding of the antibody and consequently false positive results, coupled with the fact that these are wasteful.

Figure 12 represents the titration of CD14 Pacific Blue antibody. The titration data were analysed as pseudocolour dot plots (**Fig. 12A**) and histograms (**Fig. 12B**) to determine the frequencies of stained and unstained cells as well as MFI, respectively. The frequencies of CD14⁺ cells increased with increasing antibody volume (**Fig. 12C**). The S/N ratio was highest at 1.25 μ L and 2.5 μ L, and decreased significantly at 5 μ L and 10 μ L.

(Fig. 12D). The 2.5 μ L was chosen as optimal antibody volume as this represented the saturating volume.

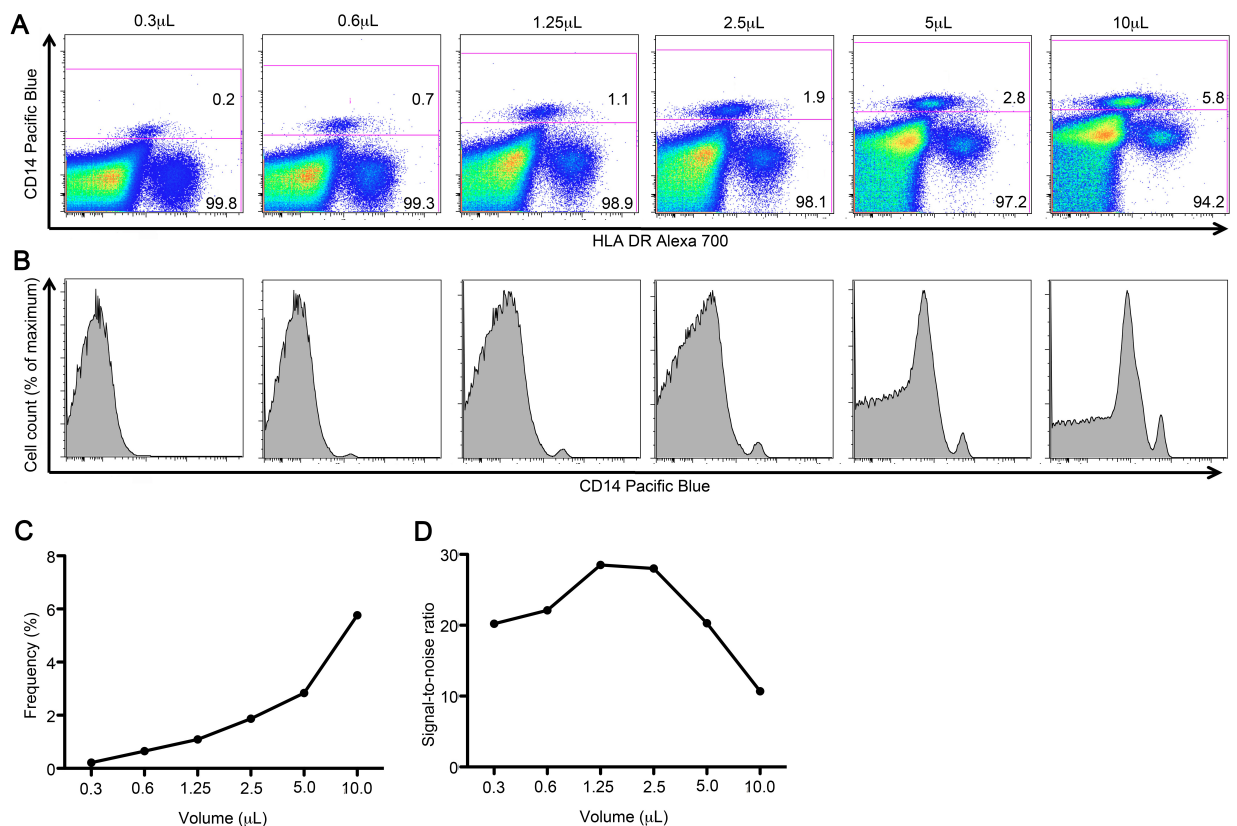


Figure 12: Titration of CD14 Pacific Blue. Frequencies of CD14⁺ and CD14⁻ cells (among innate cells without granulocytes) in unstimulated whole blood (A) Pseudocolour dot plots and (B) histograms. The values above each dot plot represent final antibody volumes. (C). Frequencies of CD14⁺ cells. (D) Signal-to-noise ratios.

2.4.3 Comparison of BD FACS Lysing and alternative lysing solutions for lysis of red blood cells

We evaluated the appropriate lysis buffer for the lysis of red blood cells in freshly collected and stimulated whole blood. We compared BD FACS Lysing solution to an alternative lysing solution made in-house. We observed that the alternative lysis buffer affected the HLA-DR staining leading to a marked reduction in frequencies of HLA-DR⁺ cells compared with BD FACS Lysing solution (Fig. 13A). This also affected CD123 staining. The frequencies of CD123⁺ cells were either markedly reduced or absent when alternative lysing solution was used compared with cells treated with BD FACS Lysing solution (Fig. 13B). We proceeded with BD FACS Lysing solution.

We did not test the possibility of fixing the cells after alternative lysis step before cryopreservation, or fixing cells before red blood cell lysis if this would have an influence on staining and identification of different cell subsets.

CD123 is an identification marker for pDC, which are major producers of IFN- α . We initially wanted to measure IFN- α production by pDC, but, in our hands, BCG or LPS did not activate pDC to express this cytokine (data not shown). We dropped pDC and IFN- α from our panels.

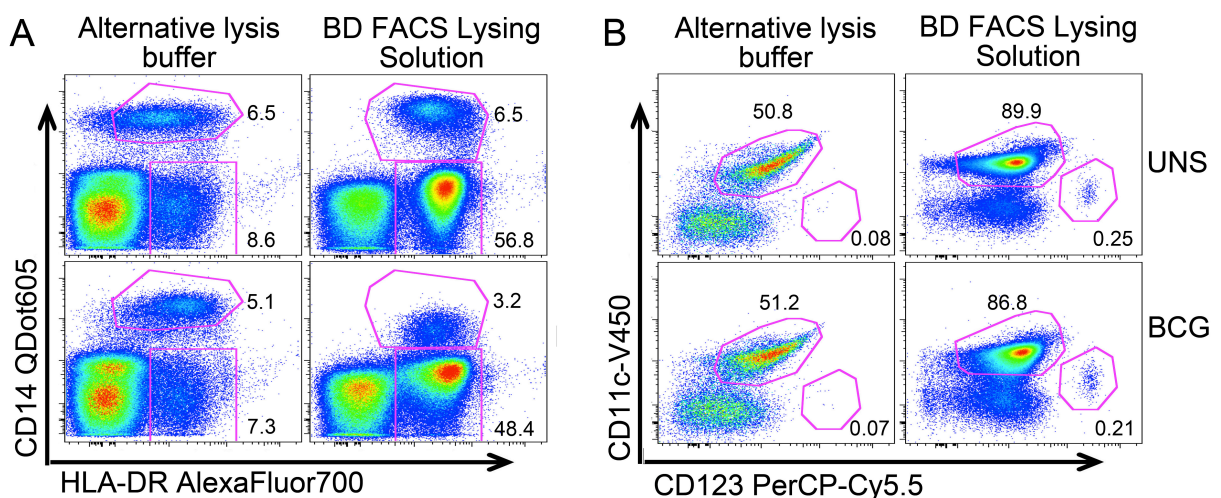


Figure 13: Comparison of lysis buffer for whole blood assay. Fresh whole blood was stimulated for 6 hours with BCG or left unstimulated. Red cells were either lysed with alternative lysis buffer or with BD FACS Lysing solution. The cells were then stained with different lineage marker antibodies. **(A)** Identification of monocytes as CD14⁺ HLA-DR⁺. The CD14⁻ HLA-DR⁺ cells are used to identify dendritic cell (DC) subsets. The numbers in panel A represent the frequencies of the specific cell subsets among total leukocytes. **(B)** Identification of DC subsets: myeloid DC, CD11c⁺CD123⁻; plasmacytoid DC, CD123⁺CD11c⁻. Numbers in panel B represent frequencies of cell subsets among HLA-DR⁺ cells.

2.4.4 Phenotypic definition of granulocytes by flow cytometry

Several studies have described flow cytometric methods for *ex vivo* characterization of peripheral blood monocytes and DCs (263-267). In these studies granulocytes are typically excluded based on their unique size and granularity, before identifying monocytes and mDC using lineage markers, such as CD14 and CD11c.

The different size and granularity of granulocytes, compared with monocytes and mDC, allows identification of these cell subsets by *ex vivo* flow cytometric analysis (263, 264).

Upon stimulation with live mycobacterium BCG-GFP, or LPS, we observed a decrease in side-scatter fluorescence of granulocytes, while the side-scatter fluorescence for mDC and monocytes increased (**Fig. 14A**). This precluded separation of mDC and monocytes from granulocytes using forward and side scatter parameters.

The CD66 isoforms a, c, d and e are members of the carcinoembryonic antigen (CEA) family of the Ig superfamily, and are exclusively expressed on granulocytes and epithelial cells (268). Staining with anti-CD66a/c/e antibody allowed identification of peripheral blood granulocytes (CD66a/c/e⁺, **Fig. 14B**). Since granulocytes express high levels of HLA-DR and low levels of CD14 and CD11c, exclusion of granulocytes was required for accurate identification of CD14⁻HLA-DR⁺CD11c⁺ mDC and HLA-DR⁺CD14⁺ monocytes. Upon granulocyte exclusion the frequency of cells falling into the HLA-DR⁺ gate decreased from 61% (IQR, 58-72%) to 9% (IQR, 7-13%, **Fig. 14C-E**). Similarly, the proportion of HLA DR⁺CD14⁻ cells expressing CD11c amongst all leucocytes decreased from 55% (IQR, 51-58%) to 1% (IQR, 0.7-1.5%) upon exclusion of CD66a/c/e⁺ cells (**Fig. 14C-E**). Using this method, a median (IQR) of 8,049 mDC (6,152-11,432), 27,218 monocytes (19,447-33,437) and 368,000 granulocytes (273,500-412,500) were collected in the respective gates.

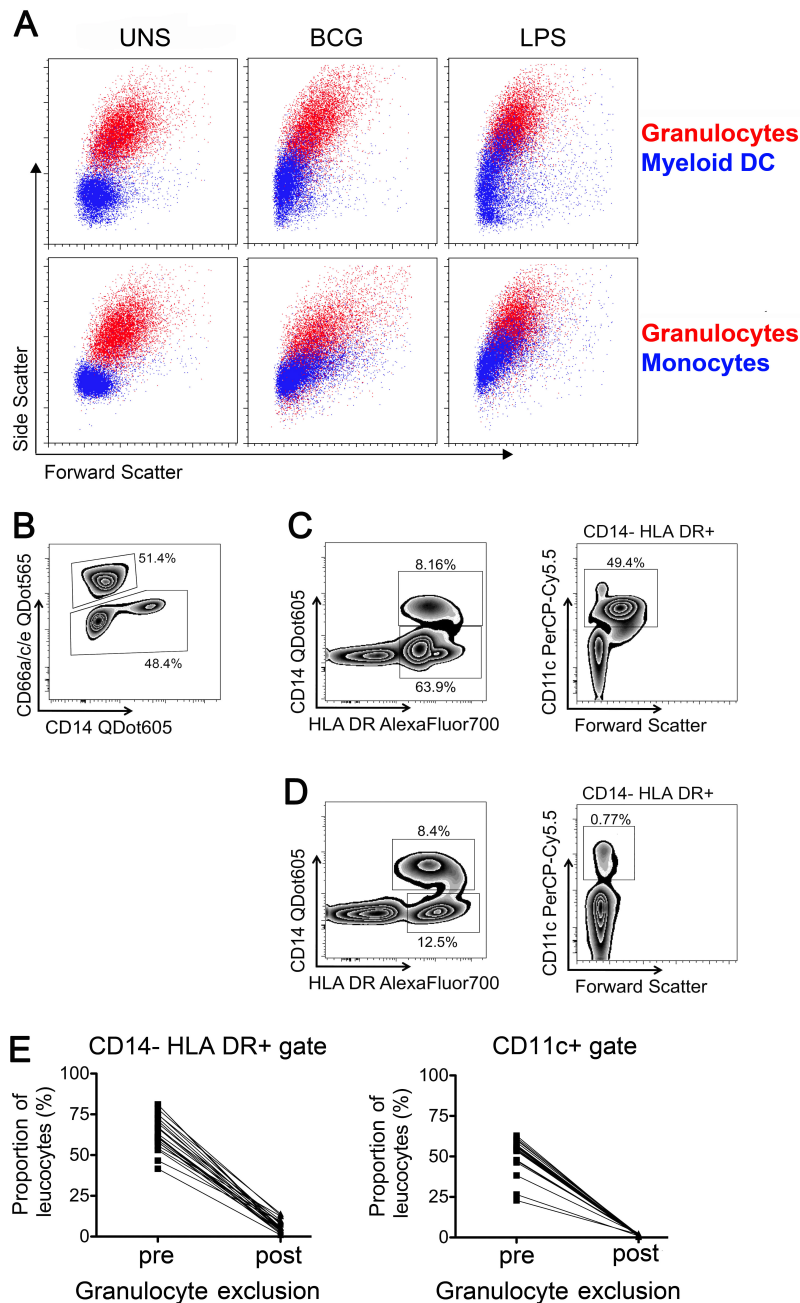


Figure 14: Optimising flow cytometric detection of innate cell subsets. (A) Forward scatter and side scatter properties of granulocytes and mDC (top row) or monocytes (bottom row), after incubation of whole blood with no antigen, BCG-GFP or LPS for 6 hours. Granulocytes were identified as CD66a/c/e⁺, monocytes as CD14⁺ and mDC as CD14⁻, HLA DR⁺ and CD11c⁺ (refer to gating strategy). (B) Identification and exclusion of granulocytes by anti-CD66a/c/e staining. (C and D). CD14 and HLA DR expression by peripheral blood innate cells (left plots), and CD11c expression by CD14⁻, HLA-DR⁺ cells (right plots), before (C) or after (D) exclusion of CD66a/c/e⁺ granulocytes. (E) Frequencies of CD14⁻ HLA-DR⁺ and CD11c⁺ mDC pre and post exclusion gating of CD66a/c/e⁺ granulocytes, in 25 adults. The frequencies of cell subsets among total leucocytes falling into the CD14⁻ HLA-DR⁺ or CD11c⁺ gates are shown in each plot (Refer to gating strategy in Figure 19).

2.4.5 Comparison of BD FACS Lysing and 10% DMSO/FCS as cryopreservation media

Batch thawing of samples cryopreserved after stimulation and fixation allows more standardised antibody staining for flow cytometry. We compared frequencies of cells stained immediately after lysis or stained after lysis and cryopreservation. Prior cryopreservation did not significantly affect the frequencies of mDC or monocyte subsets in unstimulated (**Fig. 15A**, left) or BCG-stimulated blood (**Fig. 15A**, right). Choice of cryopreservation medium may also affect cellular proteins and thus antibody staining. We evaluated the use of either FACS Lysing Solution (FLS) or 10% DMSO in FCS as cryopreservation media. Frequencies of CD14⁺ and CD11c⁺ cells in unstimulated or BCG-stimulated whole blood were also not significantly affected by the choice of cryopreservation medium (**Fig. 15B**). Although cryopreservation did appear to result in lower frequencies of IL-6 expressing monocytes and mDC in 4 donors, this difference was not significant (**Fig. 15C**). Similarly, frequencies of IL-6-expressing monocytes appeared to be higher when FACS Lysing solution was used, compared with DMSO/FCS, but this was not significant (Fig. 15C). Cryopreservation medium did not influence frequencies of IL-6-expressing mDC after incubation of whole blood with BCG. Subsequent experiments were performed with 10% DMSO/FCS as freezing medium.

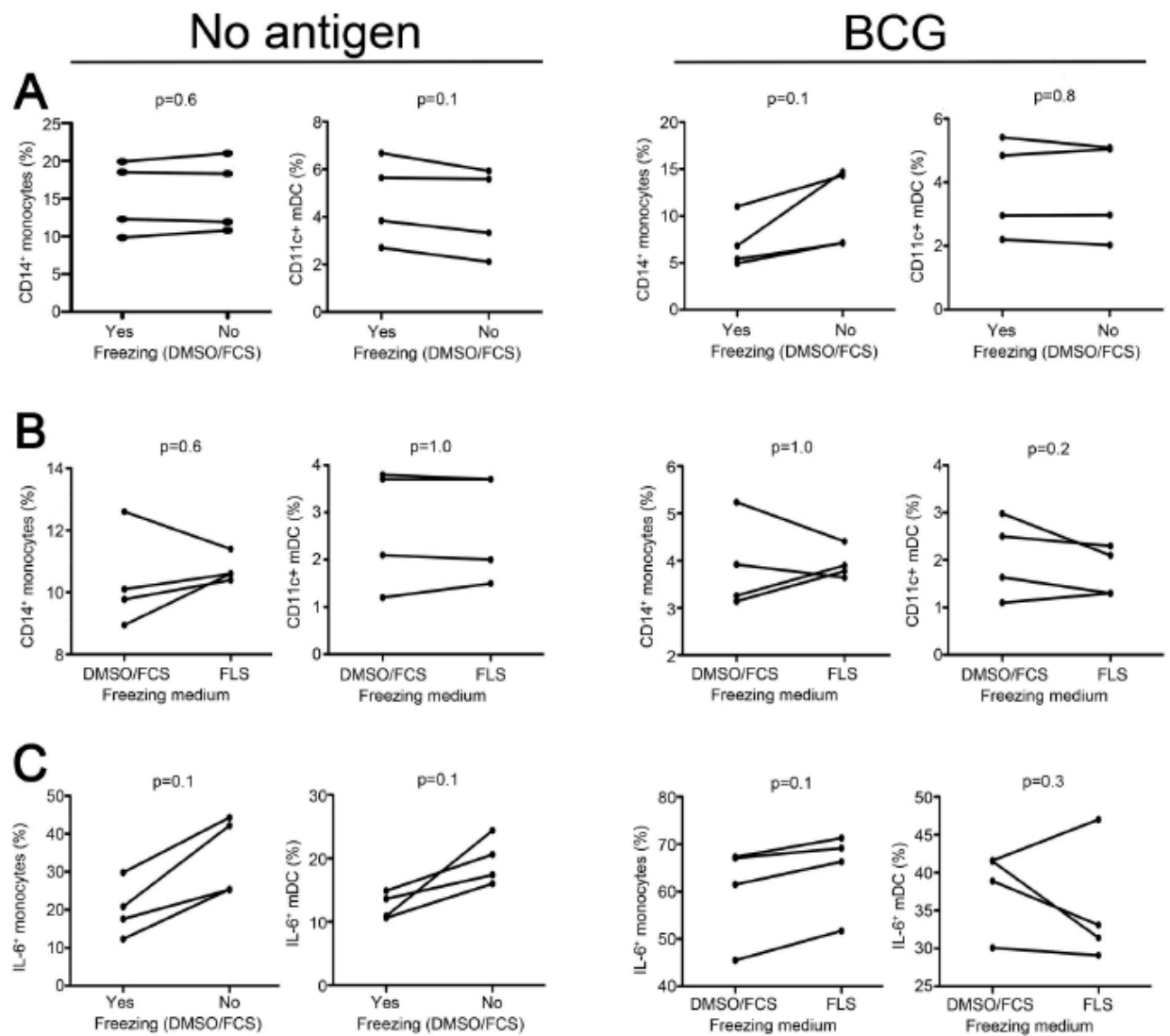


Figure 15: Effect of cryopreservation on flow cytometric analysis of innate cells. Flow cytometric delineation of innate cell subsets in whole blood incubated for 6 hours. **(A)** Frequencies of monocytes and mDC in samples either stained directly or in samples that were cryopreserved before staining. **(B)** Frequencies of monocytes and mDC after cells were cryopreserved in FACS Lysing solution (FLS), or in 10% DMSO in FCS. **(C)** Frequencies of monocytes and mDC expressing IL-6, after stimulation with BCG, detected in samples either stained directly or in samples that were cryopreserved before staining (left plots), or after cryopreservation in FLS or DMSO/FCS (right plots). The Wilcoxon signed rank test was used for statistical analysis.

2.4.6 Effect of activation on expression of lineage markers

We investigated whether innate cell activation upon pathogen recognition affects expression levels of innate lineage markers and flow cytometric delineation of monocytes and mDC. Lower frequencies of CD14⁺ monocytes were detected upon

BCG stimulation, compared with unstimulated samples. This was observed when expression of CD14 was measured by flow cytometric staining with QDot605 or Pacific Blue conjugated anti-CD14 antibodies (**Fig. 16A and B**). A decrease in median fluorescence intensity of CD14 was also observed upon BCG stimulation, albeit only when QDot605-conjugated anti-CD14 was used.

No difference in the frequency of CD11c⁺ mDC among HLA DR⁺CD14⁻ cells was observed upon BCG stimulation, and CD11c fluorescence was only moderately decreased (**Fig. 16C**). Similar results were obtained when whole blood was incubated with LPS (data not shown). Cell activation did not affect the expression of CD66a/c/e or the frequency of CD66a/c/e⁺ cells (data not shown).

The downregulation of CD14 and CD11c necessitated optimal blood processing and antibody staining conditions to identify these key lineage markers after incubation of whole blood with BCG or LPS. Because monocytes are adhesive cells, we tested whether EDTA treatment after stimulation would increase the frequency of CD14⁺ monocytes. Although we observed a higher frequency of CD14⁺ cells in most donors after EDTA treatment, compared with untreated samples, this difference was not significant (**Fig. 16D**). Regardless, EDTA treatment was included for all subsequent experiments.

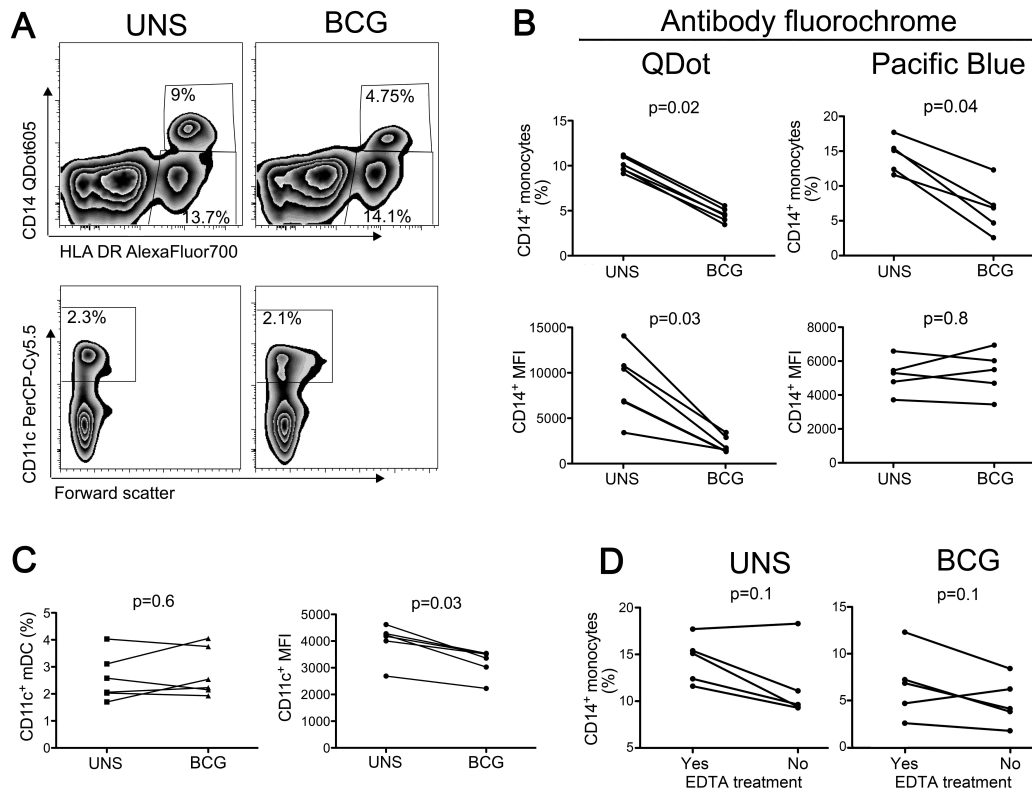


Figure 16: Cell activation downregulates CD14. Whole blood from 5 donors was incubated with no antigen or 3.5×10^5 CFU/mL BCG-GFP for 6 hours before flow cytometric analysis of cell subsets. **(A)** Representative plots showing flow cytometric detection of cells falling into the CD14⁺ and HLA DR⁺ (top plots) and CD11c⁺ (lower plots) gates. **(B)** Frequencies and CD14 MFI of monocytes detected with anti-CD14 QDot (left panels) or anti-CD14 Pacific Blue (right panels). **(C)** Frequencies of CD11c⁺ mDC (left) and MFI of CD11c mDC (right). **(D)** Monocyte frequencies were measured in whole blood that was either treated with EDTA, or in blood not treated with EDTA after 6-hour culture in the absence of antigen (left) or with BCG (right). The Wilcoxon signed rank test was used for statistical analysis.

2.4.7 Choice of staining buffer for QDot-conjugated antibodies

QDots are fluorescent nanocrystals commonly used for imaging and flow cytometric analysis (269). Performance of these fluorochromes can be sensitive to components in staining buffers, such as heavy metals (270, 271). To optimise the antibody staining method for innate cell delineation, we tested the performance of fluorochrome-conjugated antibodies in different staining buffers. We observed low fluorescence of CD66a/c/e-QDot565 and CD14-QDot605 staining when cells were incubated with a single cocktail of all 8 antibodies in PBS (with or without 1% BSA or FCS) (**Fig. 17A**; and data not shown). The low signal of these markers precluded reliable delineation of

monocyte and DC subsets, especially after BCG or LPS stimulation. By contrast, cell staining with the single antibody cocktail in BD Perm/Wash buffer resulted in higher fluorescence of the QDot-conjugates, allowing more precise gating of cell subsets. When cells were first stained with non-QDot-conjugated antibodies in PBS, followed by a second staining step with anti-CD66a/c/e-QDot565 and anti-CD14-QDot605 in BD Perm/Wash buffer, the fluorescence of CD14-QDot605 was even brighter. However, this did not enhance CD66a/c/e-QDot565 fluorescence markedly. The fluorescence of non-QDot-conjugated antibodies was not affected by staining buffer.

1mM EDTA in staining buffer has been shown to improve QDot fluorescence (269). We did not observe any improvement in fluorescence of QDot-conjugated antibodies when cells were stained in PBS containing 1mM EDTA (**Fig. 17B**). We therefore proceeded with single-step staining in BD Perm/Wash (intracellular cytokines), and PBS and Perm/Wash, two-step staining (activation markers) for all subsequent experiments.

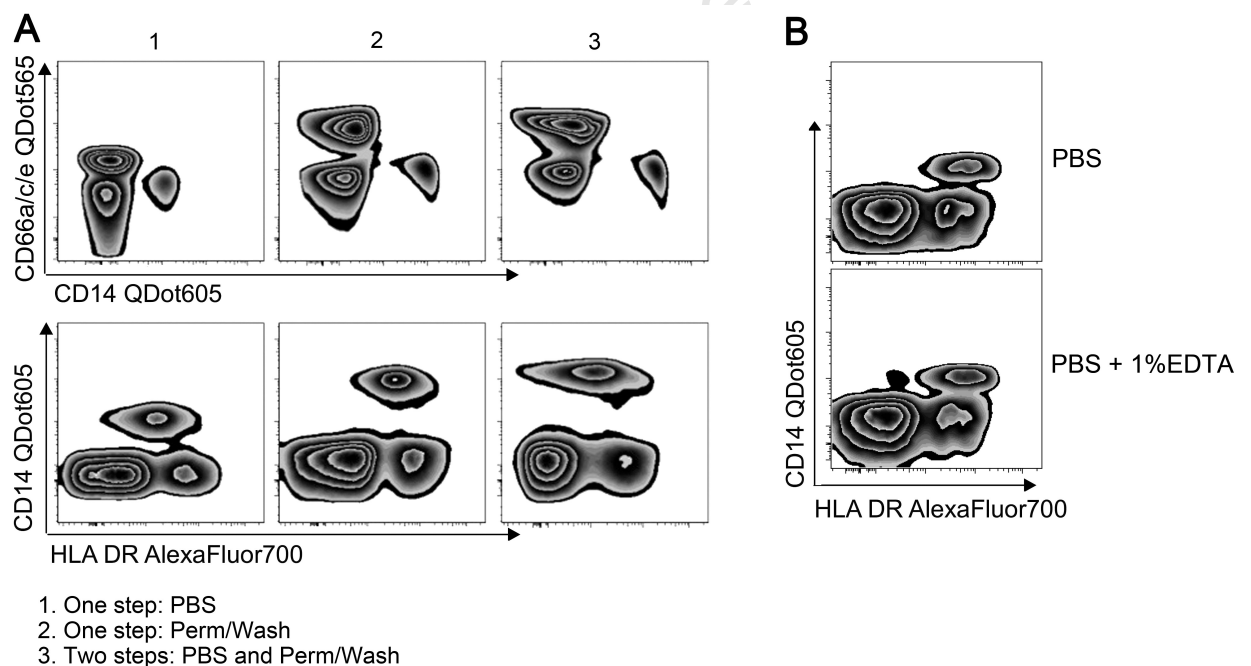


Figure 17: Comparison of buffers for QDot staining. QDot-conjugate antibody staining of whole blood from a single representative donor. **(A)** Single step staining in PBS or Perm/Wash or two-step staining in PBS (for non QDot-conjugated antibodies), then Perm/Wash (for QDot-conjugated antibodies). **(B)** Single step staining with PBS or PBS + 1% EDTA.

2.4.8 Blocking cytokine export

Intracellular cytokine staining is dependent on intracellular trapping of newly synthesised cytokines, resulting in cytokine accumulation at sufficient quantities to enable detection by flow cytometry. We compared frequencies of cytokine expressing innate cells when brefeldin A (BFA) or monensin or both were used as blocking reagents for intracellular protein transport. After staining, we detected higher but not statistically significant frequencies of monocytes expressing cytokines when BFA was used, compared with monensin alone (**Fig. 18A and B**). When BFA and monensin were used together, the results were more comparable to the use of BFA alone. Further experiments were then done using BFA

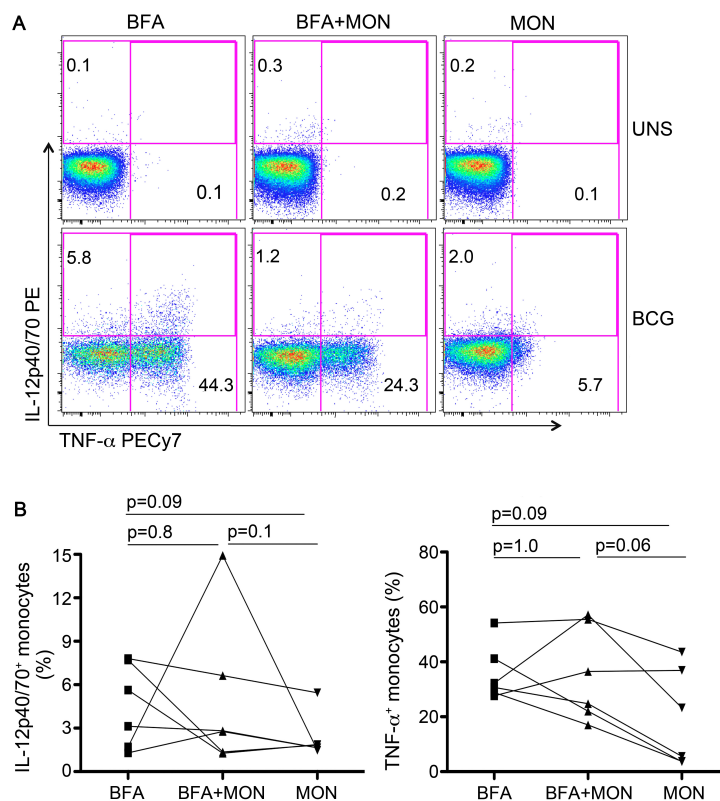


Figure 18: Comparison of BFA and monensin as blocking agents for cytokine export. (A) Whole blood was either incubated with media (UNS) or with BCG for 6 hours. BFA alone (left column), BFA and monensin (middle column) or monensin alone (right column) was added after 3 hours of incubation and cultured for a further 3 hours for cytokine accumulation. The cells were then stained and acquired on the flow cytometer. Shown are the frequencies (%) of each cell subset with the gate. **(B)** Frequencies of monocytes expressing the cytokines (IL-12 and TNF- α) when BFA, monensin or BFA and monensin were used to block cytokine export. All values are background subtracted. Wilcoxon signed rank test was used for statistical analysis. $p < 0.05$ was considered significant. MON, monensin; BFA, brefeldin A.

2.4.9 Fluorescence-minus-one analysis

FMO can be used to assess and quantify spectral overlap that can lead to false positive results. The range for spectral overlap detected in our FMO controls was 0 – 0.01 (**Table 1**). The frequencies of cells detected in each channel were consistent between FMO controls. These frequencies were similar to control samples stained with the full 8-colour antibody panel (**Table 1**). We concluded that the spectral overlap detected for all 8 channels was low and would not compromise the reliability of the panel to measure lineage markers and cytokines.

FMO	AlexaFlu or 700	QDot 605	QDot 565	PerCP -Cy5.5	APC	PE	Pacific Blue	PE- Cy7	FP
HLA- DR	0.00	38.80	37.90	33.10	32.10	31.50	34.20	28.20	31.60
CD14	4.61	0.00	4.92	4.80	4.21	4.71	4.42	4.18	4.35
CD66	51.40	52.30	0.00	52.20	51.70	52.00	49.40	51.30	51.40
CD11c	56.40	58.00	56.30	0.01	57.00	57.30	56.10	56.90	57.00
IL-6	3.05	3.00	3.07	3.12	0.01	2.96	3.08	2.86	2.85
IL-10	0.013	0.015	0.010	0.013	0.014	0.00	0.013	0.018	0.016
IL-12	0.26	0.29	0.26	0.28	0.29	0.29	0.01	0.26	0.30
TNF- α	1.71	1.72	1.57	1.58	1.61	1.68	1.63	0.00	1.72

Table 1: FMO analysis for the intracellular cytokine panel. The spectral overlap was assessed by investigating the frequencies of cells with or without each single antibody-fluorochrome within the leukocyte gate. In this analysis, the full 8-colour panel was run, as well as the 8 combinations of the panel minus a single antibody-fluorochrome (shaded boxes). FP, full panel (LPS-stimulated whole blood). The limit of sensitivity for the flow cytometry instrument used is 0.01%.

The same procedure was followed for the surface panel after stimulating whole blood with BCG-GFP. We found that the spectral overlap for the 8 channels was 0 to 0.2 (**Table 2**). We concluded that the spectral overlap detected for all 8 channels was low

and would not compromise the reliability of the panel to evaluate lineage and maturation markers.

FMO	Alexa Fluor 700	QDot 605	QDot 565	V450	APC	PE	PerCP-Cy5.5	FITC	FP
HLA-DR	0.00	49.60	49.10	51.90	50.30	51.30	49.20	58.00	49.00
CD14	4.21	0.00	3.41	3.81	3.75	3.98	4.25	3.83	3.80
CD66	48.60	49.30	0.00	49.30	49.30	48.90	49.40	52.10	50.40
CD11c	45.6	46.40	44.70	0.00	46.00	46.50	45.80	52.70	45.40
CD83	3.07	2.97	2.77	2.93	0.10	3.15	2.95	2.96	3.21
CD86	1.41	1.37	1.44	1.39	1.36	0.08	1.40	1.70	1.45
CD40	13.8	13.6	13.0	12.6	12.5	11.9	0.00	14.3	13.2
GFP	37.9	38.4	37.3	38.3	38.3	38.5	38.8	0.20	38.6

Table 2: Fluorescence-minus-one analysis for the maturation marker panel. FMO was assessed as above. In this analysis, the full 7-colour panel (plus FITC channel for GFP) was run, as well as the 7 combinations of the panel minus a single antibody-fluorochrome (shaded boxes). We used unstimulated sample as “FMO” for the GFP (FITC channel). FP, full panel (BCG-GFP-stimulated whole blood).

2.4.10 Final antibody-fluorochrome combinations

We chose antibody-fluorochrome combinations of lineage markers and cytokines in **Table 3** as the optimal 8-colour ICS antibody panel, and lineage and maturation markers in **Table 4** for the surface antibody panel.

	Fluorochrome	Marker/Antibody name (clone)	Titre/ μL	Vendor (cat#)
Lineage markers	QDot 605	CD14 (Tuk4)	0.2	Invitrogen (Q10013)
	AlexaFluor700	HLA DR (L243)	0.5	Biolegend (307626)
	PerCP-Cy5.5	CD11c (Bu15)	0.5	Biolegend (337210)
	QDot 565*	CD66a/c/e* (ASL-32)	1.25 [†]	Biolegend (342302)
Intracellular cytokines (ICS)	Pacific Blue	IL-12/23p40 (C11.5)	2.5	Biolegend (501820)
	PE	IL-10 (JES3-19F1)	2.5	Biolegend (506804)
	APC	IL-6 (MQ2-13A5)	1.0	Biolegend (501112)
	PE-Cy7	TNF- α (Mab11)	5.0	BD Pharmingen (557647)

Table 3: Description of the antibody-fluorochrome combinations of the final panel to assess intracellular cytokine expression by innate cells. Manufacturer's names, clones and catalogue numbers are also provided

*The CD66 antibody (Biolegend) is conjugated in-house to QDot 565 (Invitrogen) according to the manufacturer's protocol (**section 2.3.8**)

[†]The antibody was titrated after each conjugation before use. The titer volume was not always the same.

	Fluorochrome	Marker/Antibody name (clone)	Titre/ μ L	Vendor (cat#)
Lineage markers	QDot 605	CD14 (Tuk4)	0.2	Invitrogen (Q10013)
	AlexaFluor700	HLA DR (L243)	0.5	Biolegend (307626)
	V450	CD11c (B-Ly6)	0.5	BD Horizon (560370)
	QDot 565*	CD66a/c/e* (ASL-32)	1.25 [†]	Biolegend (342302)
Maturation markers	PerCP-CY5.5	CD40 (5C3)	0.5	Biolegend (334316)
	PE	CD86 (IT2.2)	2.5	BD Pharmingen (555665)
	APC	CD83 (HB15e)	0.3	Biolegend (305312)

Table 4: Description of the antibody-fluorochrome combinations of the final panel to assess maturation marker expression by innate cells. Manufacturer's names, clones and catalogue numbers are also provided.

*The CD66 antibody (Biolegend) is conjugated in-house to QDot 565 (Invitrogen) according to the manufacturer's protocol (**section 2.3.8**).

[†]The antibody was titrated after each conjugation before use. The titer volume was not always the same.

2.4.11 Gating strategy

We then aimed to develop a flow cytometry data analysis strategy to address our research questions. A gating strategy must be defined during the panel development process to facilitate a focused and objective analysis. The gating strategy is guided by the research questions, which are developed prior to the selection of antigens for the panel. **Figure 19** below shows the gating strategy used to identify innate cell subsets and determine their function.

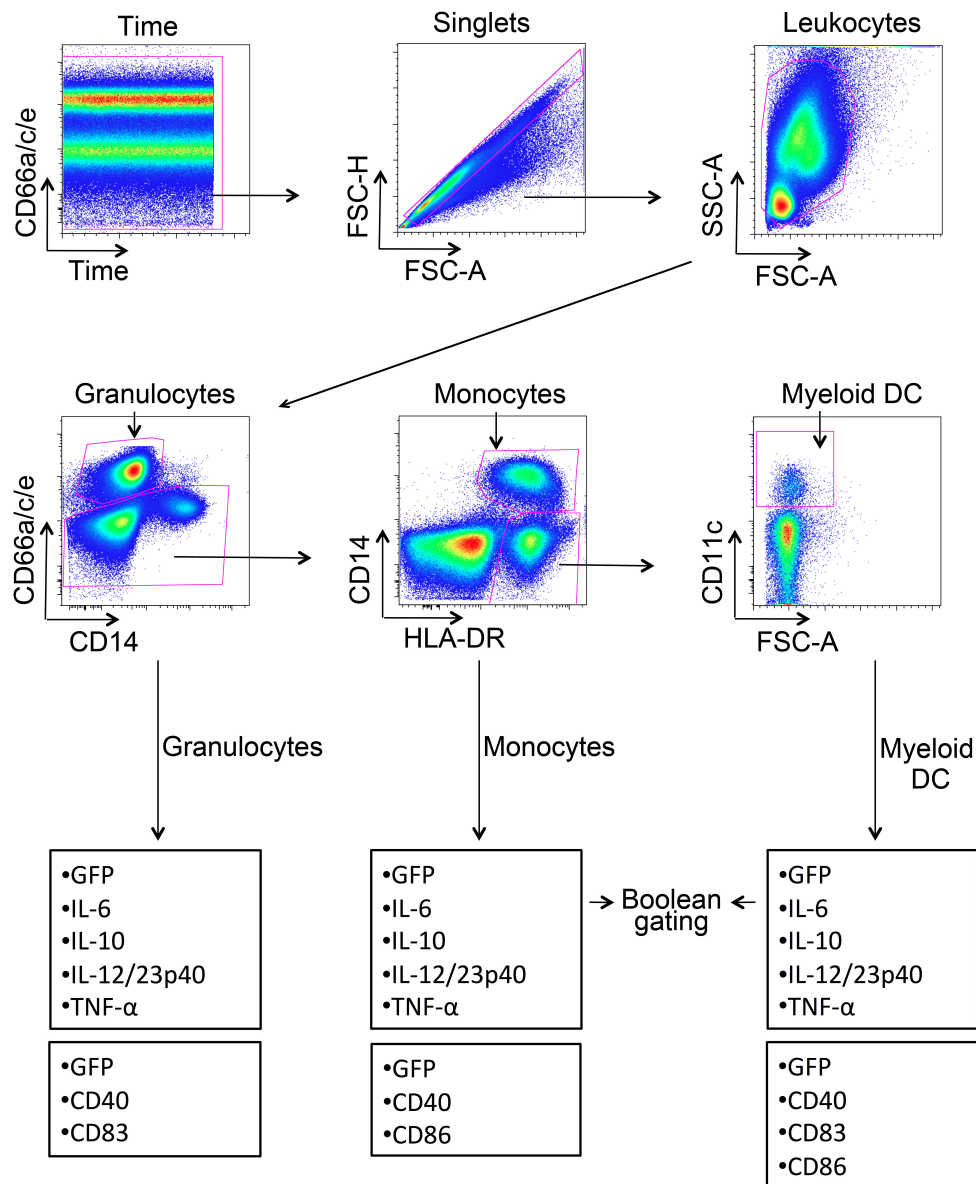


Figure 19: Gating strategy used for flow cytometry analysis. Time gate is used to ensure uniform fluorescence over time during acquisition. Doublets are excluded from the analysis by gating on FSC-height vs FSC-area. Total leukocytes are identified by the SSC-area vs FSC-area gate. Granulocytes are identified as CD66a/c/e⁺ cells. The CD66a/c/e⁻ population is used to identify monocytes (CD14⁺HLA-DR⁺) by gating on CD14 vs HLA-DR. The CD14⁺HLA-DR⁺ cells are used to identify mDC as CD11c⁺ cells. Expression of intracellular cytokines or maturation markers is then determined on granulocytes, monocytes and mDC. Intracellular cytokines are analysed as follows: (1) Total IL-6, IL-10, IL-12/23p40 and TNF-α expression (ii) combinatorial cytokines profiles for IL-6, IL-12/23p40 and TNF for mDC and monocytes, (iii) MFI of IL-6, IL-10, IL-12/23p40 and TNF-α. Activation markers or co-stimulatory molecules are analysed as (i) MFI, and (ii) stimulation index or fold induction, i.e ratio of MFI of stimulated samples (BCG and LPS) over MFI of unstimulated samples.

2.4.12 Inclusion of BCG-GFP

Upon pathogen recognition, activated innate cells may bind to and/or phagocytose the microbe and express cytokines and chemokines (272). Alternatively, activation and cytokine expression may occur in the absence of pathogen binding or phagocytosis. We used a GFP-expressing BCG to allow detection of innate cells that have acquired (bound and/or internalised) BCG, by flow cytometry in which 500 000 – 1 000 000 events were acquired (**Fig. 20A**). We first determined the optimal dose of BCG that would induce responses by innate cells. We observed that the concentration of BCG inoculum was an important determinant of innate cell response to BCG. The proportion of GFP⁺ monocytes and granulocytes reached a plateau at an inoculum concentration of 3.5×10^5 CFU/mL of whole blood, whereas the proportion of GFP⁺ mDC appeared to reach a maximum at a lower inoculum dose of 1.7×10^5 CFU/mL (**Fig. 20B**, upper panel). Surprisingly, expression of the pro-inflammatory cytokine IL-6 was remarkably sensitive to the mycobacterial inoculum concentration (**Fig. 20A and B**). IL-6 expression by monocytes, mDC and granulocytes peaked at a mycobacterial inoculum of 3.5×10^5 CFU/mL and markedly decreased at higher doses (**Fig. 20A-B**). The dose of 3.5×10^5 CFU/mL was chosen for subsequent experiments.

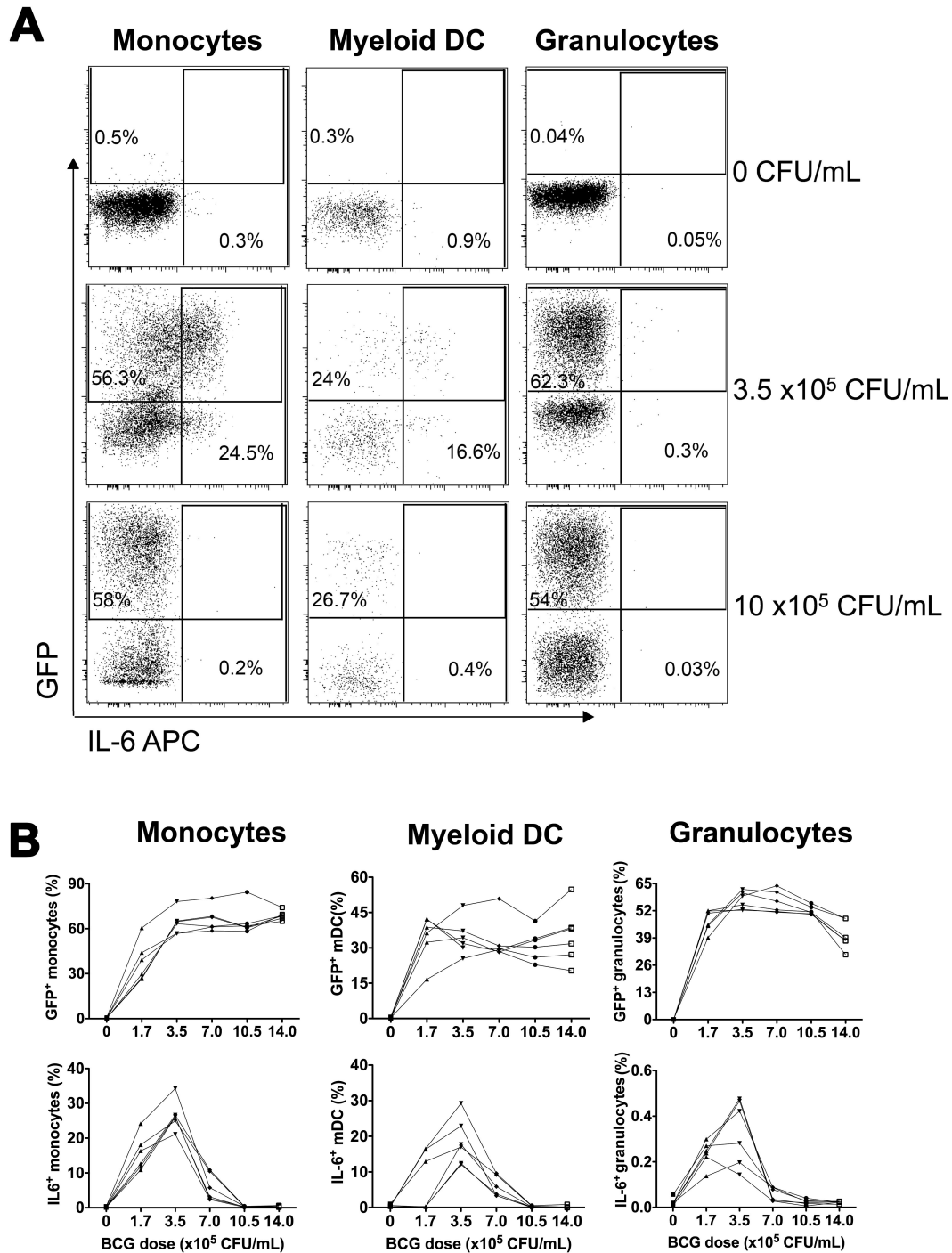


Figure 20: Measurement of BCG acquisition by innate cells. (A) Representative dot plots of IL-6 expression by GFP⁺ or GFP⁻ monocytes, mDC or granulocytes, after incubation without BCG-GFP (upper row), or with 3.5x10⁵ CFU/mL (middle row), or 10 x 10⁵ CFU/mL of BCG-GFP (bottom row). The frequencies of cells falling into each of the 2 boolean gates are shown. (B) Frequencies of GFP⁺ monocytes, mDC, or granulocytes (upper row) after titration of BCG-GFP into whole blood from 5 donors. (Bottom row) Frequencies of total IL-6 expressing monocytes, mDC or granulocytes at different BCG-GFP inocula.

We also evaluated the binding and internalisation of BCG by pDC, HLA-DR⁺CD123⁺CD11c⁻ cells (**Fig. 21A**). We observed that while myeloid dendritic cells efficiently bound or internalised BCG-GFP, plasmacytoid dendritic cells did not (**Fig. 21B**)

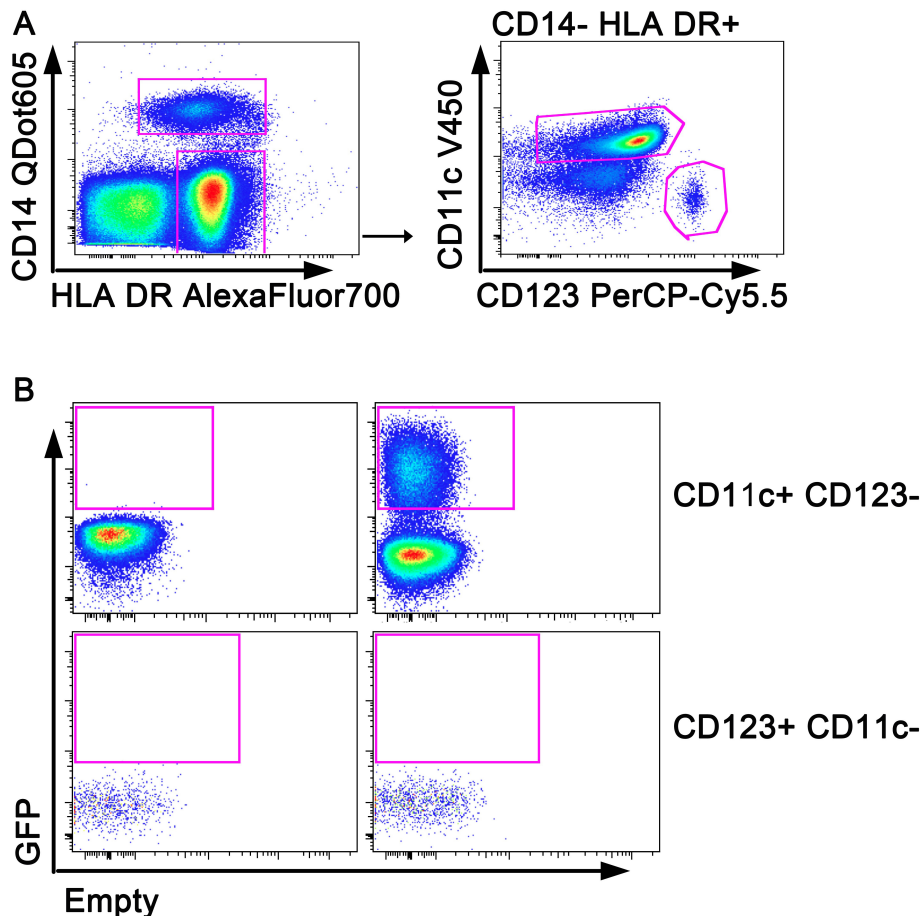


Figure 21: Acquisition of BCG-GFP by dendritic cell subsets. (A). Identification of dendritic cell subsets within the CD14⁺HLA-DR⁺ gate. (B) Plots showing binding and/or internalisation of BCG-GFP by myeloid (CD11c⁺CD123⁻) and plasmacytoid DC (CD123⁺CD11c⁻). Granulocytes were not separated in this experiment so the CD11c⁺ gate also includes mDC and granulocytes, which can both acquire BCG as shown in **Figure 20**. The plots are representative of three adult donors.

We next determined the proportion of GFP⁺ cells among each innate cell subset, as well as the proportion of each subset among BCG-containing cells. A median proportion of 45% (IQR, 37-50%) of granulocytes were GFP⁺ (**Fig. 22A**). A lower proportion of monocytes were GFP⁺ (Median, 36%; IQR, 31-38%) while mDC displayed the lowest proportion of GFP⁺ cells (median, 26%; IQR, 23-30%) (**Fig.22A**). A similar, but markedly

more pronounced picture was observed when we assessed the relative proportions of these innate cell subsets among GFP⁺ cells. Granulocytes comprised 87% (IQR, 82-89), monocytes comprised 7% (IQR, 5-9%), while mDC contributed 1% (IQR, 0.8-1.4%) of all GFP⁺ innate cells (**Fig. 22B**). Granulocytes were thus the peripheral blood innate cell subset with the highest capacity to acquire BCG.

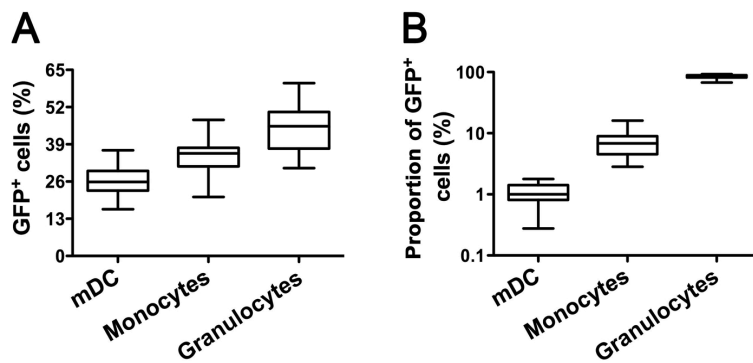


Figure 22: Comparison of mDC, monocyte or granulocyte acquisition of mycobacteria. Whole blood from in 25 donors was incubated with 3.5×10^5 CFU/mL BCG-GFP for 6 hours before flow cytometric analysis of cell subsets. **(A)** Proportion of GFP⁺ cells among each innate cell subset. **(B)** Proportion of each innate cell subset among GFP⁺ cells. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range.

2.4.13 Effect of BCG acquisition on intracellular cytokine expression

We applied the optimised whole blood innate ICS assay to compare cytokine expression between innate cells that bound and/or internalised BCG (GFP⁺) and GFP⁻ negative cells (**Fig. 23**). A substantial proportion of GFP⁻ monocytes and mDC expressed the pro-inflammatory cytokines IL-6, IL-12/23p40 and TNF- α , in various combinations, albeit at lower frequencies than GFP⁺ monocytes and mDC (**Fig. 24A and B**). Notably, monocytes expressing IL-12/23p40 alone were observed at a higher frequency in GFP⁻, compared with GFP⁺ cells (**Fig. 24A**). Monocytes expressing IL-6 alone and mDC co-expressing IL-6 and TNF- α were the dominant cytokine-expressing subsets in both GFP⁺ and GFP⁻ cells (**Fig. 24A and B**).

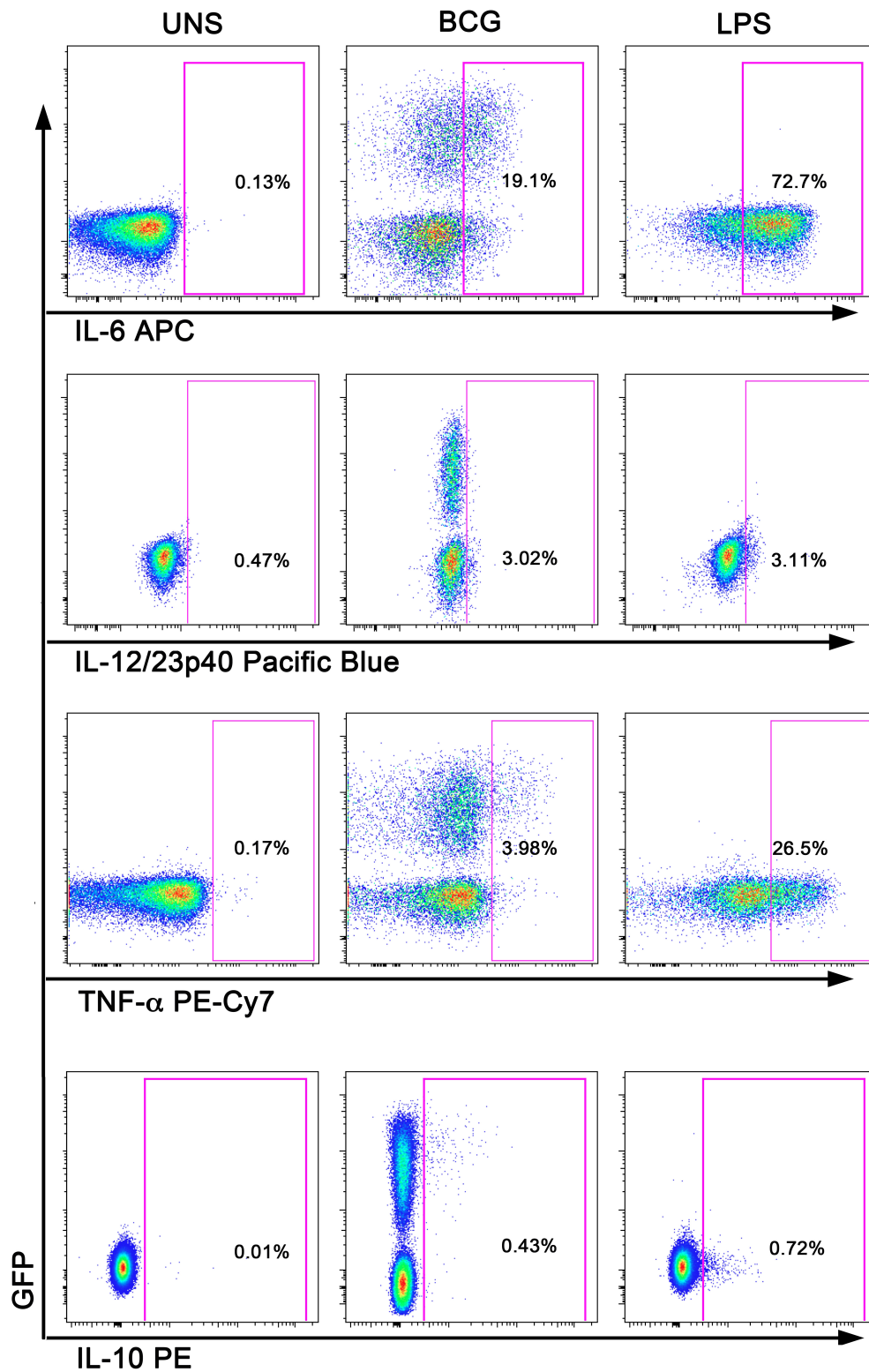


Figure 23: Cytokine expression by monocytes. Whole blood was incubated with BCG-GFP, LPS or media for 6 hours. Monocytes were then selected as above and cytokine expression determined. Shown are flow plots from a representative adult donor.

Upon LPS stimulation, monocytes expressing IL-6 alone also comprised the dominant subset, while cells co-expressing IL-6 and TNF- α were prominent (**Fig. 24C**). By contrast, similar frequencies mDC expressed IL-6 alone or co-expressed IL-6 and TNF- α (**Fig. 24D**).

IL-10-expressing cells were detected at very low frequencies compared with IL-6, TNF- α and IL-12/23p40, and IL-10 co-expression with these pro-inflammatory cytokines was generally low. Again, IL-10-expressing monocytes and mDC were observed at higher proportions amongst GFP⁺ cells, compared with GFP⁻ cells (**Fig. 24E and F**). LPS stimulation induced higher frequencies of IL-10-expressing monocytes, compared with BCG (**Fig. 24E**). BCG binding or internalisation induced a higher frequency of cells expressing cytokines.

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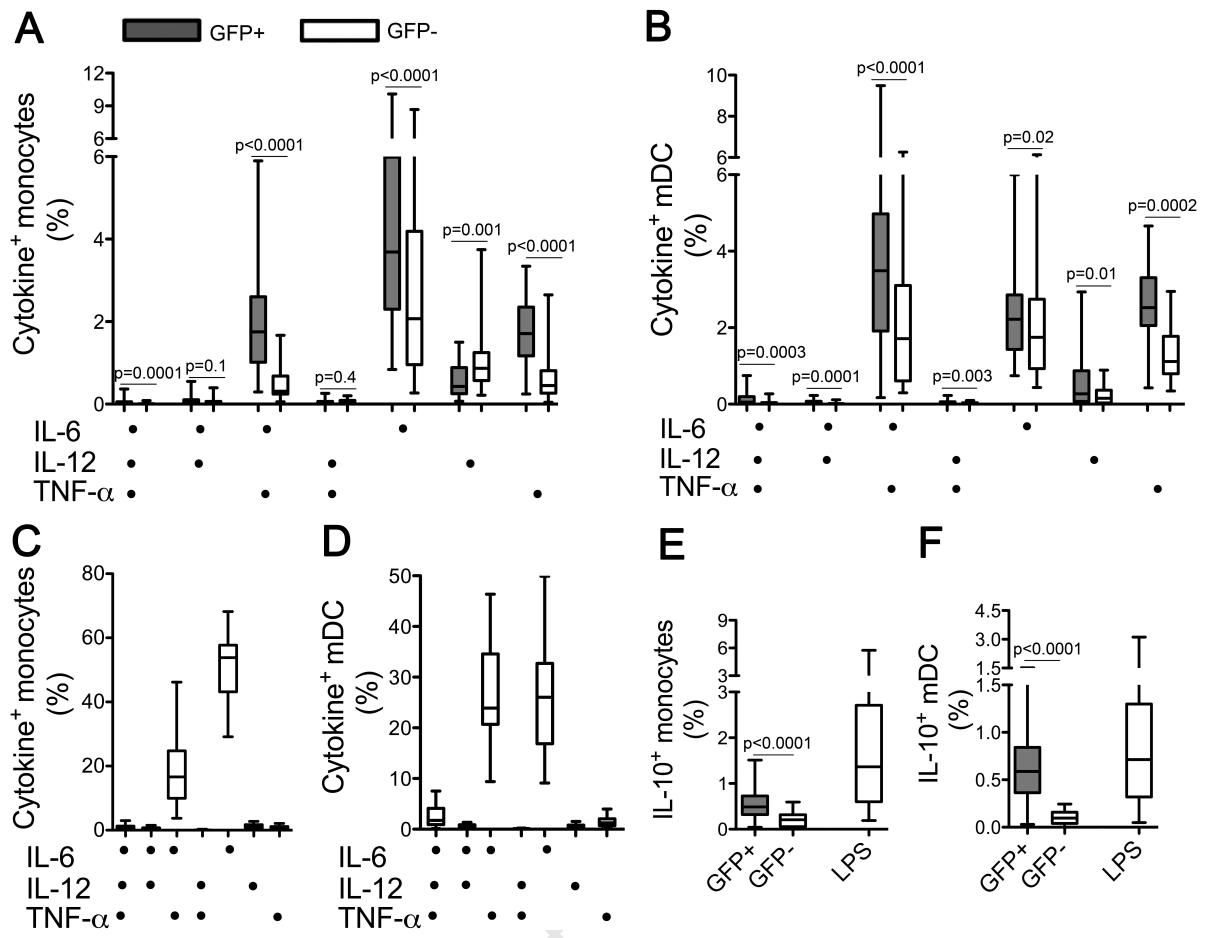


Figure 24: Differential cytokine expression by GFP⁺ and GFP⁻ innate cells. Whole blood from 25 donors was incubated with 3.5×10^5 CFU/mL BCG-GFP for 6 hours before flow cytometric analysis of cell subsets. Cytokine co-expression patterns in monocytes (A) or mDC (B) that have phagocytosed BCG (GFP⁺) or not (GFP⁻). Cytokine co-expression patterns in monocytes (C) or mDC (D) after LPS stimulation. Frequencies of IL-10-expressing monocytes (E) or mDC (F) after whole blood incubation with BCG-GFP or LPS. Differences between GFP⁺ and GFP⁻ cells were calculated with the Wilcoxon signed rank test. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range.

2.4.14 Effect of BCG acquisition on expression of maturation markers

We next evaluated whether GFP⁺ and GFP⁻ mDC expressed similar levels of maturation markers (**Fig. 25A**). Upon stimulation, there was an increase in expression of CD40 and CD83 but CD86 expression was not altered (**Fig. 25B**, left panels). We observed that CD40 expression was higher in GFP⁻ compared with GFP⁺ mDC. CD83 expression was

higher in GFP⁺ mDC, while CD86 expression was similar between GFP⁺ and GFP⁻ mDC.

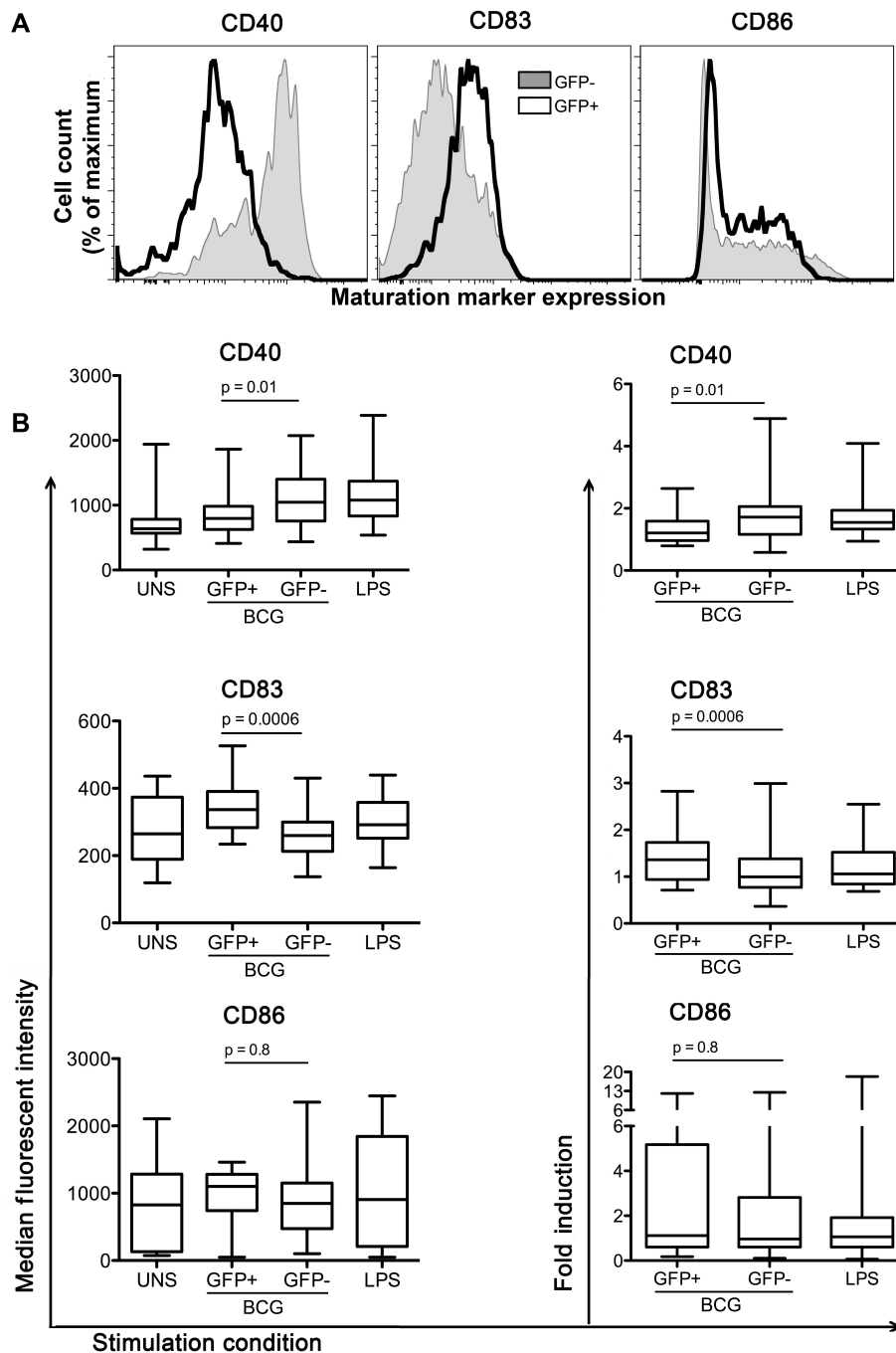


Figure 25: Expression of activation and co-stimulatory molecules by myeloid dendritic cells. (A). Expression of CD40, CD83, and CD86 was determined on BCG-GFP⁺ and BCG-GFP⁻ mDC after stimulation of whole blood with BCG-GFP. **(B).** MFI and fold induction over unstimulated for CD40, CD83 and CD86 expression. Expression of these markers was also determined in whole blood stimulated with LPS. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range. Wilcoxon signed ranked test was used for statistical analysis. n=25

We also evaluated CD40 expression on monocytes (**Fig. 26A**). GFP⁻ monocytes expressed more CD40 compared to GFP⁺ monocytes (**Fig. 26B and C**). CD86 expression in monocytes was higher in GFP⁺ compared with GFP⁻ cells (**Fig. 26D and E**). Importantly, CD86 expression was down regulated upon stimulation (**Fig. 26E**). CD83 is not an activation marker of monocytes and was not evaluated.

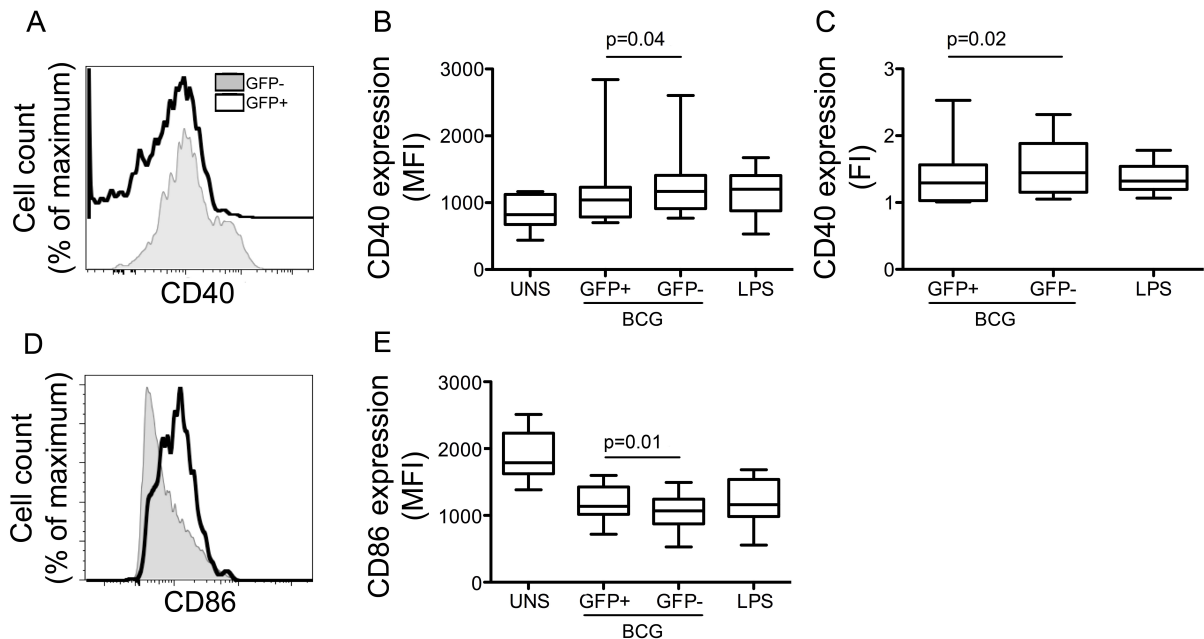


Figure 26: CD40 expression by monocytes. (A). Representative histogram showing expression of CD40 by BCG-GFP⁺ and BCG-GFP⁻ cells. (B). MFI of CD40 expression. (C). Fold induction over unstimulated (UNS) of CD40 expression. Expression of these markers was also determined in whole blood stimulated with LPS. (D) Representative histogram showing expression of CD86 by BCG-GFP⁺ and BCG-GFP⁻ cells. (E) Median fluorescent intensity (MFI) of CD86 expression. Wilcoxon signed ranked test was used for statistical analysis. In B, C and E, horizontal lines represent the median, boxes represent the IQR and whiskers represent the range. n=25

2.5 Discussion

We developed and optimised an assay for measuring intracellular cytokine expression by peripheral blood innate cells in response to viable mycobacteria using very small volumes of blood. Our method presents a number of important variables for optimal performance of this innate cell ICS assay: 1. Exclusion of granulocytes is required for reliable flow cytometric delineation of mDC and monocytes in whole blood; 2. Anti-CD66a/c/e antibody staining allows flow cytometric identification and analysis of granulocytes among activated innate cells; this was not possible using FSC and SSC parameters; 3. Innate cells that have not bound or phagocytosed mycobacteria express cytokines, and this cytokine expression is exquisitely sensitive to the dose of mycobacterial inoculum; 4. Fluorescent antibody staining buffer and cell activation are important determinants of performance and outcomes of the ICS assay.

We show that the increase in monocyte and mDC granularity, and decrease in granulocyte granularity, after whole blood stimulation with BCG or LPS precludes discernment of granulocytes from monocytes and mDC. Further, granulocyte expression of the mDC lineage marker, CD11c, necessitated exclusion of granulocytes to identify peripheral blood mDC. We selected CD66a/c/e as a marker to phenotype granulocytes as it is exclusively expressed by these cells in peripheral blood. We show that anti-CD66a/c/e antibody allows identification and exclusion of granulocytes, and subsequent identification of monocytes and mDC using key lineage markers. Our results highlight that innate assays using whole blood should take into account the marked changes that occur upon innate cell activation, and co-expression of key lineage markers by different innate cell subsets. In our hands, the changes in granularity ruled out identification of granulocytes by SSC and FSC parameters, which is the most common method for phenotyping innate cells *ex vivo* (263, 264).

Our results also underscore an important consideration when identifying DC subsets in whole blood, since no single marker is expressed exclusively by all DC subsets. The most common DC identification methods enumerate HLA DR⁺ and CD11c⁺ DCs after excluding T cells (CD3), B cells (CD19 or CD20), NK cells (CD56 or CD16), and monocytes (CD14) using lineage markers (263, 265-267). However, exclusion gating of lineage marker-positive cells may lead to exclusion of immature DC, which express low

levels of CD14 and CD16 (267). We propose a combination of markers that allows identification of unstimulated or activated granulocytes, monocytes and mDC without these confounders, while allowing simultaneous analysis of cytokine expression patterns of these cells using a single antibody cocktail. Wang *et al.* (267) also showed that CD66a/c/d/e antibody-containing lineage cocktails allowed detection of higher frequencies of DCs, compared with cocktails containing anti-CD14 antibodies.

As we were working in the context of BCG vaccination, we used live BCG in order to generate data that could be easily translated to *in vivo* situations as live BCG is used in vaccination to prevent TB. Incubation of whole blood with BCG or LPS led to marked downregulation of CD14 and moderate downregulation of CD11c. Our results that EDTA treatment did not change CD14⁺ cell frequencies significantly suggest that greater adherence of monocytes upon activation was an unlikely contributor to this finding. Downregulation of CD14 and CD11c was previously reported upon stimulation with high doses of TLR7/8 or TLR4 ligands (273). CD14 downregulation is also described in response to histamines in monocytes (274), LPS and *E. coli* in rabbit alveolar macrophages (275). However, *M.tb* infection has been shown to upregulate the expression of CD14 on monocytes (276). Given the role of CD14 as the TLR4 co-receptor for LPS binding, the downregulation is likely due to macropinocytosis-mediated internalisation upon TLR4 stimulation (277, 278). Lower frequencies of CD14⁺ cells after stimulation may also be due to shedding of CD14 or monocyte death. A spontaneous decrease in CD14 without stimulation has been reported, which could be due to internalisation of membrane-bound CD14 followed by processing and secretion of soluble CD14, or the rapid recycling of CD14-TLR4-MD2 complexes between the plasma membrane and the Golgi apparatus (279). Incubation of PBMC with *E. coli* or Group B streptococcus also leads to a reduction in viable monocytes (272). Similarly, monocytes are known to rapidly die through either classical apoptosis or alternative cell death processes after phagocytosis of mycobacteria (280). Notably, HLA DR⁺CD11c⁺CD14^{-dim} cell population may also contain CD14⁻CD16⁺ monocytes (281). Peripheral blood frequencies of CD16⁺ monocytes were reported to increase during *M.tb* infection, but these cells were more susceptible to apoptosis, and, unlike CD14⁺ monocytes, did not differentiate *in vitro* into monocyte-derived-macrophages (282). We could not delineate these monocyte sub-populations, as we did not measure CD16 expression in our analyses.

The significant reduction in CD14⁺ fluorescence necessitated optimisation of staining conditions for detection of monocytes. QDot-conjugated antibodies (CD14 and CD66a/c/e) performed best when antibody staining was performed in BD Perm/Wash buffer. Fluorescence of QDot nanocrystals is sensitive to staining buffers, and depends on concentrations of heavy metals (270, 271). Low concentrations of cupric ions were recently shown to eliminate QDot fluorescence (269). The latter study showed that 1mM EDTA completely protected the fluorescent properties of these nanocrystals. However, in our hands staining in 1mM EDTA/PBS did not result in enhanced QDot fluorescence.

Analysis of cytokines at the intracellular level requires accumulation of the cytokine at measurable levels. This accumulation is achieved by blocking the export of cytokines from the cells. This is achieved by use of BFA or monensin. Monensin is a sodium ionophore derived from *Streptomyces cinnamonensis* that inhibits trans-Golgi transport by collapsing intracellular Na⁺ and H⁺ gradients necessary for protein transport (283). BFA is a lactone synthesised from palmitate by certain fungi and blocks protein secretion and transport from the endoplasmic reticulum (ER) to the Golgi apparatus (283). BFA usually blocks cytokine export earlier than monensin (283). We tested the use of BFA and monensin for the optimal blocking of cytokine export in our assay and found that BFA performed better than monensin. This was in agreement with Jensen *et al.* (273) who showed that BFA was a better blocker of cytokine export than monensin in response to stimulation of PBMC with CpG, a TLR9 ligand. Nylander and Kallies (283) also showed that BFA was better blocker in a mouse model.

Incubation of innate cells with GFP-expressing BCG permits evaluation of the frequency of cells that have phagocytosed or bound BCG to their surface BCG. BCG acquired by innate cells can either be intracellular or surface-bound. This allows comparison of cytokine expression by cells that have acquired BCG (BCG-GFP⁺) with cells that have not (BCG-GFP⁻). Importantly, our GFP-based assay system did not allow discrimination between cells that have phagocytosed BCG and cells with surface-bound mycobacteria. Although not investigated here, we anticipate that the proportion of cells with internalised BCG markedly exceeds those with surface-bound BCG, as was previously shown for human epithelial cells (284). Interestingly, while the proportion of GFP⁺ cells increased with greater bacterial inocula, the proportion of functional, IL-6 expressing cells peaked at an inoculum of 3.5×10^5 CFU/mL. IL-6 expression of these cells dropped rapidly at higher bacterial loads. Our data highlight that titrating the mycobacterial

inoculum when measuring innate cell cytokine expression is an important optimisation step. Also, this suggests that vaccine doses should be carefully chosen to induce optimal responses after vaccination. The exact mechanism for the lower cytokine expression at high bacterial inocula is not clear, but may be related to the known inhibitory effect of polar lipids, such as phenolic glycolipids (PGL), found in the cell wall of BCG, increase cell death or increase active suppression of cell function by increased mycobacterial dose (285, 286). PGL derived from *M.tb* H37Rv or BCG was shown to inhibit the production of TNF- α and IL-6 by murine bone marrow derived macrophages (BMM) in a dose-dependent manner (285). In humans, monocytes prestimulated with *M. leprae*-derived PGL-1 exhibited increased cytokine production, and loss in production of vascular endothelial growth factor (VEGF) when stimulated with LPS (287). It is unknown whether this sensitivity of *in vitro* innate cell cytokine expression to mycobacterial dose applies to innate cell behavior *in vivo*. Such sensitivity would imply that infection with high doses of pathogen might lead to suboptimal inflammatory responses.

We observed cytokine expression by a considerable proportion of GFP⁻ cells. A higher frequency of GFP⁻ monocytes expressed IL-12/23p40 alone, compared with GFP⁺ monocytes. The significance of this observation is unknown. Cytokine expression by GFP⁻ innate cells is likely due to bystander activation by cytokines secreted by phagocytic cells, or other cells that can directly recognise mycobacteria, such as NK or $\gamma\delta$ T cells. This could also be due to passive or active secretion of lipids or other mycobacterial components from cells, or apoptotic neutrophils or macrophages that have been taken up by other cells including DCs in which GFP expression may have been lost (288). GFP⁻ cells also expressed higher (CD40), similar (CD86) or lower (CD83) levels of activation markers compared with GFP⁺ cells. Upon incubation with BCG, there was downregulation of CD86 expression on monocytes. This may be a mechanism of immunoregulation. Previous studies have also reported impaired maturation of monocyte-derived DCs by low expression of CD80 upon stimulation with mycobacteria (289).

Granulocytes were the major peripheral blood phagocytes of BCG, although little or no cytokine response was detected in these cells. Granulocytes that phagocytosed BCG might have undergone apoptosis and taken up by other cells such as DCs and

macrophages. This may explain the lack of cytokine expression by granulocytes. Our assays did not allow for investigation of apoptosis by innate cells and this will be investigated in the future. This finding that granulocytes were the major cells that phagocytosed BCG is consistent with a recent study showing that neutrophils were the predominant *M.tb*-infected cell subset in sputum and bronchoalveolar lavage from patients with multidrug resistant TB (203). However, these data do not accord with several murine studies that invariably showed alveolar macrophages and DCs to be the predominant populations of BCG-infected lung cells *in vivo* (290, 291). Despite being present at high numbers in lungs of infected mice, granulocytes were not infected to the same extent (290). These differences may simply be due to distinct immune characteristics or presentation of TB infection/disease in mice and humans (203). The different markers and methods for identification of cell subsets may also underlie the discrepant outcomes; for example, we identified granulocytes as CD66a/c/e⁺ while Humphreys *et al.* (290) identified these cells in mice as CD11b^{+Hi}CD11c⁻.

A limitation of the flow cytometric assay described here was the absence of a viability marker to exclude dead cells. Stimulation with BCG or LPS was performed on fresh whole blood, which reduced the likelihood of cell death typically observed when culturing thawed cells. In line with this, we observed no or only very minor evidence of artifactual antibody staining and/or autofluorescence. Although not tested here, a staining step with a fixation-resistant viability dye may be incorporated after red cell lysis, but before cell fixation, to allow exclusion of dead cells, as we recently described for another whole blood assay (292). Another limitation is that we were unable to identify all innate cell subsets and measure their responses to mycobacteria. Monocytes and dendritic cells exist in different subsets, expressing different lineage markers and we were not able to include all possible markers to identify the different subsets. For monocytes we chose CD14 as a lineage marker because 80-90% of monocytes in blood express this marker (13). Other subsets of monocytes express CD16 and using both CD14 and CD16 in the same antibody panel have allowed identification of the different subsets of monocytes in blood (281). Importantly, upon activation, we detected significantly lower frequencies of CD14-expressing monocytes, and we were unable to explain what happened with these monocytes and their function in response to mycobacteria. Similarly we used CD11c as a marker to identify mDC. Two subsets of mDC exist in blood – mDC1 (CD11c⁺CD1c⁺CD141⁻) and mDC2 (CD11c⁺CD141⁺CD1c⁻)

(18). Because other innate cells such as granulocytes can also express CD11c, we introduced CD66a/c/e in our antibody panels to identify and exclude granulocytes before analyzing mDC. Plasmacytoid DCs (CD123⁺CD11c⁻) exist in blood and are important in antiviral immunity. pDC also exist in two subsets, CD123⁺CD2⁺ and CD123⁺CD2⁻ (293). We identified these cells in whole blood but could not determine their major function – IFN- α production – because in our hands, BCG stimulation did not result in expression of this cytokine by pDC.

To summarise, we developed innate cell assays that measure expression of cytokine, activation markers and co-stimulatory molecules by flow cytometry after stimulation with LPS or mycobacteria. These assays may be applicable to studying innate cell responses to any fluorescent pathogen, and can be performed on blood volumes as low as 200 μ L per condition, making this particularly suitable for pediatric studies. In **Chapter 3**, we apply these assays to determine maturational changes in innate responses over the first 9 months of life.

Chapter 3: Maturation changes in innate immune responses over the first 9 months of life.

3.1 General introduction

Bacterial and viral diseases continue to be the major cause of morbidity and mortality in early life (294). The most common causes of death in infants are due to respiratory and diarrheal diseases (295-297). Older children and adults are less susceptible to these diseases (295). The 'immature' neonatal immune system has been suggested to underlie this high susceptibility (296). To lessen the burden of infectious diseases in newborns and infants, new vaccines that are effective, safe and induce long-term protection when administered in the first few days or weeks of life need to be developed. In order to develop such vaccines, we need to understand the immunological basis for increased susceptibility of newborns and infants to infections (294, 298).

Multiple studies have shown differences in DC and monocyte/macrophage function between cord blood and blood from adults (299). To summarise, in comparison to adults, cord blood monocytes and DCs have low expression of co-stimulatory molecules and are relatively unresponsive to LPS or IFN- γ (300). Cord blood monocytes also have reduced capacity to differentiate into DCs (299). Cord blood monocyte-derived DCs (MDDC) display lesser cytokine production in response to TLR and CD40 signaling (299). The mechanisms underlying low responses by the neonatal innate system are not yet fully understood, but most ascribe observations to intrinsic immaturity of the innate immune system.

BCG is the only vaccine currently used for the prevention of TB. This vaccine is highly protective against severe forms of childhood TB, but not against pulmonary TB, at all ages. Innate cells recognise BCG through pattern recognition receptors, including TLRs 2, 4 and 8 (53, 54, 57, 301). Recognition of BCG may result in its phagocytosis and/or production of cytokines. Previous studies have shown that BCG induces the production of cytokines such as IL-10, IL-12, TNF- α and IL-6 in cord blood mononuclear cells (302, 303). Although BCG has been safely given to millions of infants world-wide, many variables surrounding the optimal use of this vaccine remain unknown. As discussed in **Chapter 1**, several factors may influence responses to BCG vaccination.

To address one of these variables, age of vaccination, our group recently completed a project to compare T cell immunity induced by BCG given at birth or given at 10 weeks of age (143). We showed that infants who were vaccinated at 10 weeks had a more optimal vaccination-induced CD4⁺ T cell response, compared with those vaccinated at birth. Delaying BCG administration to 10 weeks of age resulted in (i) a higher specific effector T cell response 10 weeks after vaccination, (ii) a higher specific memory T cell response at 1 year of age, and (iii) a lesser proportion of specific effector memory T cells and a higher proportion of antigen-specific CD45RA⁺CCR7⁺ memory T cells at one year of age. Differential innate immune responses, in particular DC and monocyte function, during the first 10 weeks of life may account for the more optimal immune response to BCG when administered later in life. We hypothesised that monocytes and DCs of newborns, after exposure to *M. bovis* BCG, will exhibit low expression of the co-stimulatory molecules and pro-inflammatory cytokines (IL-12, TNF- α and IL-6), and more anti-inflammatory cytokine, IL-10.

Recent studies substantiate this hypothesis (3, 304). The innate immune responses between newborns (cord blood) and infants or adults were compared. Whole cord blood or peripheral blood was incubated with various TLR ligands, including PAM3 (TLR2/1), poly I:C (TLR3), LPS (TLR4), 3M 002 (TLR8) and 3M 003 (TLR7/8). Adults produced increased levels of IL-12 and TNF- α , compared with newborns, while blood cells from newborns produced higher levels of IL-10 and IL-6. Here we extend these observations to also investigate differences in innate immune responses to mycobacteria between newborns and infants at 10- and 36 weeks of age.

To our knowledge, no published data exists about changes in innate immune responses to BCG in newborns and infants within the first year of life. Most studies investigating changes in innate responses in newborns and infants within the first year of life have measured soluble cytokines secreted in response to purified TLR ligands (248, 305-308). To our knowledge, no study has characterised intracellular expression by different innate cell subsets.

3.2 Objectives and hypothesis

Our objective was to investigate differences in cytokine production and expression of maturation markers, of mDC, monocytes and granulocytes over the first 9 months of life.

We hypothesised that in response to mycobacterial stimulation:

1. Production of pro-inflammatory cytokines by innate cell subsets would increase with age.
2. Production of anti-inflammatory cytokine, IL-10, by innate cell subsets would decrease with age.
3. Expression of maturation markers by innate cell subsets would increase with age.

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3.3 Materials and methods

3.3.1 Study design and participants

This was a cross-sectional study with four groups: newborns, infants at 10 and 36 weeks of age and adults.

1. Newborns born to HIV-negative women through elective caesarian section (i.e., not in active labour). This group was necessary to evaluate innate responses at birth.
2. Healthy 10-week old infants (age range: 8-12 weeks), who have been routinely vaccinated with BCG at birth. We selected this time point because this corresponds to the time at which infants received BCG vaccine in our previous delayed BCG study (143). This also coincides with the Expanded Programme on Immunisation (EPI) schedule for diphtheria, tetanus, pertussis (DTP) vaccine, hepatitis B vaccine (HBV) and haemophilus influenza group B (Hib) vaccine.
3. Healthy 36-week old infants (age range: 34-38 weeks), who have been routinely vaccinated with BCG at birth. We selected this time point as this coincides with EPI schedule for measles vaccine.
4. Healthy adults (age range: 18-50 years). We selected adults as control group because several studies have compared innate responses between newborns and adults.

We aimed to enroll 25 *M.tb* uninfected individuals into each group. We have shown that this sample size is sufficient to measure differences in immune responses between individuals (143, 234). The University of Cape Town research ethics committee approved the protocol for the study.

3.3.2 Recruitment of participants

Pregnant women who were scheduled to undergo elective caesarian section were informed about the study verbally at clinics during antenatal visits. If the pregnant woman expressed interest in the study and was screened for HIV infection, a research team worker explained the study to her, and obtained consent. The consent forms were available in English, Xhosa and Afrikaans; the person taking consent conducted the

consent in the mother's language. If an interpreter was used this was clearly noted on the consent form. If a subject was illiterate or a non-writing individual, an impartial, preferably non study personnel, witness sat in on the consenting process and signed as witness if satisfied that everything in the consent form was explained fully to the mother in her home language. The mother then agreed by means of a thumbprint.

Infants who had received BCG at birth were recruited at the SATVI clinical site in Worcester. If a mother (and/or father) expressed interest in the study, the study procedure was discussed in detail by a SATVI field research worker, and the consent forms provided. The mother (or father or legal guardian) was given a chance to discuss this with his/her partner/other family members and think it over, before formally signing consent. In the case where the mother (or legal guardian) was illiterate, an impartial witness sat in for the consenting as above, if there was need.

Healthy adult volunteers were recruited at the SATVI clinical site in Worcester. Written informed consent was obtained. In the case where the volunteer was illiterate, an impartial witness assisted in the consent process, as above.

The consent process for infants and adults included consent for rapid HIV testing, i.e., pre-test counseling was provided. Two copies of the consent form were completed and signed; one was retained by the mother/parent, the other was stored in a locked cabinet in the SATVI Office.

3.3.3 Exclusion criteria

3.3.3.1 Newborns

1. Newborns born to HIV positive mothers or whose mothers were not screened for HIV infection by the state-run program to prevent mother to child transmission (MTCT) of HIV. HIV exposure or infection of infants may affect innate immune responses as well as the BCG-induced immune response.
2. Mothers who did not give birth through elective caesarian section.
3. Significant pregnancy complications.
4. Any acute or chronic disease in the mother, including TB.

3.3.3.2 *Ten- and 36-week old infants*

1. BCG not administered within 48 hours of birth, as is routine.
2. Known HIV exposure.
3. Clinically significant anaemia. If the study personnel suspected anaemia, a finger prick haemoglobin examination was done. A Hgb<9.5 g/dL led to exclusion. The infant was referred for evaluation and treatment of anaemia.
4. Any acute disease (a cold, acute diarrhea, etc.) at the time the blood draw was scheduled.
5. Any chronic disease after birth, e.g., congenital heart disease and chronic lung disease.
6. Exposure to TB disease in the household.
7. Acute disease, such as newborn respiratory disease and sepsis in the first month of life, from which the infant had recovered prior to the time of blood draw, was not an exclusion criterion.

3.3.3.3 *Adult control group*

1. Any acute or chronic disease.
2. Immunosuppressive medication.
3. Lactation or pregnancy (women).
4. Exposure to TB disease in the household.

3.3.4 Blood collection

For newborns, the placenta was taken after delivery, and a needle attached to a blood collection bag, both heparinised, was inserted into the cord and cord blood was allowed to flow into the bag by gravity. A maximum volume of blood obtainable was collected.

A maximum of 8mL or 20mL of blood was collected from each infant or adults, respectively, into heparinised tubes.

3.3.5 TLR ligands and bacteria, and antibodies

Ultrapure lipopolysaccharide (LPS, TLR4 ligand, 100ng/mL final concentration), isolated from *Salmonella minnesota*, and CpG (TLR9 ligand, 15µg/mL final concentration) were obtained from InvivoGen. The lipopeptides fibroblast stimulating lipopeptide 1 (FSL-1, TLR2/6 ligand, 300ng/mL) and PAM3 (Pam3CSKKKK, N-palmitoyl-S-[2,3-bis-(palmitoyloxy)-propyl]-(R)-cysteinyI-(lysyl)3-lysine, TLR2/1 ligand, 300ng/mL final concentration) was obtained from EMC Microcollections. Viable BCG expressing green fluorescent protein (BCG-GFP, Pasteur strain; 3.5×10^5 CFU/mL final concentration, donated by Muazzam Jacobs, University of Cape Town) was cultured in our laboratory. Bacteria and TLR ligands were prepared at 10 times the final concentration in RPMI 1640, and 20µL was added into 96 round bottom well plates. Polymixin B (BioChemika, 10mg/mL; Steinheim, Germany) was added to the antigen wells containing the lipopeptides to minimise any possible effects of LPS contamination.

The panels of antibodies optimised and described in Table 2 (Chapter 2) were used in this study.

3.3.6 Whole blood assay, staining and flow cytometric acquisition

6-hour blood stimulation. Heparinised blood was collected from each participant and 180µL added to wells of a 96-well plate containing 20µL of LPS, bacteria or RPMI 1640 (media). The cultures were incubated at 37°C, 5% CO₂ in humidified conditions for 6 hours. For blood stimulated for 6 hours, BFA to block export of cytokine was added to each well after 3 hours and the plate was incubated for 3 additional hours. After a total

incubation of 6 hours, EDTA was added (2mM final concentration, Sigma-Aldrich) to detach adherent cells, and the plates were incubated for 10 minutes at room temperature. Red blood cells were lysed at room temperature for 10 minutes with BD FACS Lysing solution. This lysing step was repeated to ensure complete red cell lysis. Fixed white cells were cryopreserved in 10% DMSO in heat inactivated fetal calf serum (10% DMSO/FCS). The cells were then thawed and stained for 1 hour in Per/Wash with antibodies for lineage markers and cytokines (**Table 3 in Chapter 2**) and 1 million or all event acquired on the flow cytometer.

18-hour blood stimulation. Whole blood was stimulated as above without addition of BFA. After 18 hours, supernatants were removed and white cells harvested in EDTA and cryopreserved as described above. The cells were thawed and stained with antibodies for lineage and maturation markers (**Table 4 in Chapter 2**). Cells were first stained in PBS with non QDot-conjugated antibodies for 1 hour followed by washing in Perm/Wash and second step staining in Perm/Wash with QDot-conjugated antibodies and 1 million or all event acquired on the flow cytometer.

The detailed whole blood assay and staining protocols are attached to this thesis as appendices.

3.3.7 Secreted cytokine measurement

Supernatants from whole blood stimulated for 18 hours were thawed. Concentrations of IL-12p40, IL-12p70, IL-10, IL-6 and TNF- α were measured for all samples by Milliplex MAP Multiplex Immunoassay (which is based on Luminex MAP technology) according to the manufacturer's instruction. Volumes of 200 μ L 5-fold serial dilutions of the standard in assay buffer were made to give standard range of 10000, 2000, 400, 80, 16 and 3.2 pg/mL. A sample volume of 25 μ L, either diluted 2- or 25-fold was used. The plate was read in a Bio-Plex machine. Standard curve values were considered as outliers and excluded if the observed/expected \times 100 ((obs/exp)*100) was outside the range of 100 ± 30 , according to the manufacturer's instruction. Cytokine values below the lowest level of detection (out of range (OOR) $<$) were assigned a zero value.

3.3.8 Data analysis

Flow cytometry data were analysed as described in **Chapter 2** (see **section 2.3.12**). Bio-Plex software on Bio-Plex machine was used to evaluate levels of soluble cytokines. The Kruskal-Wallis test followed by Mann-Whitney U test, or the Wilcoxon matched pairs test, were used for statistical analyses.

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3.4 Results

Twenty-five participants were enrolled into each group. All infants and adults were of mixed ethnicity while newborns at birth were of mixed ethnicity (Coloured), Black African and Caucasian origin (**Table 5**).

Age group	Cord Blood	10 weeks	36 weeks	Adult
Total screened for <i>M.tb</i>		30	33	100
Total Positive		3	4	37
Total negative		27	29	63
Total Enrolled	25	25	25	25
Gender	M (13) F (12)	M (11) F (14)	M (13) F (12)	M (10) F (15)
Ethnicity	C (n=10) W (n=12) B (n=3)	C (n=25) W (n=0) B (n=0)	C (n=25) W (n=0) B (n=0)	C (n=25) W (n=0) B (n=0)

Table 5: Characteristic of study participants. M, Male; F, Female; C, Coloured; B, Black African; W, Caucasian.

3.4.1 Pro-inflammatory cytokine responses by monocytes

Pro-inflammatory cytokines mediate the recruitment of cells to the site of infection or inflammation. We evaluated the expression of TNF- α , IL-6 and IL-12/23p40 by monocytes after incubation of whole blood with BCG, LPS or media (UNS) (**Fig. 27A**). Frequencies of monocytes expressing these cytokines and the amount of cytokine expressed per cell measured as MFI were evaluated. Further, we evaluated the

integrated MFI (iMFI), as a product of frequency and MFI. This represents the total functional response of cytokine producing cells (309). Because there were low frequencies of monocytes expressing IL-12/23p40, we compared the levels between blood incubated with no stimulant or with BCG or LPS. The levels of IL-12/23p40 expressed in response to BCG and LPS were significantly higher compared with those in unstimulated samples (**Fig. 27B**).

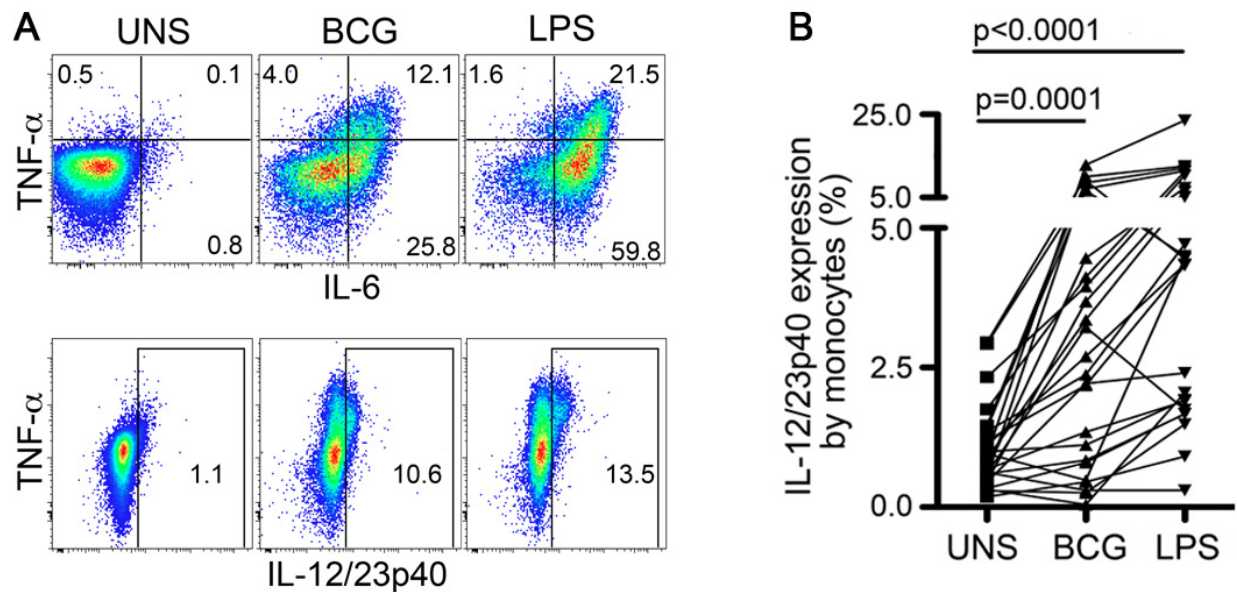


Figure 27: Pro-inflammatory cytokine expression by monocytes. Monocytes were identified as described in gating strategy (**Chapter 2, section 2.4.11; Figure 19**) and cytokine expression determined by flow cytometry. **(A)** Shown are dot plots from an infant at 36 weeks of age. The numbers represent frequencies (%) of monocytes expressing the cytokine. **(B)** Comparison of frequencies of monocytes expressing IL-12/23p40 between stimulation conditions. Wilcoxon matched pairs test was used for statistical analyses.

In response to BCG, there was an increase with age of IL-12/23p40 expression by monocytes, when we evaluated frequencies, MFI and iMFI (**Fig. 28A**). In response to TLR4 ligand (LPS), the same results were obtained (**Fig. 28B**). The levels of IL-12/23p40 expressed by monocytes were also higher in adults compared with newborns in response to BCG and LPS (**Fig. 28A and B**).

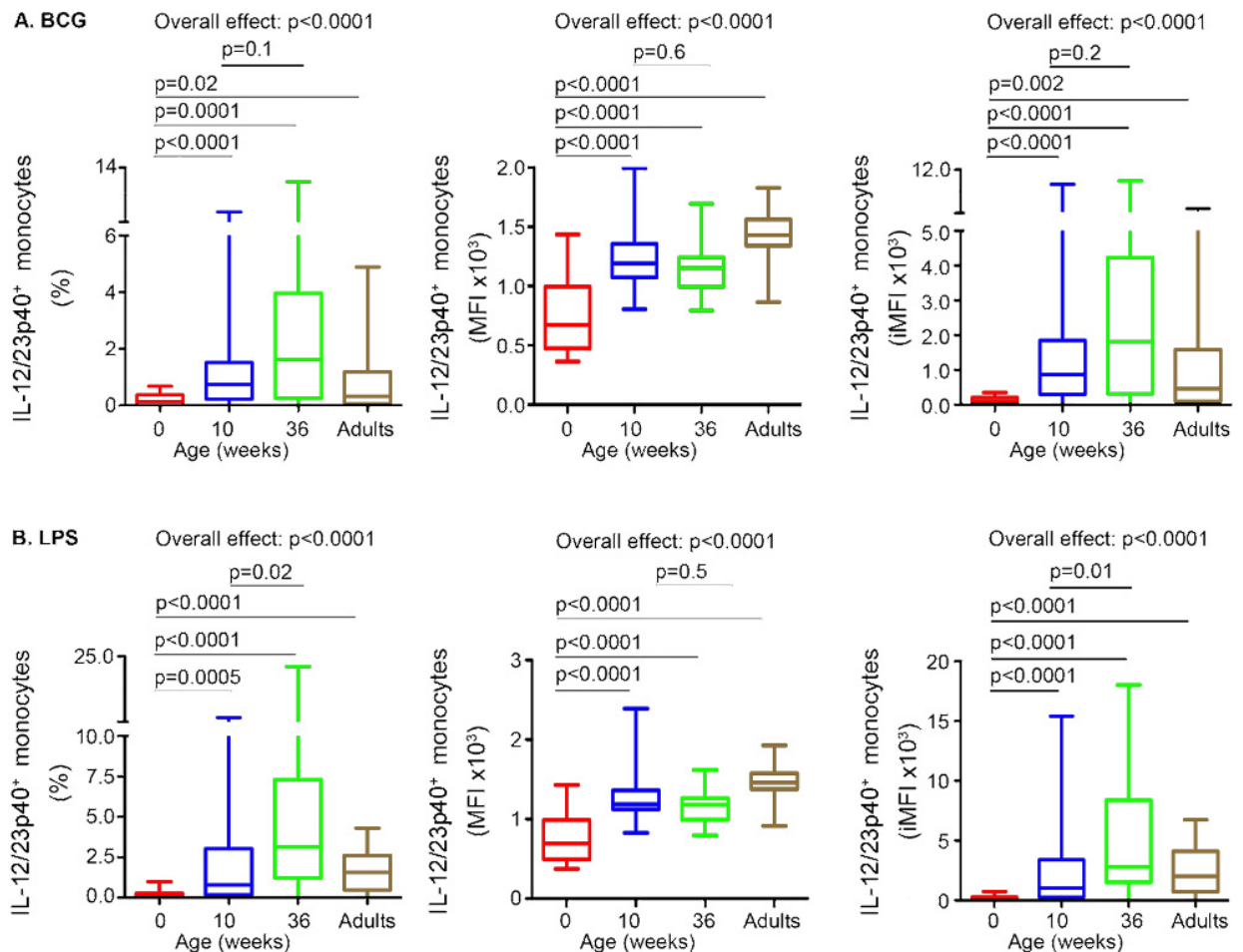


Figure 28: IL-12/23p40 expression by monocytes. IL-12/23p40 expression was determined after incubation of whole blood with BCG (A) or LPS (B). Monocytes were identified as described in gating strategy (Chapter 2, section 2.4.11; Figure 19). Kruskal-Wallis (overall effect over the first 9 months of life) followed by Mann-Whitney tests were used for statistical analyses. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range.

In response to BCG and LPS, TNF- α expression by monocytes also increased with age (Fig. 29A). However, the amount of TNF- α expressed on a per cell basis, measured as MFI, was not different between newborns and infants in response to BCG (Fig. 29B). The levels of TNF- α expressed by monocytes were higher in adults compared with newborns in response to BCG and LPS.

Levels of IL-6 expressed by monocytes also increased with age in response to both BCG and LPS (Fig. 30A and B), although the MFI of IL-6 expressing monocytes was similar in newborns and infants in response to BCG (Fig. 30A). Levels of IL-6

expressed by monocytes were only higher in adults compared with newborns in response to LPS but not BCG.

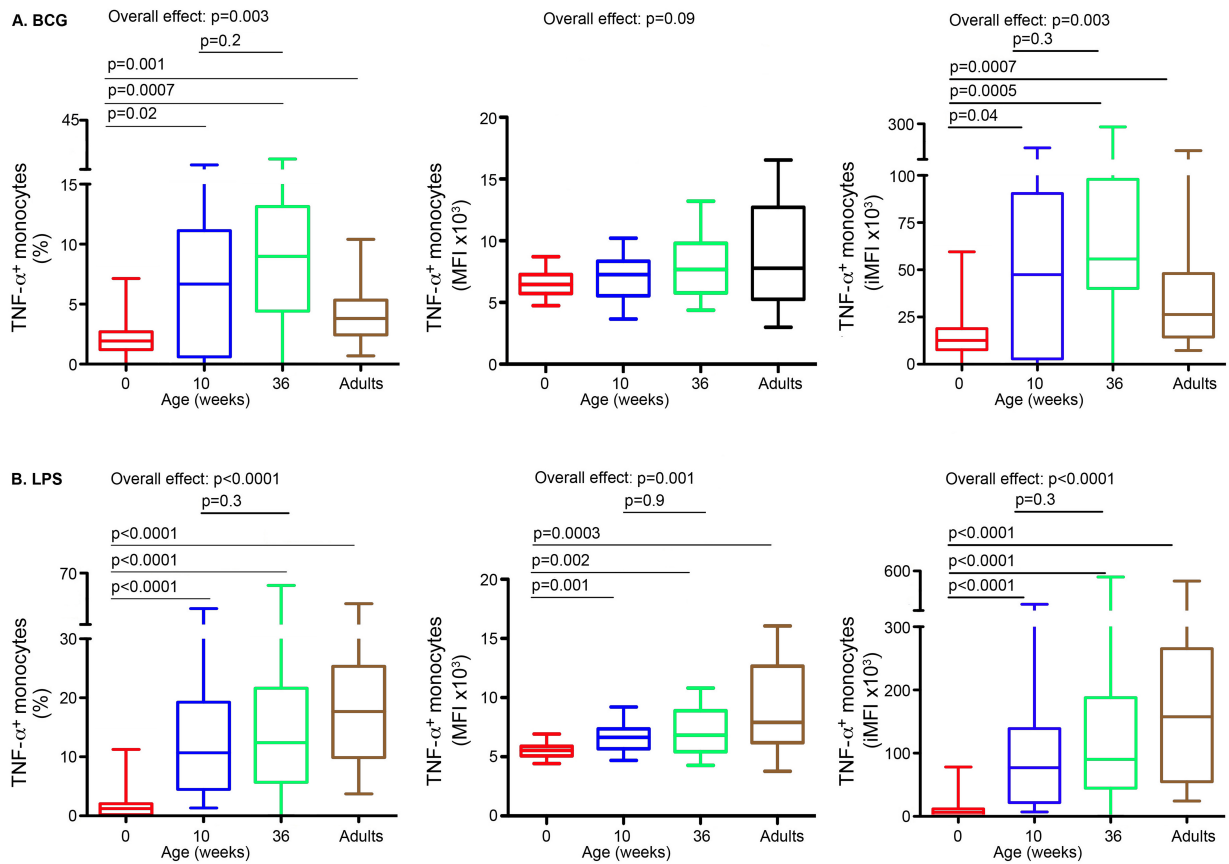


Figure 29: TNF- α expression by monocytes. TNF- α expression was determined after incubation of whole blood with BCG (A) or LPS (B). Monocytes were identified as described in gating strategy (Chapter 2, section 2.4.11; Figure 19). Kruskal-Wallis (overall effect over the first 9 months of life) followed by Mann-Whitney tests were used for statistical analyses. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range.

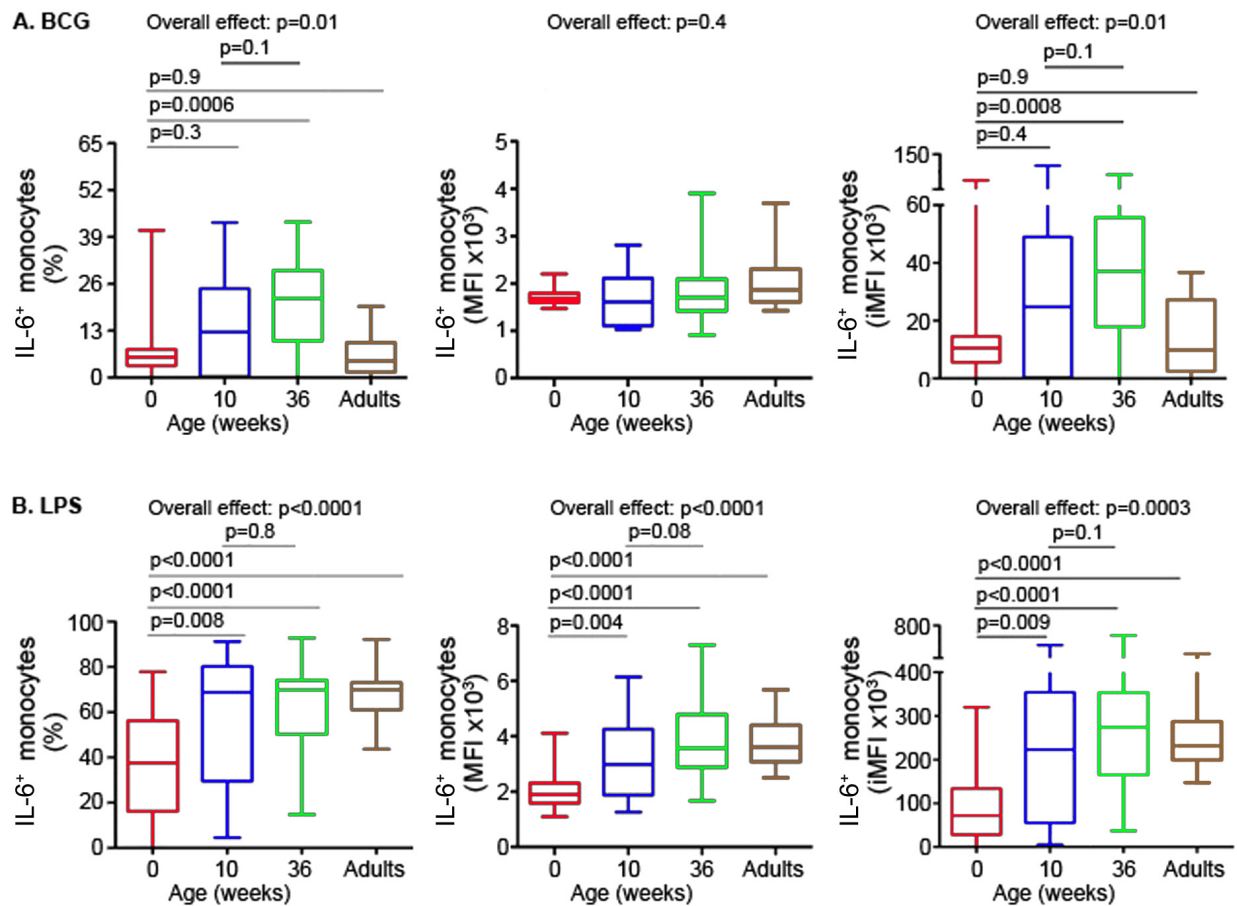


Figure 30: IL-6 expression by monocytes. IL-6 expression was determined after incubation of whole blood with BCG (A) or LPS (B). Monocytes were identified as described in gating strategy (Chapter 2, section 2.4.11; Figure 19). Kruskal-Wallis (overall effect over the first 9 months of life) followed by Mann-Whitney tests were used for statistical analyses. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range.

We also evaluated co-expression of pro-inflammatory cytokines by monocytes in response to BCG and LPS (Fig. 31A). Monocytes expressing IL-6 and TNF- α (TNF- α^+ IL-6 $^+$) or IL-6 alone (L-6 $^+$) in response to BCG or LPS were the dominant subsets (Fig. 31A). Again frequencies of these IL-6 $^+$ and TNF- α^+ IL-6 $^+$ monocytes were lower in newborns compared with infants (Fig. 31A). We evaluated the frequencies of monocytes expressing one (monofunctional), two (bifunctional) or three (polyfunctional) cytokines in any combination. Upon BCG stimulation, lower frequencies of monocytes in newborns were monofunctional, bifunctional or polyfunctional, compared with infants (Fig. 31B). In response to LPS lower frequencies in newborns were polyfunctional or bifunctional compared with infants (Fig. 31C). There were similar frequencies of

monofunctional monocytes in newborns and infants (**Fig. 31C**). The frequencies of monocytes expressing cytokines in any combination were similar in newborns compared with adults in response to BCG but lower in newborns in response to LPS (**Fig. 31B and C**).

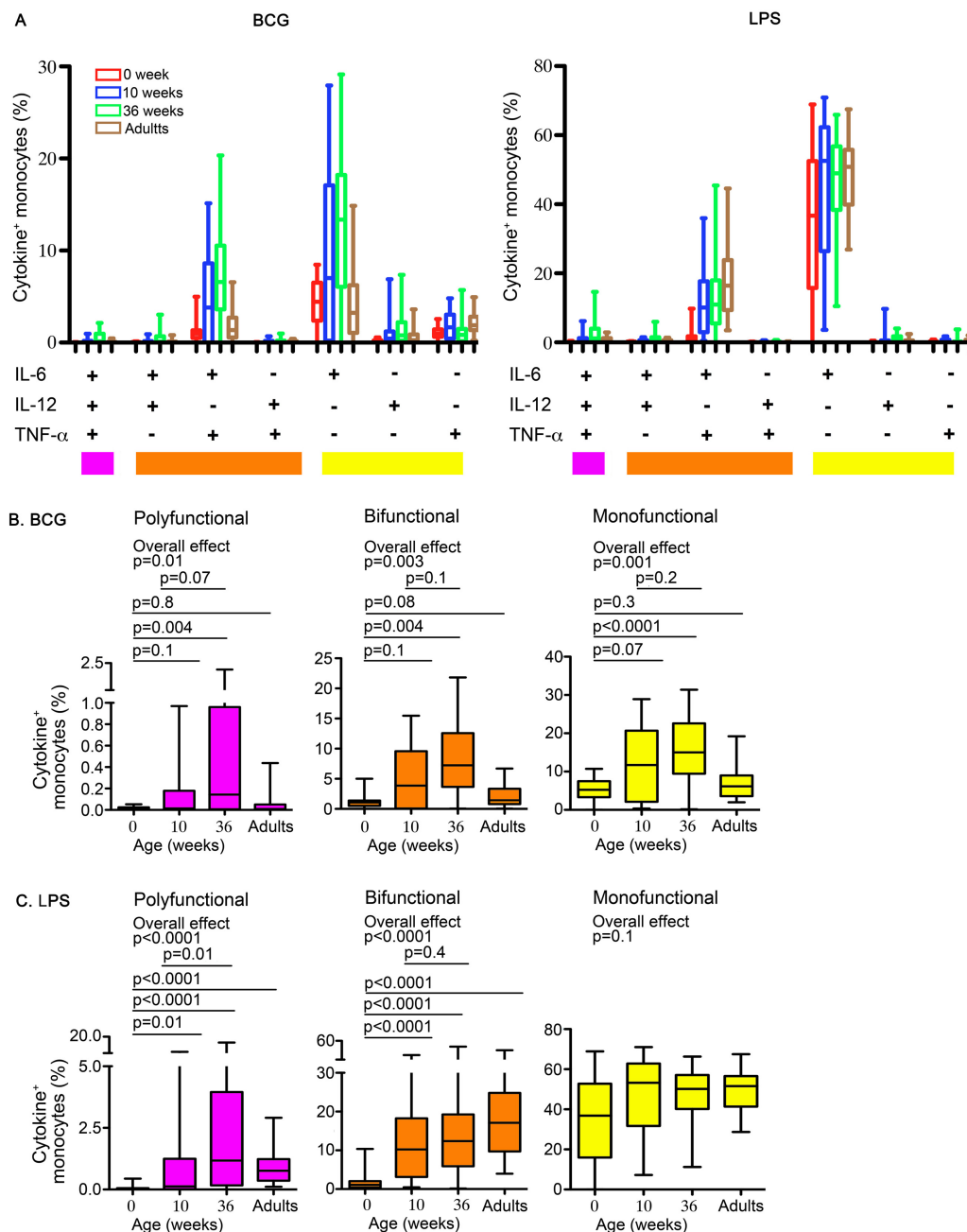


Figure 31: Monocytes expressing multiple cytokines. (A) Monocytes expressing cytokines in various combinations. The ability of monocytes to express 3 (purple), 2 (orange) or 1 (yellow) cytokine in any combination after incubation of whole blood with BCG (B) or LPS (C). Kruskal-Wallis (overall effect over the first 9 months of life) followed by Mann-Whitney tests were used for statistical analyses. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range.

3.4.2 Pro-inflammatory cytokine responses by mDC

mDC are also major producers of pro-inflammatory cytokines. We determined the expression of pro-inflammatory cytokines by mDC after whole and cord blood was incubated with no stimulant or with BCG or LPS, (**Fig. 32A**). The levels of IL-12/23p40 expressed by mDC in response to BCG and LPS were significantly higher compared with those in unstimulated samples (**Fig. 34B**).

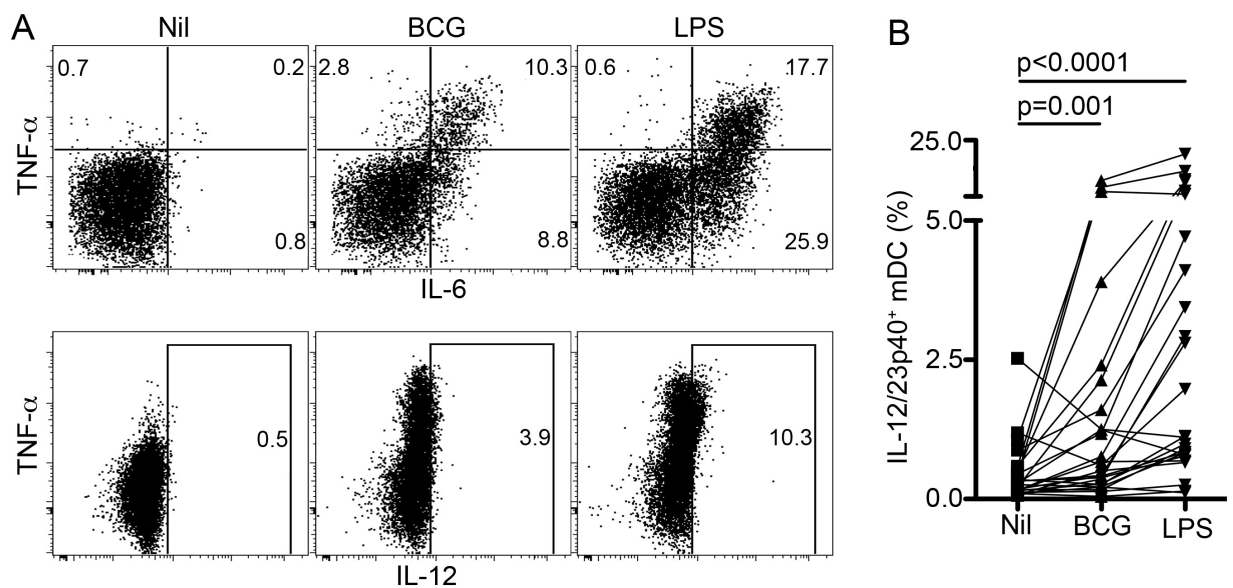


Figure 32: Pro-inflammatory cytokine expression by myeloid dendritic cells. Whole blood was incubated with no stimulant or with BCG or LPS for 6 hours. mDC were identified as described in gating strategy (**Chapter 2, section 2.4.11; Figure 19**) and cytokine expression determined by flow cytometry. (**A**). Shown are dot plots from an infant at 36 weeks of age. The numbers represent frequencies (%) of DCs expressing the cytokine. (**B**). Comparison of frequencies of mDC expressing IL-12/23p40 between stimulation conditions. Wilcoxon matched pairs test was used for statistical analyses.

Upon BCG stimulation, the frequencies of mDC expressing IL-12/23p40 were similar in newborns and infants (**Fig. 33A**). The MFI of mDC expressing IL-12/23p40 increased with age (**Fig. 33A**), while the iMFI of IL-12/23p40 expressing mDC was similar at all ages (**Fig. 33A**). LPS stimulation resulted in lower frequencies, MFI and iMFI of mDC expressing IL-12/23p40 (**Fig. 33B**). The frequencies, MFI and iMFI of mDC expressing

IL-12/23p40 were also lower in newborns compared with adults in response to LPS, but only MFI was lower in newborns in response to BCG (**Fig. 33A and B**).

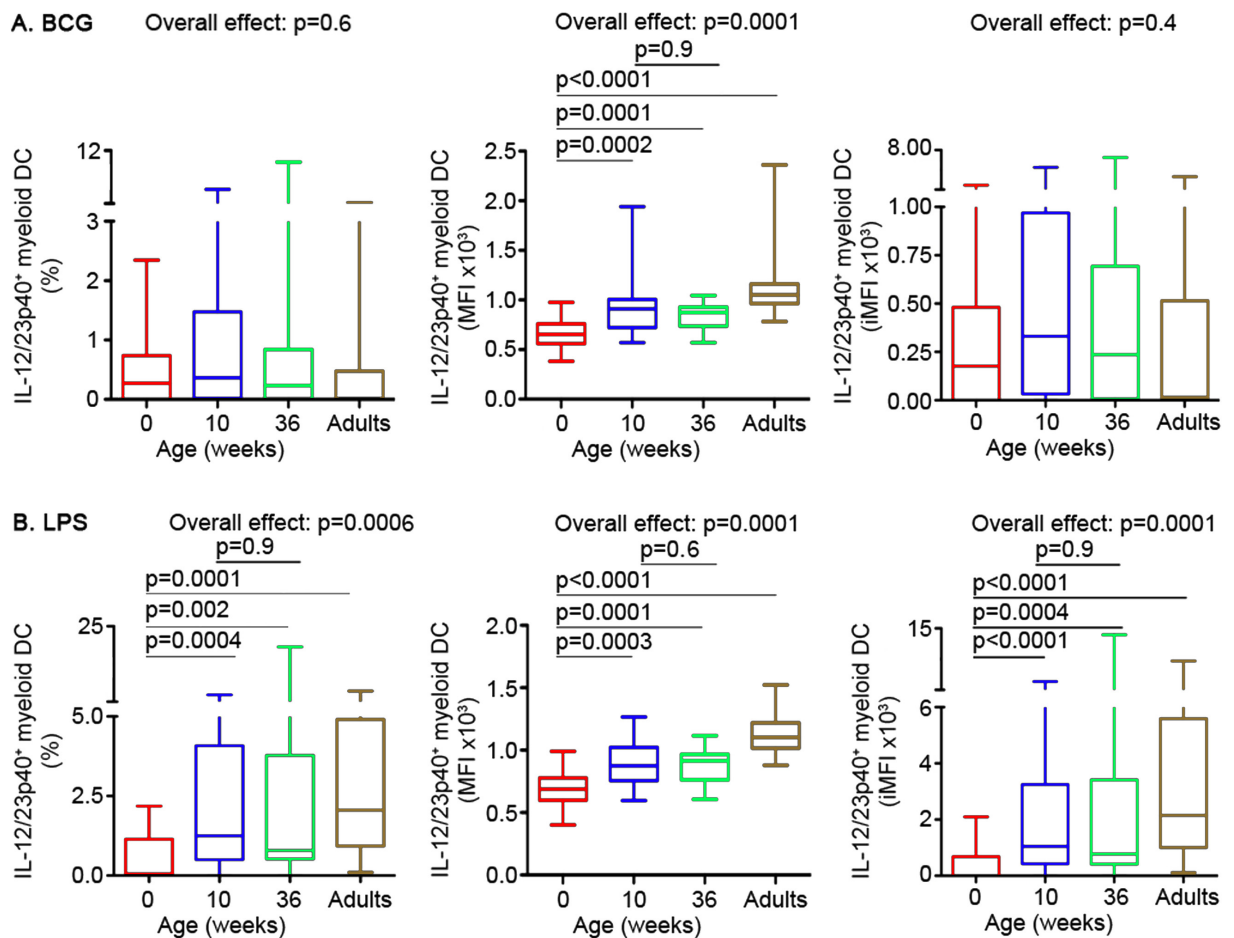


Figure 33: IL-12/23p40 expression by myeloid dendritic cells. IL-12/23p40 expression was determined after incubation of whole blood with BCG (**A**) or LPS (**B**). Myeloid DCs were identified as described in gating strategy (**Chapter 2, section 2.4.11; Figure 19**). Kruskal-Wallis (overall effect over the first 9 months of life) followed by Mann-Whitney tests were used for statistical analyses. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range.

When whole or cord blood was incubated with BCG, we observed similar frequencies, MFI and iMFI of mDC expressing TNF- α in newborns compared with infants (**Fig. 34A**). The levels were also similar in adults compared with newborns. However, LPS stimulation resulted in an increase with age of frequencies, MFI and iMFI of mDC expressing TNF- α (**Fig. 34B**).

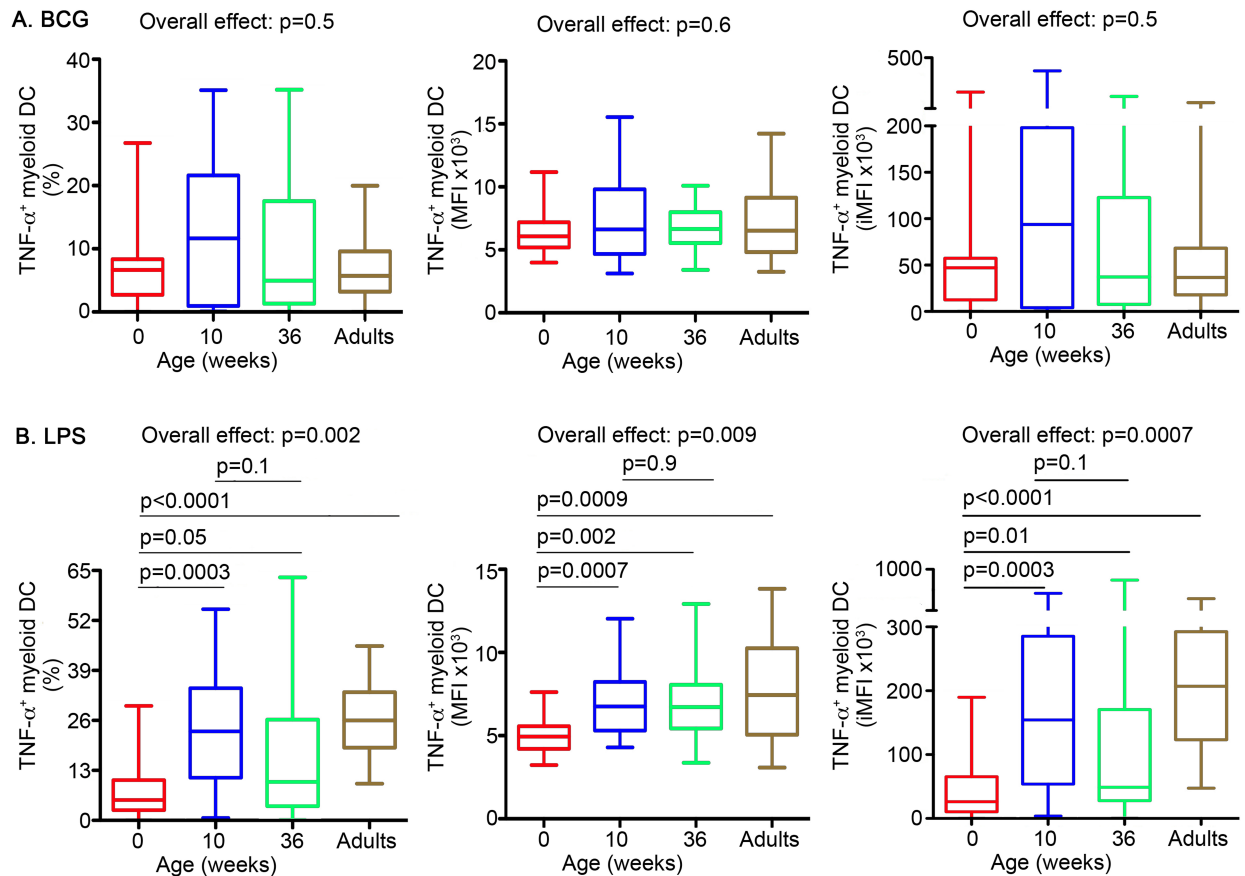


Figure 34: TNF- α expression by myeloid dendritic cells. TNF- α expression was determined after incubation of whole blood with BCG (A) or LPS (B). Myeloid DCs were identified as described in gating strategy (Chapter 2, section 2.4.11; Figure 19). Kruskal-Wallis (overall effect over the first 9 months of life) followed by Mann-Whitney tests were used for statistical analyses. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range.

We also observed similar frequencies, MFI and iMFI of mDC expressing IL-6 in newborns compared with infants in response to BCG (Fig. 35A).

In response to LPS, there were similar frequencies of mDC expressing IL-6 in newborns compared with infants (Fig. 35B). On a per cell basis, LPS stimulation resulted in an increase with age of mDC expressing IL-6. The iMFI of IL-6 expressing mDC was similar in newborns compared with infants (Fig. 35B). The frequencies, MFI and iMFI of mDC expressing IL-6 were lower in newborns compared with adults in response to LPS but the frequencies of mDC expressing IL-6 were higher in newborns compared with adults in response to BCG ($p=0.04$) (Fig. 35A and B).

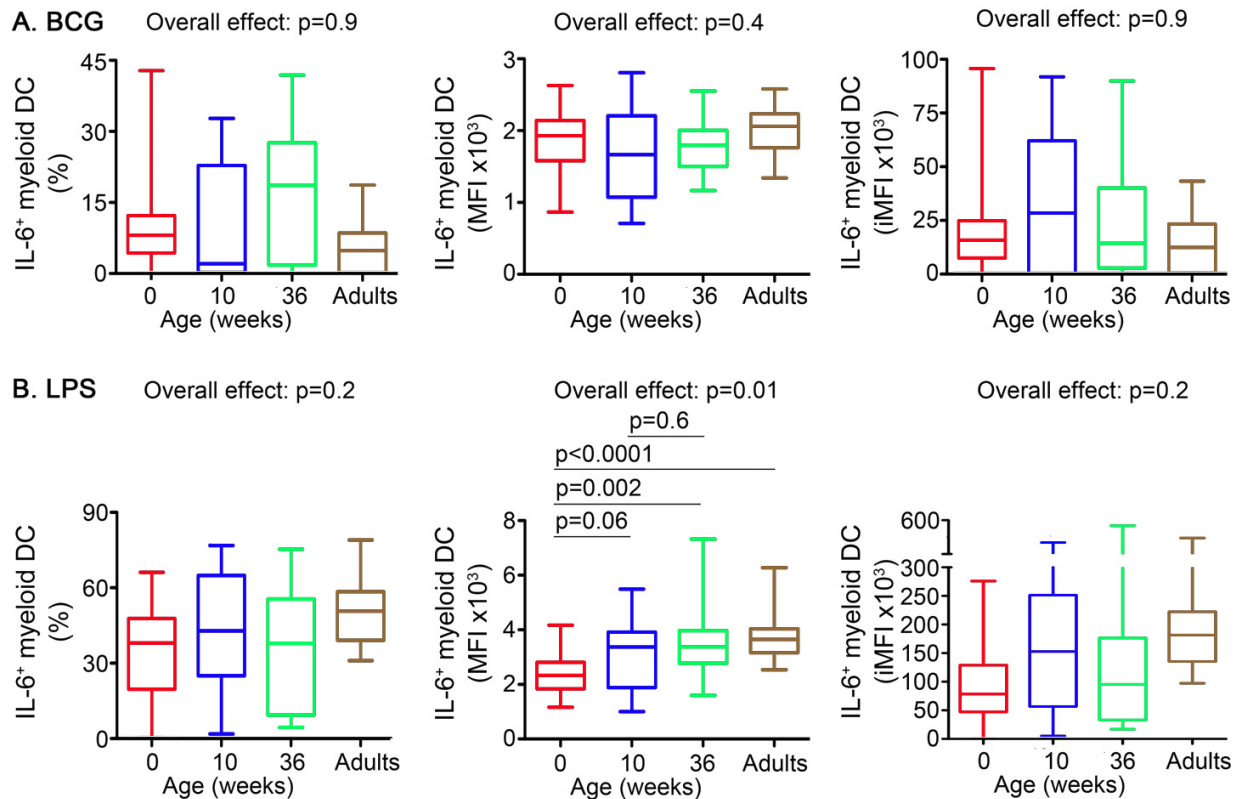


Figure 35: IL-6 expression by myeloid dendritic cells. IL-6 expression was determined after incubation of whole blood with BCG (A) or LPS (B). Myeloid DCs were identified as described in gating strategy (Chapter 2, section 2.4.11; Figure 19). Kruskal-Wallis (overall effect over the first 9 months of life) followed by Mann-Whitney tests were used for statistical analyses. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range.

We next evaluated co-expression of pro-inflammatory cytokines by mDC in response to BCG and LPS (Fig. 36A). In response to BCG, the dominant subsets of mDC co-expressed IL-6 and TNF- α (IL-6⁺TNF- α ⁺), while in response to LPS, the dominant subsets expressed either IL-6 and TNF- α or IL-6 alone. There were similar frequencies of mDC that were polyfunctional, bifunctional or monofunctional in newborns compared with infants in response to BCG (Fig. 36B). However, LPS stimulation resulted in an increase in frequencies of mDC that were polyfunctional or bifunctional with age. In contrast, LPS stimulation resulted in a decrease in mDC frequencies that were monofunctional with age (Fig. 36C).

BCG stimulation resulted in similar frequencies of mDC that were polyfunctional, bifunctional and monofunctional, while LPS stimulation resulted in lower frequencies of

mDC that were polyfunctional or bifunctional in newborns compared with adults (**Fig. 36 A and B**).

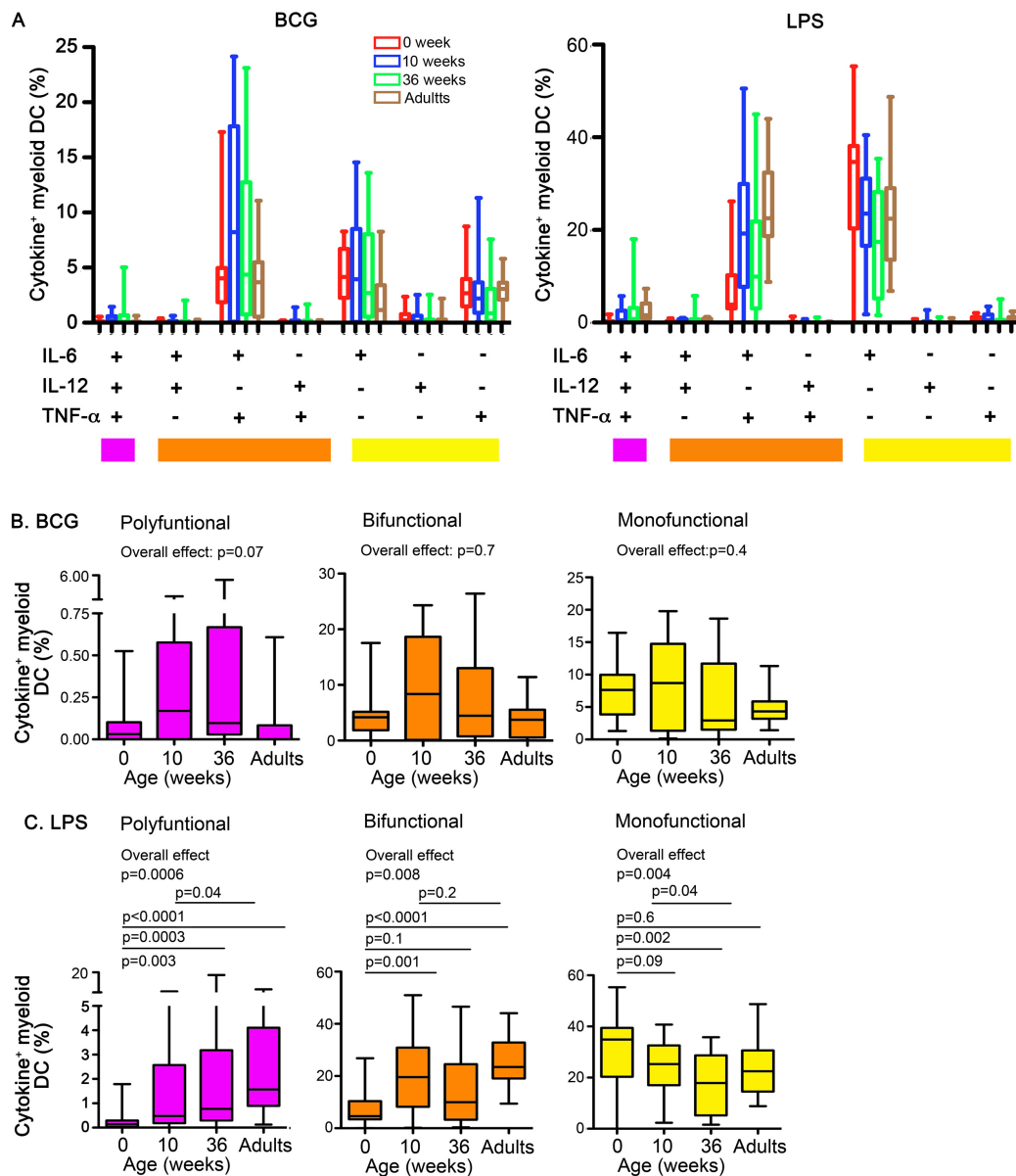


Figure 36: Myeloid DC expressing multiple cytokines. (A) mDC expressing cytokines in various combinations. The ability of mDC to express 3 (purple), 2 (orange) or 1 (yellow) cytokine in any combination after incubation of whole blood with BCG (B) or LPS (C). Kruskal-Wallis (overall effect over the first 9 months of life) followed by Mann-Whitney tests were used for statistical analyses. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range.

In summary, in response to BCG, pro-inflammatory cytokine expression by monocytes increased with age while expression by mDC was similar in newborns and infants. LPS stimulation resulted in a general increase with age of pro-inflammatory cytokine expression by monocytes and mDC.

3.4.3 Pro-inflammatory cytokine responses by granulocytes

Granulocytes, and especially neutrophils, are among the first cells to respond or arrive at the site of infection. Although these are effector cells that can kill and eliminate the infecting pathogen, these cells also produce inflammatory mediators that can mediate recruitment of other cells. We also evaluated cytokine expression by granulocytes. Very low frequencies of granulocytes expressed cytokines, TNF- α and IL-6, compared with mDC and monocytes (**Fig. 37A**). Stimulation with BCG or LPS did not result in an increase in frequencies of granulocytes expressing IL-12/23p40 or IL-10 (data not shown). No age-related differences were observed in the frequencies of granulocytes expressing TNF- α in response to BCG and LPS (**Fig. 38B**). However, frequencies of granulocytes expressing IL-6 increased with age in response to both BCG and LPS (**Fig 37C**). Also, the frequencies of granulocytes expressing IL-6 were higher in adults compared with newborns in response to BCG and LPS.

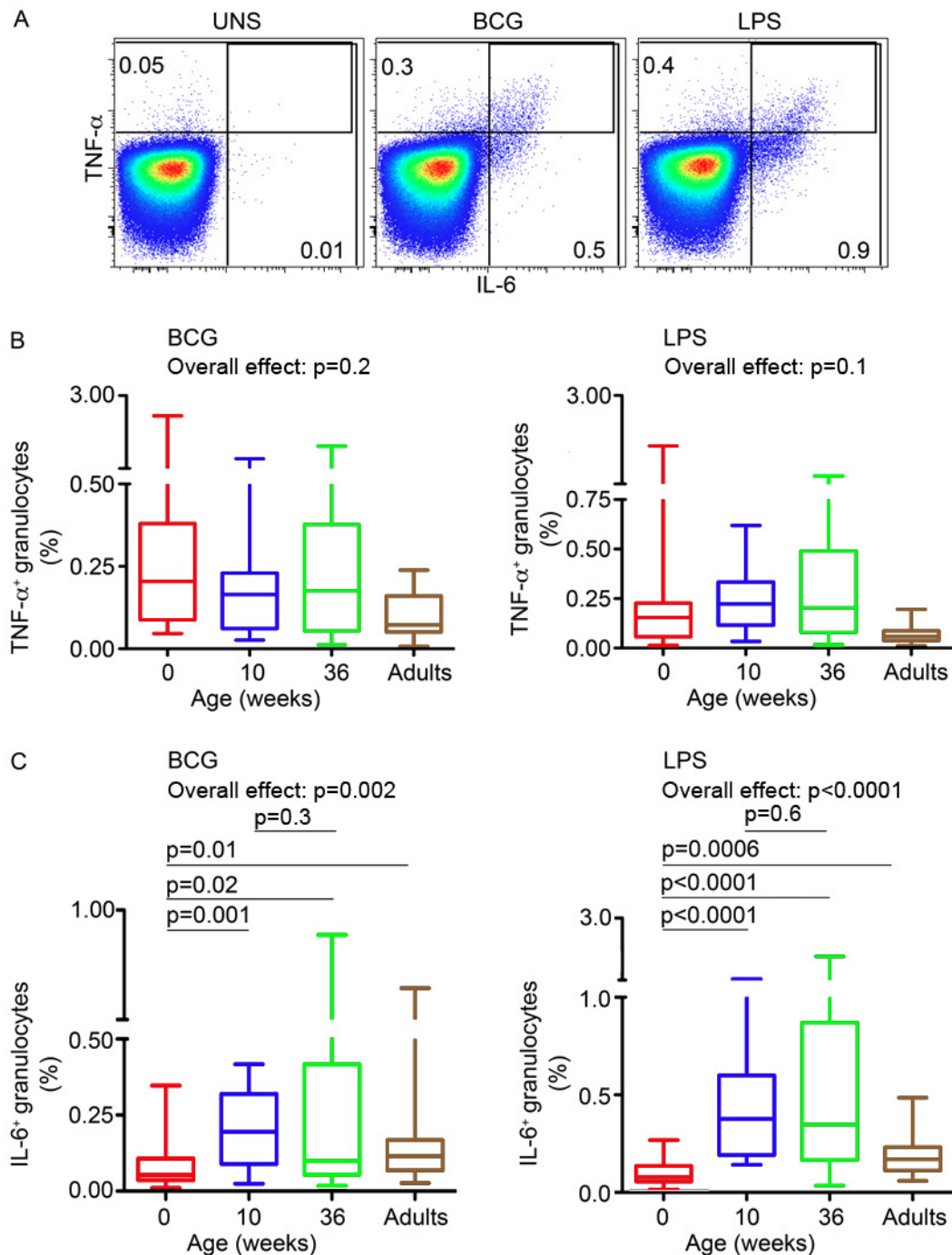


Figure 37: Cytokine expression by granulocytes. (A) Dot plots showing expression of IL-6 and TNF- α when whole blood was incubated with no stimulant or with BCG or LPS. Shown are frequencies (%) of granulocytes expressing each cytokine. Comparison of TNF- α (**C**) and IL-6 (**C**) expression after incubation of whole blood with BCG or LPS. Granulocytes were identified as described in gating strategy (section 2.4.11; Figure 19). Kruskal-Wallis (overall effect over the first 9 months of life) followed by Mann-Whitney tests were used for statistical analyses. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range.

3.4.4 Soluble pro-inflammatory cytokine secretion

3.4.4.1 Pro-inflammatory cytokine secretion in response to mycobacteria, TLR2/1 and TLR4 ligands

In order to determine the total cytokine responses by innate cells, we quantified the levels of cytokines secreted in supernatants from whole or cord blood incubated for 18 hours with no stimulant, BCG or ligands for TLR 2/1 (PAM3), TLR4 (LPS), TLR2/6 (FSL-1) and TLR9 (CpG). We first determined the levels of cytokines in whole or cord blood incubated with no stimulant. Levels of IL-12p40 and IL-6 secreted in unstimulated blood were similar in newborns and infants, while levels of TNF- α moderately increased with age (**Fig. 38A**). Background levels of TNF- α and IL-6 secreted were higher in adults, compared with newborns.

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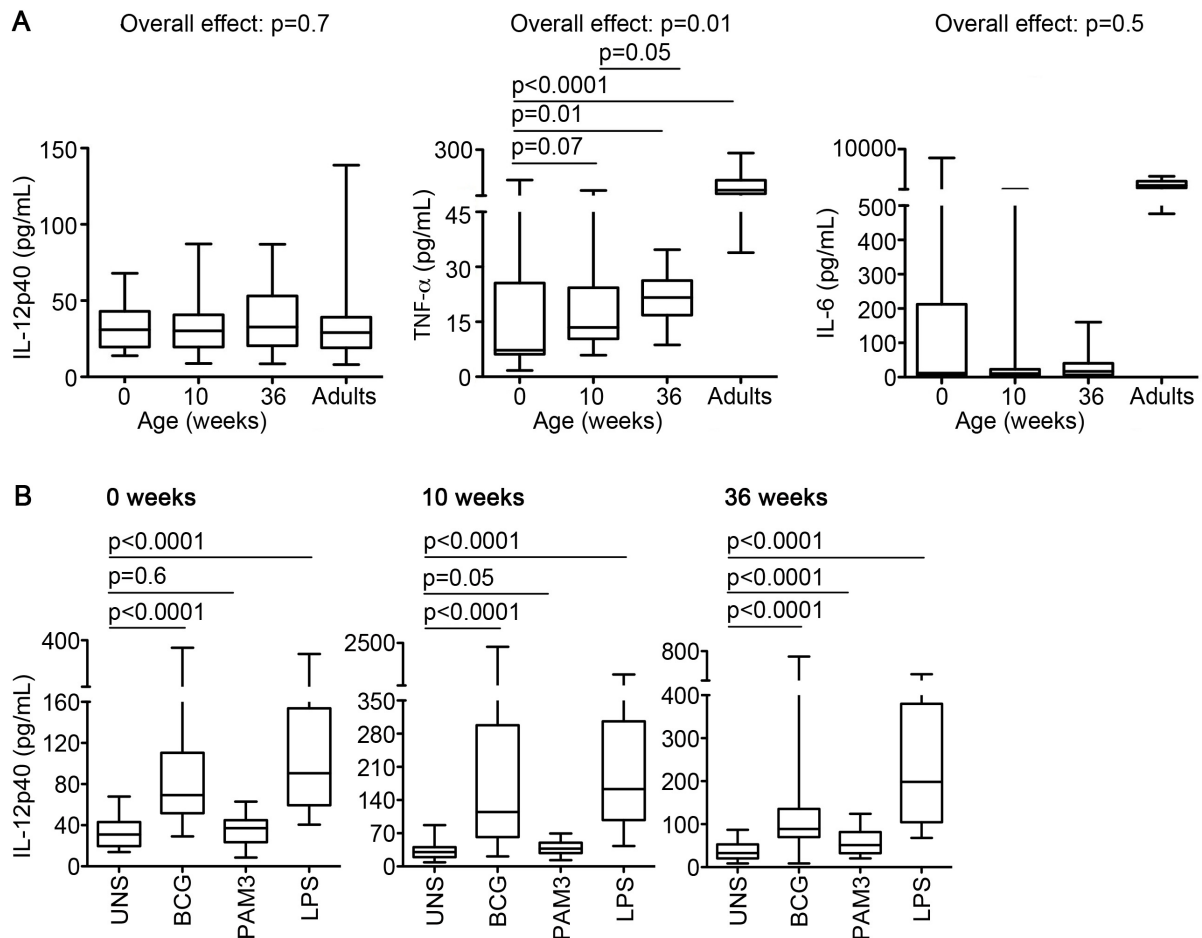


Figure 38: Background and stimulated pro-inflammatory cytokine levels. Whole or cord blood was incubated with no stimulant for 18hrs and supernatants harvested and cytokines levels determined by Milliplex MAP Immunoassay. **(A).** Comparison of background levels of IL-12p40, TNF- α and IL-6 in newborns, infants and adults. Kruskal-Wallis (overall effect over the first 9 months of life) followed by Mann-Whitney tests were used for statistical analyses. **(B).** Comparison of IL-12p40 levels between stimulation conditions. Wilcoxon matched pairs test was used for statistical analyses. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range.

In response to stimulation, IL-6 and TNF- α were secreted at high concentrations; IL-12p40 was secreted at low but readily detectable concentrations, while IL-12p70 was low and undetectable in most samples. We evaluated whether stimulation resulted in an increase in secreted levels of cytokines. BCG and LPS stimulation resulted in higher secretion of IL-12p40 in all age groups compared with unstimulated samples (**Fig. 38B**). However, PAM3 stimulation resulted in higher IL-12p40 levels only in infants but not in newborns. Stimulation resulted in significant increases in secreted levels of TNF- α and IL-6 (data not shown).

Levels of IL-12p40 were similar in newborns and infants, while levels of TNF- α and IL-6 increased with age in response to BCG stimulation (**Fig. 39A**). Levels of these pro-inflammatory cytokines secreted in adults were similar to those in newborns in response to BCG.

BCG is a complex organism with ligands for several PRRs, which can elicit different responses. To investigate responses mediated by specific TLR ligands, we also evaluated cytokine production in response to specific ligands for TLRs. In response to PAM3 and LPS, secretion of pro-inflammatory cytokines increased with age (**Fig. 39B and C**).

Levels of IL-12p40 were similar in newborns and adults, while levels of TNF- α were higher in adults in response to LPS. Of note, the levels of IL-6 secreted were higher in newborns compared with adults upon stimulation with PAM3 and LPS. Levels of pro-inflammatory cytokines were higher in infants compared with adults in response to BCG, PAM3 or LPS.

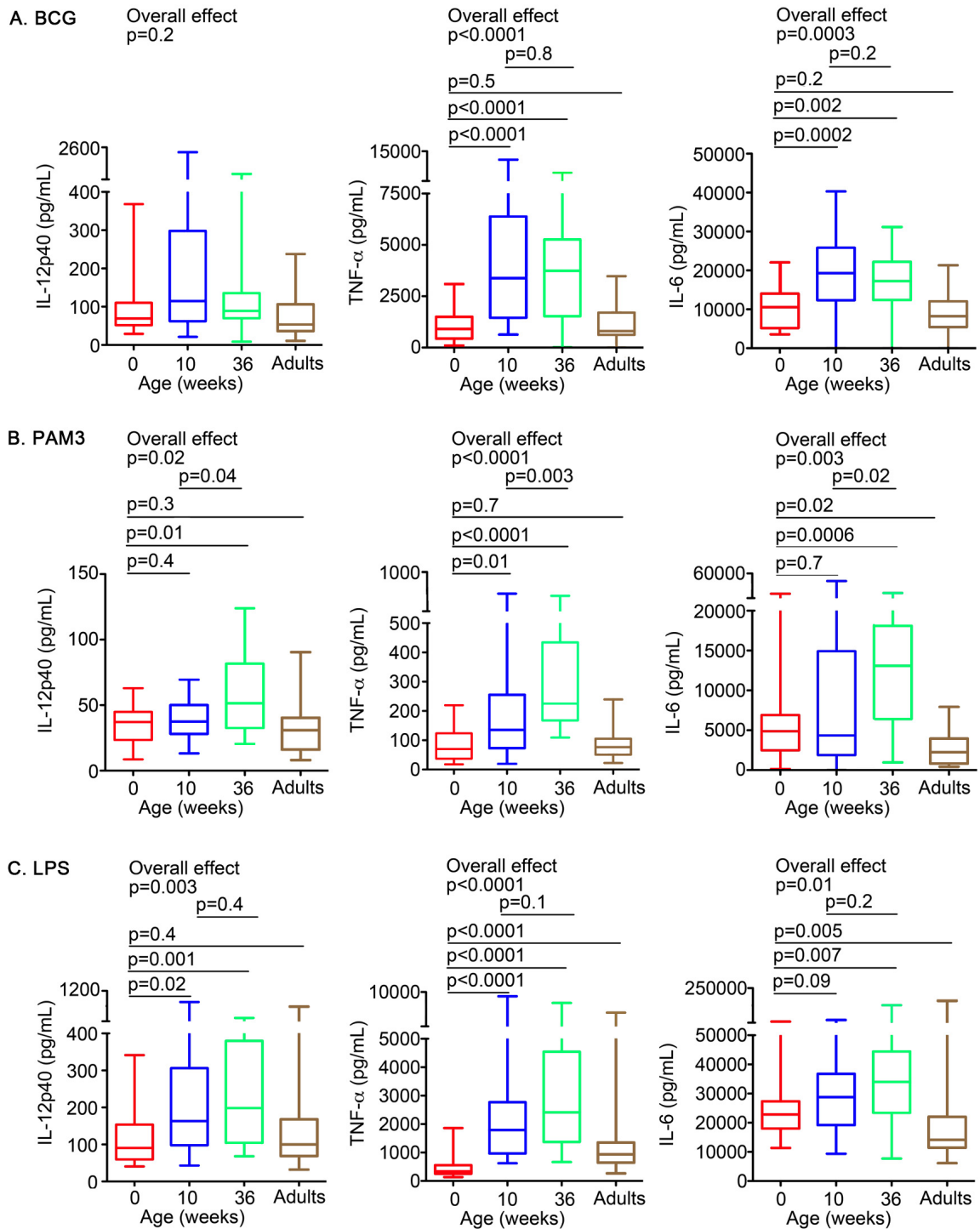


Figure 39: Production of pro-inflammatory cytokines in supernatants of whole blood. Production of TNF- α , IL-6, and IL-12/p40 after incubation of whole or cord blood with BCG (A) PAM3 (B) and LPS (C) was determined by Milliplex MAP Immunoassays. Statistical analyses were done using Kruskal-Wallis (overall effect over the first 9 months of life) followed by Mann-Whitney tests. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range.

3.4.4.2 Pro-inflammatory cytokine secretion upon stimulation with TLR2/6 and TLR9 ligands

High levels of cytokines were secreted in response to stimulation with BCG, ligands for TLR2/1 and TLR4. By comparison, very low levels of pro-inflammatory cytokines were secreted in response to ligands for FSL-1 and CpG. Levels of IL-12p40, IL-6 and TNF- α secreted after stimulation with FSL-1 were similar in newborns and infants (**Fig. 40A**). Compared with unstimulated levels of cytokines, CpG stimulation did not result in any appreciable amount of cytokine secretion; but the levels were similar for IL-12p40 and IL-6 in newborns and infants while the levels of TNF- α increased with age (**Fig. 40B**).

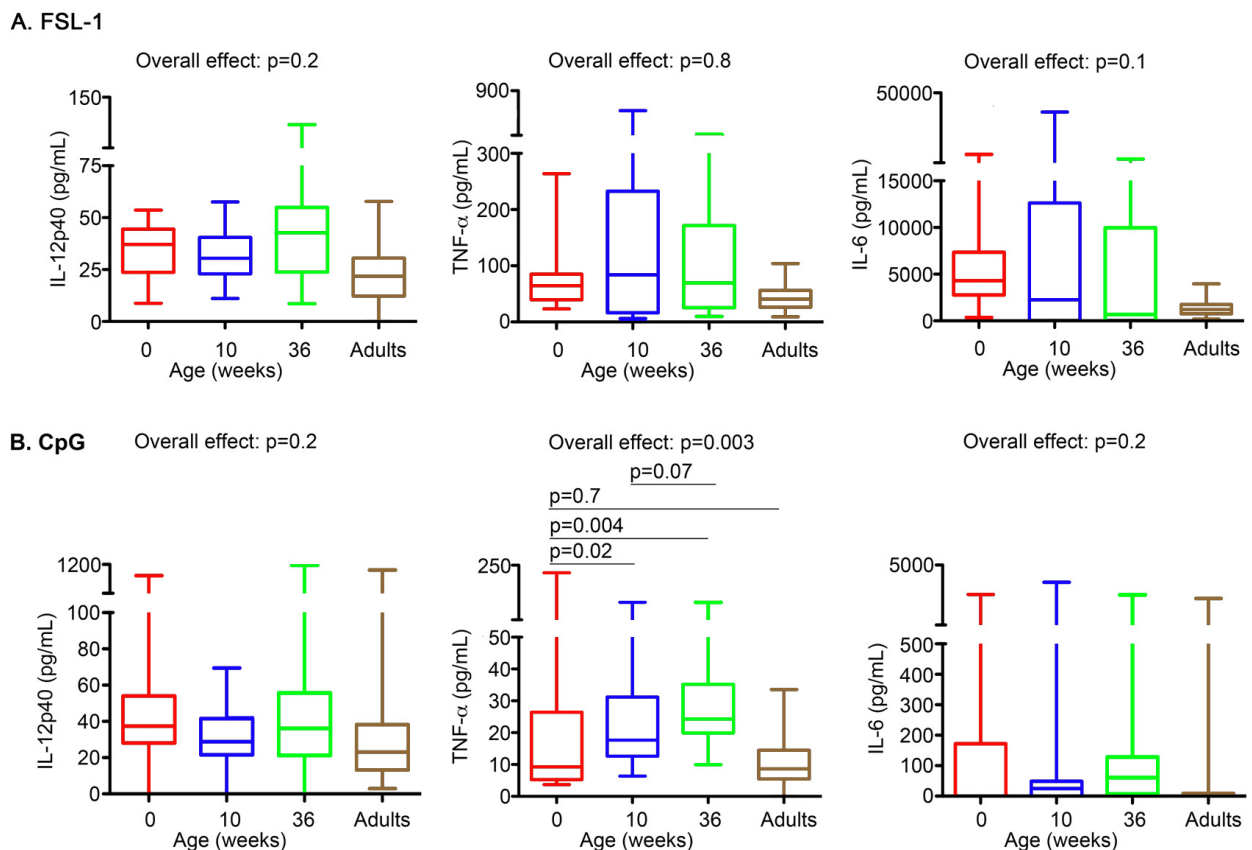


Figure 40: Production of pro-inflammatory cytokines in supernatants of whole blood. Production of IL-12/p40, TNF- α and IL-6 after incubation of whole or cord blood with TLR2/6 ligand, FSL-1 (**A**) and TLR9 ligand, CpG (**B**), was determined by Milliplex MAP immunoassays. Statistical analyses were done using Kruskal-Wallis (overall effect over the first 9 months of life) followed by Mann-Whitney tests. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range.

3.4.5 Anti-inflammatory cytokine responses

3.4.5.1 IL-10 expression by monocytes and mDC

Excessive inflammation, which may lead to pathology, can be regulated or suppressed by anti-inflammatory mediators, such as IL-10 (96). We evaluated the frequencies of IL-10 expressed by monocytes and mDC in whole blood incubated with no stimulant or with BCG and LPS (**Fig. 41A**). Stimulation with BCG or LPS resulted in higher frequencies of monocytes and mDC expressing IL-10 (**Fig. 41B and C**)

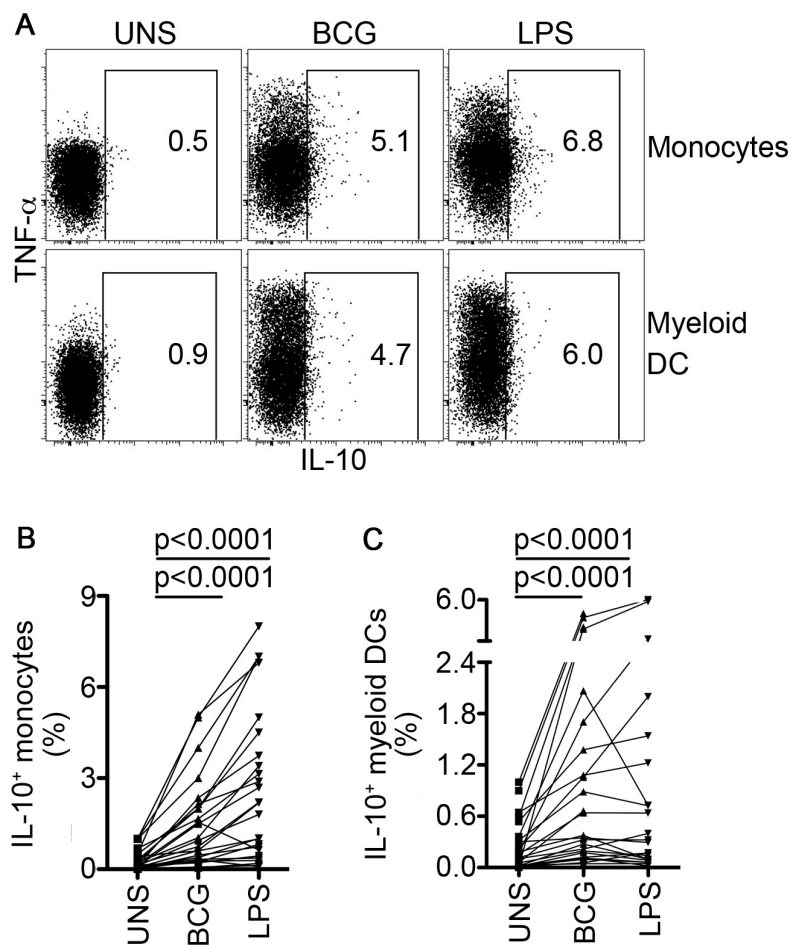


Figure 41: IL-10 expression by monocytes and myeloid DCs. Whole was incubated with BCG, LPS or incubated with no stimulant and IL-10 expression was determined by flow cytometry. (**A**). Shown are dot plots from an infant at 36 weeks of age with frequencies (%) of monocytes or DCs IL-10 expressing. Summary of IL-10 expression by monocytes (**B**) and mDC (**C**). Monocytes and mDC were identified as described in gating strategy (**section 2.4.11; Figure 19**). Wilcoxon matched pairs test was used for statistical analyses.

We observed an increase in frequencies of monocytes expressing IL-10 in response to BCG with age (**Fig. 42A**). On the contrary the MFI of monocytes expressing IL-10 in

response to BCG decreased with age (**Fig. 42A**). The iMFI of monocytes expressing IL-10 was similar in newborns and infants. Adults had higher frequencies, and similar MFI and iMFI of monocytes expressing IL-10 compared with newborns (**Fig. 42A**).

LPS stimulation resulted in an increase in frequencies, and decrease in MFI of monocytes expressing IL-10 with age (**Fig. 42B**). The iMFI of monocytes expressing IL-10 was similar in newborns and infants (**Fig. 42B**).

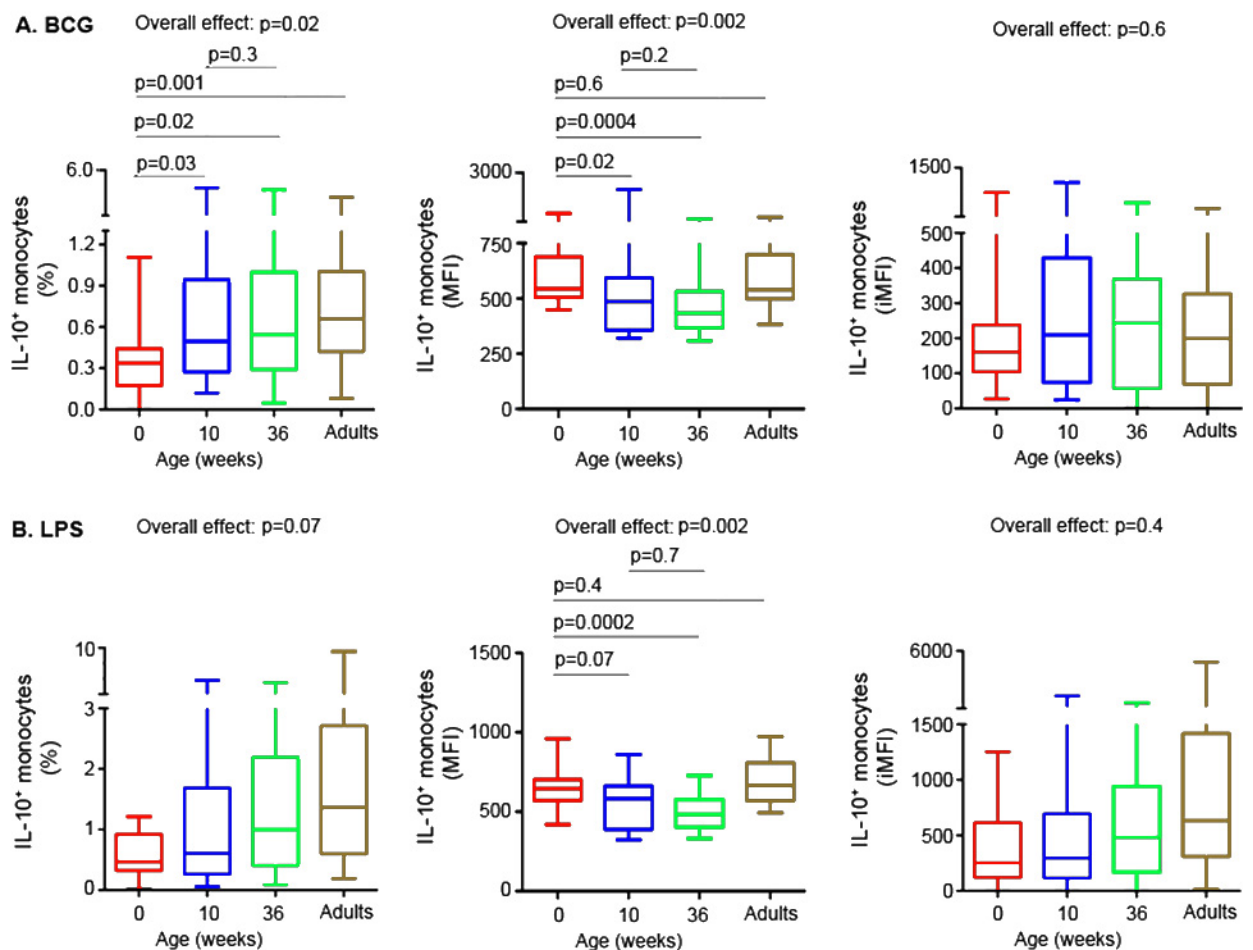


Figure 42: IL-10 expression by monocytes over the first 9 months of life. IL-10 expression was determined after incubation of whole blood with BCG (**A**) or LPS (**B**). Monocytes were identified as described in gating strategy (**section 2.4.11; Figure 19**). Kruskal-Wallis (overall effect over the first 9 months of life) followed by Mann-Whitney tests were used for statistical analyses. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range.

In response to BCG and LPS, we observed similar frequencies, MFI and iMFI of mDC expressing IL-10 in newborns and infants (**Fig. 43A and B**). There were similar

frequencies and iMFI of mDC expressing IL-10. However, we observed higher MFI of mDC expressing IL-10 in adults compared with newborns in response to BCG and LPS ($p=0.01$ and $p=0.005$, respectively) (**Fig. 43A and B**).

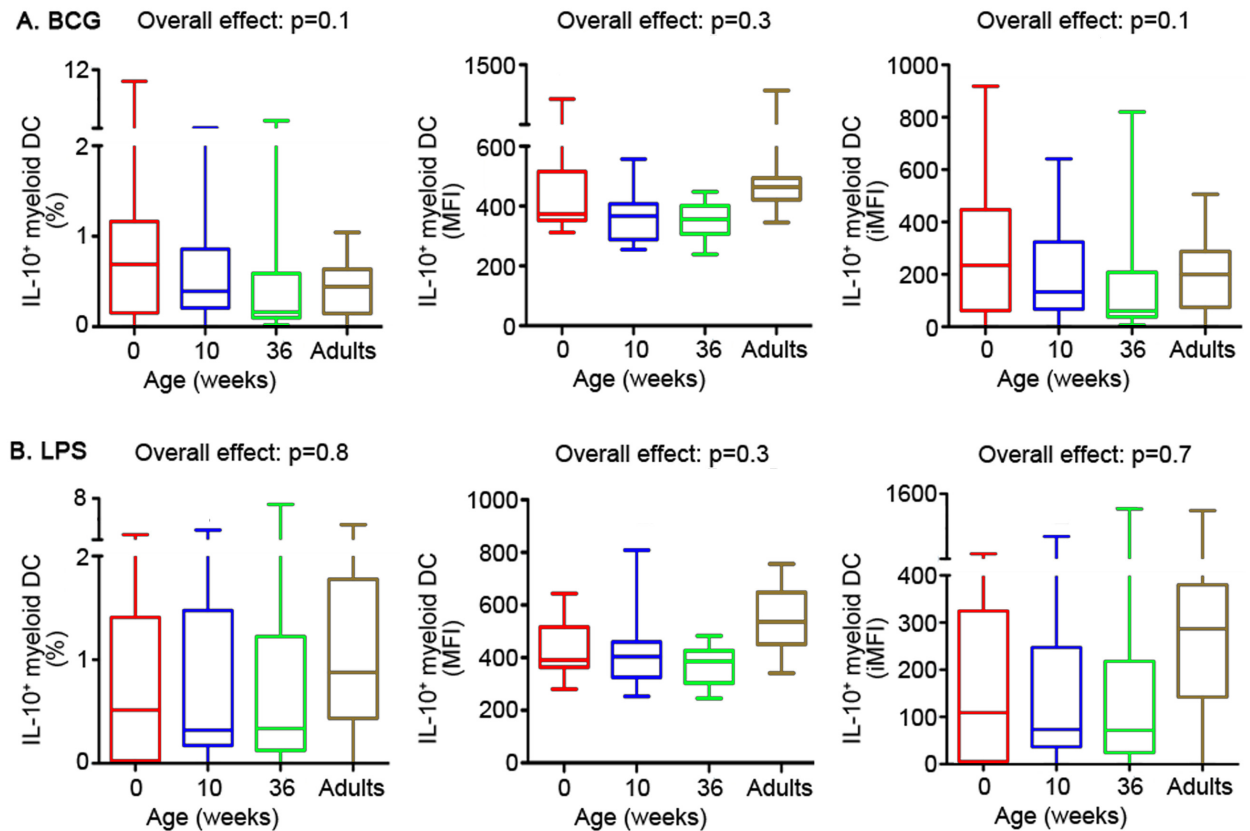


Figure 43: IL-10 expression by myeloid DC over the first 9 months of life. IL-10 expression was determined after incubation of whole blood with BCG (**A**) or LPS (**B**). Myeloid DCs were identified as described in gating strategy (**section 2.4.11; Figure 19**). Kruskal-Wallis (overall effect over the first 9 months of life) followed by Mann-Whitney tests were used for statistical analyses. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range.

3.4.5.2 Soluble IL-10 secretion

The levels of IL-10 expressed by monocytes and mDC in response to BCG and LPS, measured by intracellular staining, were mostly similar in newborns and infants. We also determined the soluble IL-10 levels when whole or cord blood was incubated with no stimulant or with BCG and TLR ligands. The levels of IL-10 in unstimulated samples were low and similar in newborns and infants (**Fig. 44**). IL-10 was secreted at high levels in response to BCG, PAM3, LPS, and FSL-1 ligands, while CpG stimulation

resulted in low levels of IL-10 secretion, which were similar to unstimulated levels (**Fig. 44**). Levels of IL-10 secreted were similar in newborns and infants in response to BCG. IL-10 levels increased with age in response to PAM3. LPS resulted in similar IL-10 levels in newborns and infants at 10 weeks but higher in infants at 36 weeks of age. Of note, stimulation with FSL-1 resulted in a decrease with age of levels of IL-10 secreted. Stimulation with CpG resulted in low levels of IL-10 secreted in newborns (**Fig. 44**). The levels of IL-10 secreted in adults were mostly similar compared with newborns except for PAM3 and CpG stimulation where the levels were different.

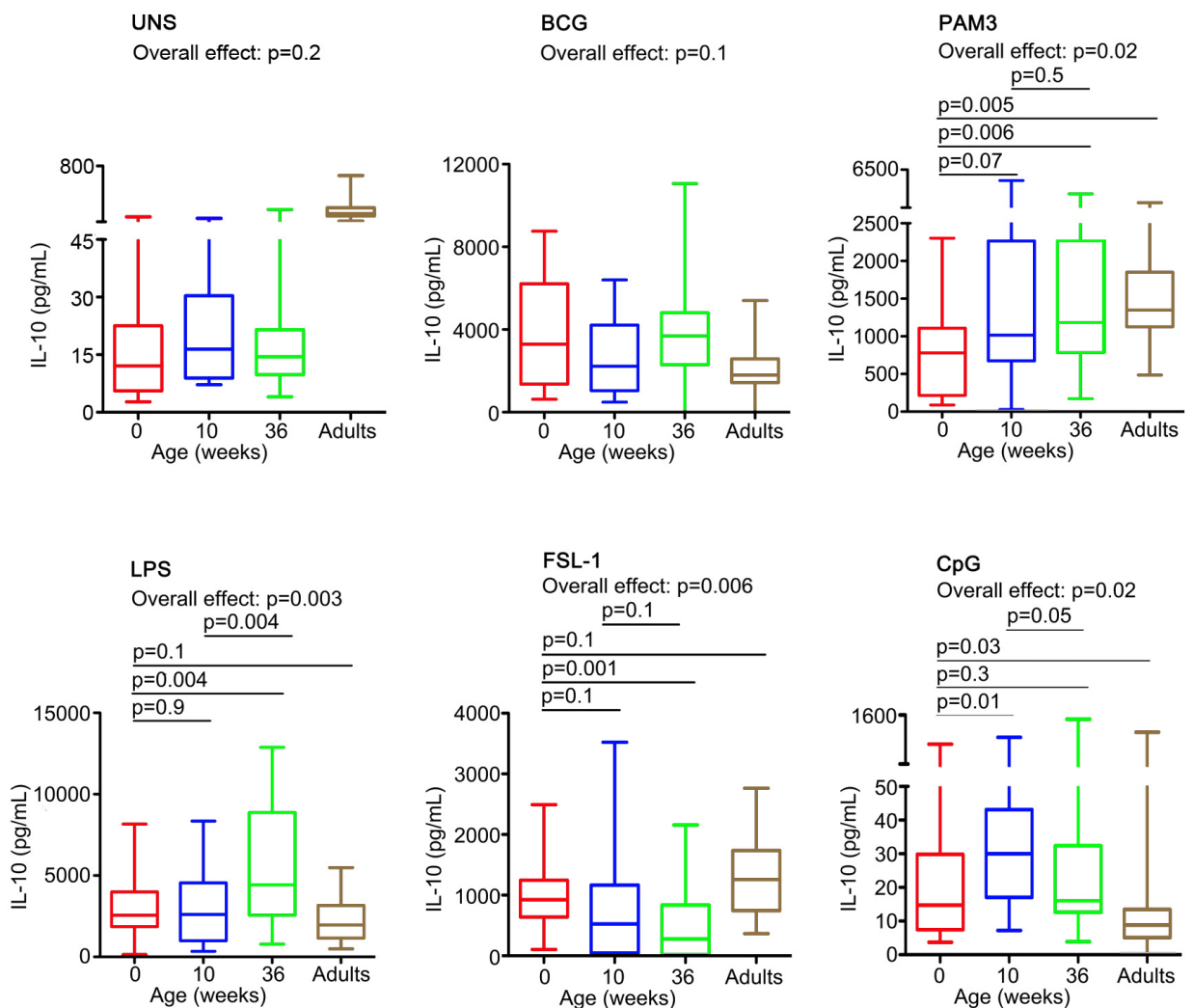


Figure 44. IL-10 secretion over the first 9 months of life. The levels of IL-10 secreted in supernatants of whole or cord blood incubated with no stimulant or stimulated with BCG and TLR ligands were assayed by luminex bead assay. Kruskal-Wallis (overall effect over the first 9 months of life) and Mann-Whitney tests were used for statistical analyses. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range.

3.4.6 Maturation of innate cells with age

Upon recognition of mycobacteria, innate cells undergo a process of activation, which involves cytokine production and expression of maturation markers. This may enhance antigen presentation by innate cells such as DCs, and may shape the development of the subsequent adaptive immune response. To measure activation of mDC, monocytes and granulocytes upon stimulation with BCG or LPS, we quantified expression of CD40, CD83 or CD86.

3.4.6.1 Maturation of mDC

We observed an increase in expression of CD40 at all age groups in response to stimulation (**Fig. 45A**). Stimulation resulted in an increase in CD83 expression in newborns but not infants, while CD86 expression upon stimulation was higher in newborns and infants at 36 weeks of age but not infants at 10 weeks of age (**Fig. 45B**).

We then evaluated the stimulation index of maturation marker expression as MFI of stimulated divided by MFI of unstimulated samples. In response to BCG, upregulation of CD40 expression increased with age (**Fig. 45C**). In contrast, in response to BCG, upregulation of CD83 expression on mDC was higher in newborns compared with infants, while upregulation of CD86 expression on mDC was higher in newborns compared with infants at 10 weeks of age (**Fig. 45C**). In response to LPS, upregulation of CD40 and CD86 expression did not change with age, while CD83 expression was higher in newborns compared with infants (**Fig. 45D**). CD40 and CD86 upregulation was similar in adults and newborns, while CD83 upregulation was lower in adults, compared with newborns, in response to BCG and LPS.

Taken together, these results indicate that mDC in newborns and infants matured differently in response to BCG and LPS.

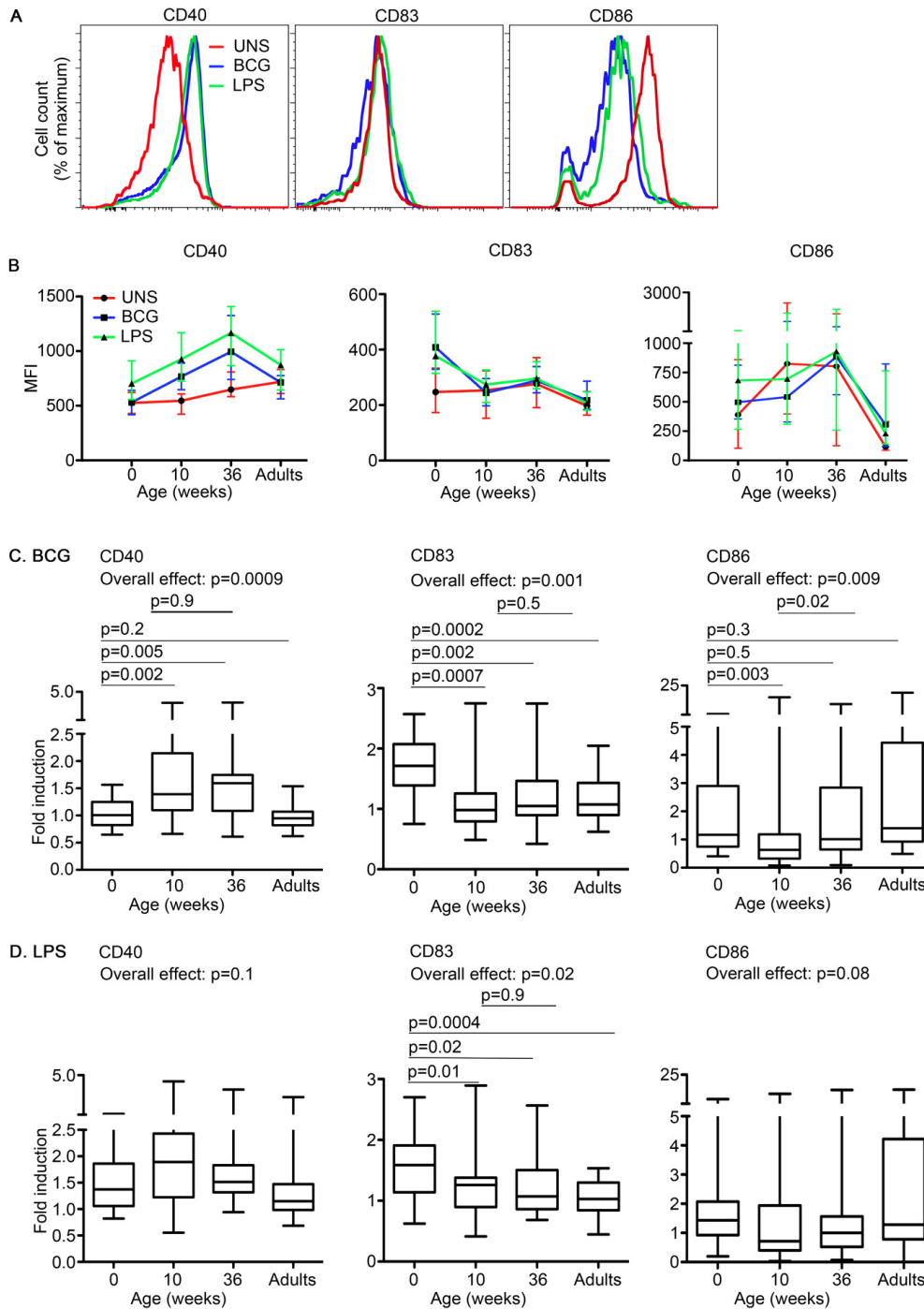


Figure 45: Expression of maturation markers on myeloid dendritic cells. (A). Histograms showing expression of maturation markers from an infant at 10 weeks of age. **(B).** Amount of maturation marker expressed by mDC when whole or cord blood was incubated with no stimulant or with BCG or LPS. Data presented as median with interquartile range (IQR). Fold induction of maturation markers on mDC in response to BCG **(C)** and LPS **(D)**. Kruskal-Wallis (overall effect over the first 9 months of life) followed by Mann-Whitney tests were used for statistical analyses. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range.

3.4.6.2 Maturation of monocytes

We also evaluated the expression of CD40 and CD86 on monocytes when whole or cord blood was incubated with no stimulant or with BCG and LPS (**Fig. 46A**). We observed that in response to BCG and LPS, there was expression of CD40 above unstimulated levels in infants but not in newborns (**Fig. 46B**). However, in response to BCG and LPS, expression of CD86 on monocytes was below background levels in infants but similar in newborns.

When we evaluated fold induction over unstimulated samples, we observed that in response to BCG and LPS, there was an increase with age of CD40 expression (**Fig. 46C and D**). There was no upregulation of CD86 expression in newborns, while infants downregulated CD86 expression, compared with newborns in response to BCG and LPS (**Fig. 46C and D**).

In response to BCG, there was similar expression of CD40 and CD86 in newborns and adults (**Fig. 46C**). In response to LPS, CD40 expression was slightly higher in adults, while CD86 expression was downregulated in adults compared with newborns (**Fig. 46D**). CD83 is not a monocyte activation marker and was not evaluated.

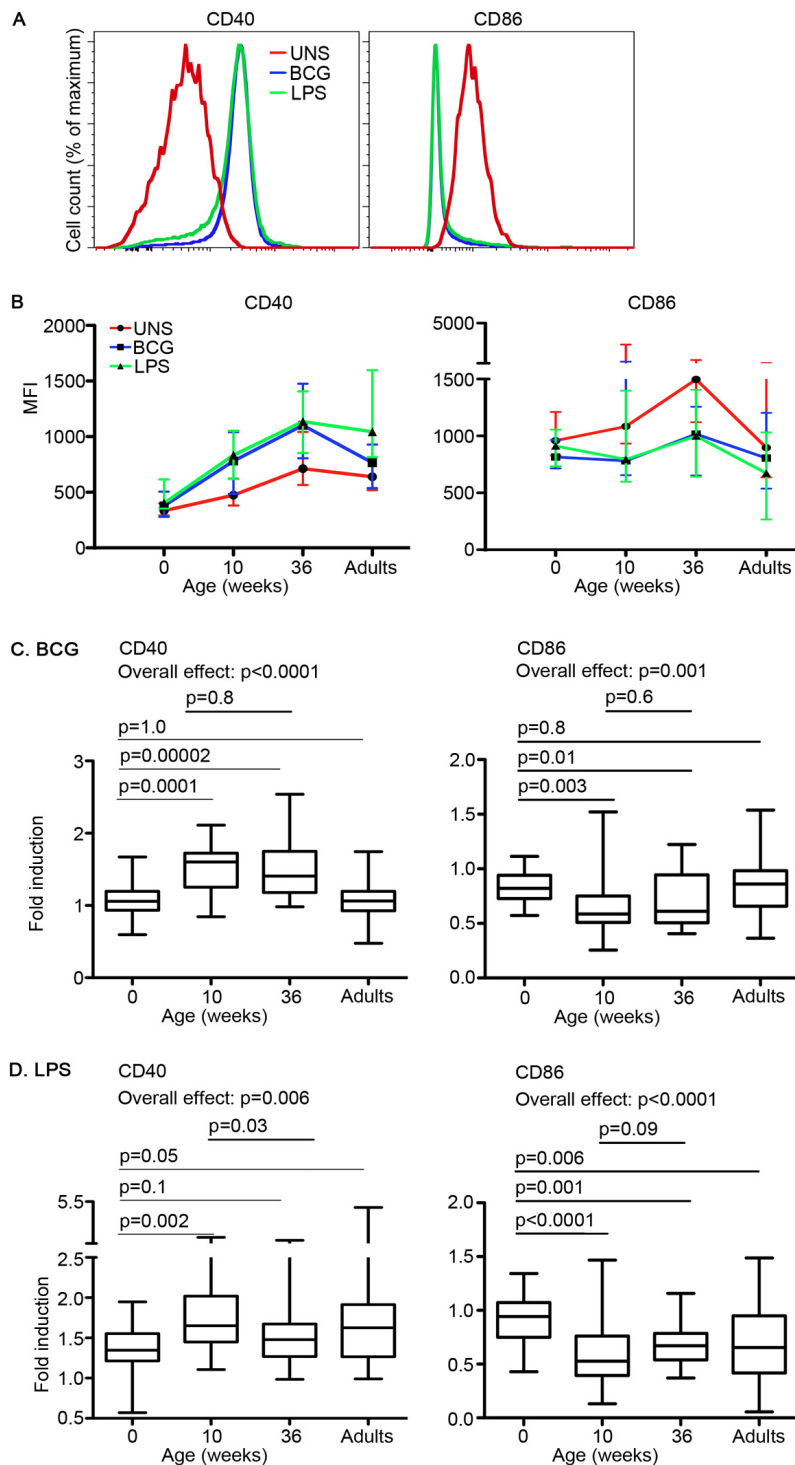


Figure 46: Expression of maturation markers on monocytes. (A). Histograms showing expression of maturation markers from an infant at 10 weeks of age. **(B).** Amount of maturation marker expressed by monocytes when whole or cord blood was incubated with no stimulant or with BCG or LPS. Data presented as median with IQR. Fold induction of maturation markers on monocytes in response to BCG **(C)** and LPS **(D)**. Kruskal-Wallis (overall effect over the first 9 months of life) followed by Mann-Whitney tests were used for statistical analyses. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range.

3.4.6.3 Maturation of granulocytes

To characterise the maturation of CD66⁺ granulocytes, we evaluated the expression of CD83, CD86 and CD40 after whole blood was incubated with no stimulant or incubated with BCG or LPS (**Fig. 47A**). We observed that in response to BCG or LPS, CD83 expression was above background in newborns and infants (**Fig. 47B**). When we evaluated the fold increase over unstimulated samples, we observed that upregulation of CD83 increased with age in response to BCG or LPS (**Fig. 47C**). Adults exhibited lower capacity to upregulate CD83 expression compared with newborns in response to BCG or LPS. CD40 and CD86 expression on granulocytes did not change upon activation with BCG or LPS at all age groups (data not shown).

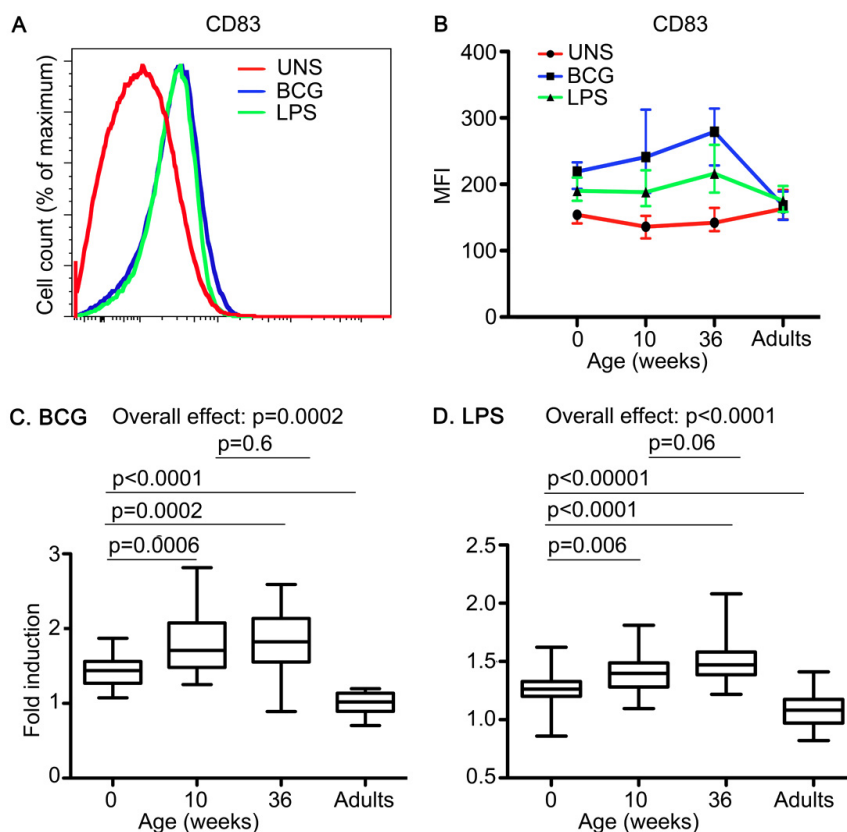


Figure 47: Expression of CD83 on granulocytes. (A). Histograms showing expression of maturation markers from a representative infant at 10 weeks of age. (B). Amount of CD83 expression on granulocytes when whole or cord blood was incubated with no stimulant or with BCG or LPS. Data presented as median with IQR. Fold induction of CD83 on granulocytes in response to BCG (C) and LPS (D). Kruskal-Wallis (overall effect over the first 9 months of life) followed Mann-Whitney tests were used for statistical analyses. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range.

3.4.7 Acquisition of mycobacteria

The use of BCG-GFP allows determination of the frequencies of cells that acquired (phagocytosed or bound) BCG by gating on GFP⁺ cells (**Fig. 48A**). BCG that has been acquired by innate cells can either be intracellular or surface-bound. We evaluated whether changes in the ability to acquire BCG over the first 9 months of life may underlie the changes in expression of cytokines or maturation markers by innate cells. We observed a decrease with age in frequencies of mDC and monocytes that acquired BCG (**Fig. 48B**). By contrast, similar frequencies of granulocytes acquired BCG in newborns and infants. The frequencies of mDC, monocytes and granulocytes that acquired BCG were similar in adults and newborns.

We then evaluated the relative proportions of mDC, monocytes and granulocytes that have acquired BCG among all cells that were GFP⁺. We observed an increase with age of proportions of GFP⁺ mDC among GFP⁺ cells (**Fig. 48C**). Monocyte proportions were similar in all age groups while for granulocytes, these proportions decreased with age, although higher at 36 weeks compared with 10 weeks of age. There were similar proportions of mDC and monocytes, and higher proportions of granulocytes that acquired BCG in adults compared with newborns. We then evaluated whether the frequencies of monocytes that have acquired BCG correlated with frequencies expressing cytokines. We observed correlations between frequencies of monocytes expressing TNF- α or IL-6 and the frequencies that have acquired BCG but no correlation with frequencies of monocytes expressing IL-12/23p40 (**Fig. 48D**). Taken together, changes in the acquisition of BCG could not fully explain the changes in cytokine or maturation marker expression by innate cells.

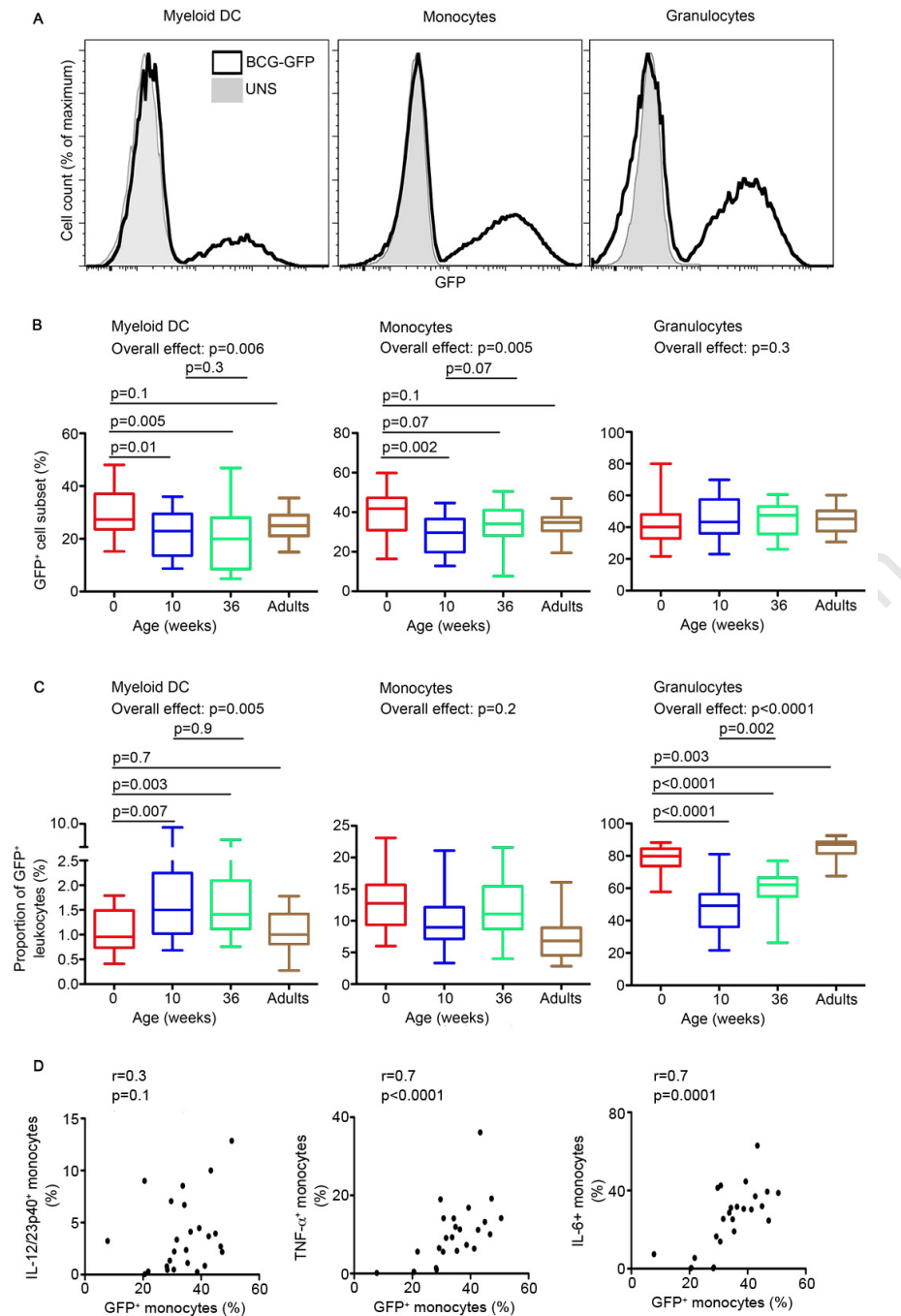


Figure 48: Binding and internalization of BCG by mDC, monocytes and granulocytes in whole blood. (A) Histograms representing GFP expression by mDC monocytes and granulocytes an infant at 36 weeks of age. (B) Frequency of cells expressing GFP among each cell subset. (C) Proportion of each cell subset expressing GFP among total GFP-expressing leukocytes. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range. (D) Correlation between frequencies of monocytes that have acquired BCG and frequencies of monocytes expressing cytokines in infants at 36 weeks of age. Statistical analyses were done using the Kruskal-Wallis test (overall effect over the first 9 months of life) followed by Mann-Whitney test, or the Spearman correlation test.

3.4.8 Innate cell counts and frequencies in blood

Since the acquisition of BCG by innate cells did not correlate with the trends observed with cytokine production or expression of maturation markers, we determined if cell counts or frequencies in blood could account for the differences. We observed that the number of mDC increased with age (**Fig. 49A**). By contrast, monocyte and granulocyte counts decreased with age. When we determined the relative proportions of mDC, monocytes and granulocytes among all leukocytes, the trends were similar to those observed with cell counts (**Fig. 49B**). The proportions of monocytes among total leukocyte population did not correlate with the frequencies of monocytes expressing TNF- α and IL-6, but correlated with frequencies of monocytes expressing IL-12/23p40 (**Fig. 49C**). Also, the proportions of mDC did not correlate with frequencies of mDC expressing any of the cytokines (data not shown). The proportions of cells among total leukocytes correlated with the proportion of GFP⁺ cells among the total GFP⁺ leukocytes (**Fig. 49D**). Taken together, cell counts and frequencies could not account for the trends observed with expression of cytokines and maturation markers by innate cells.

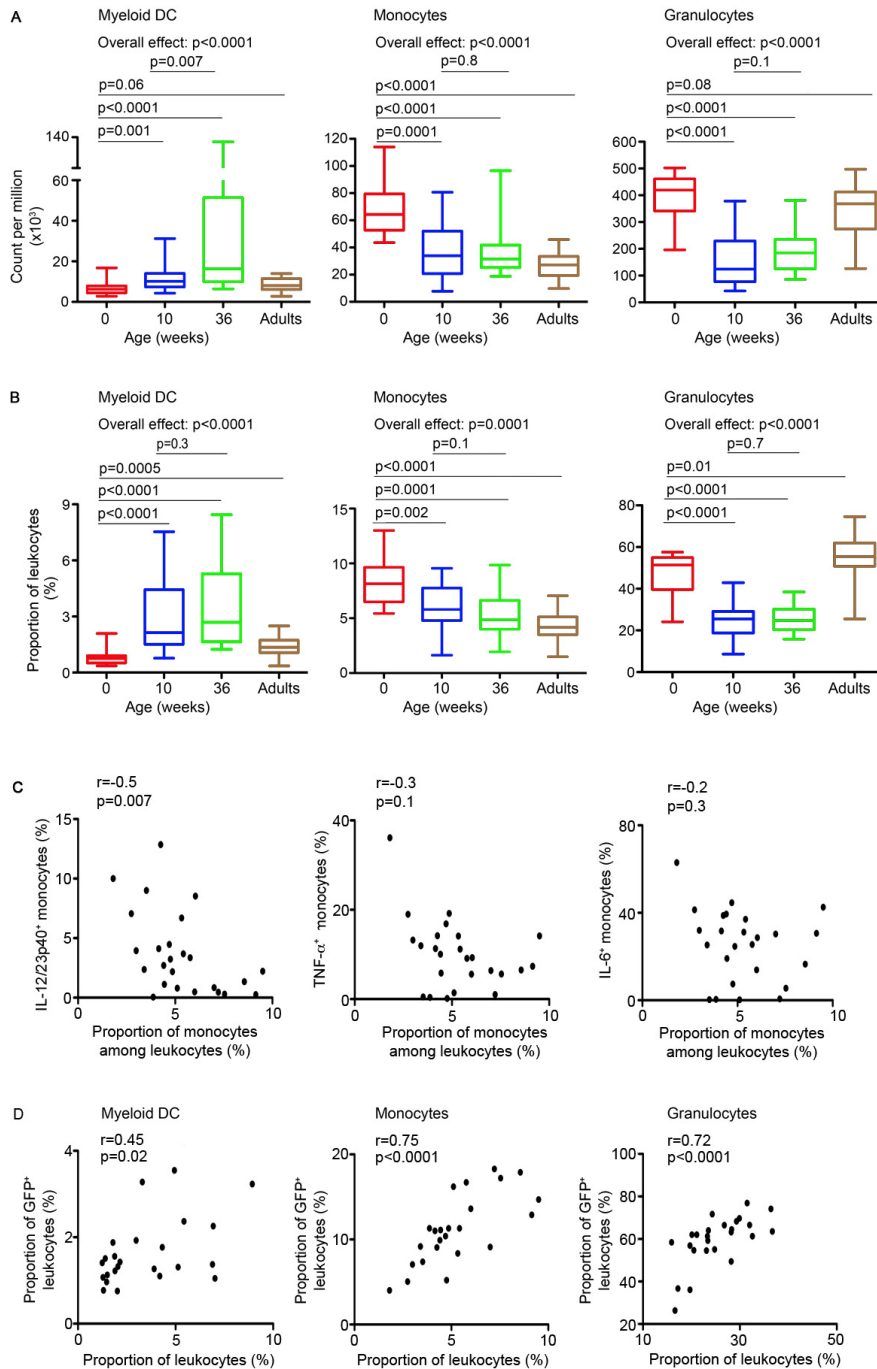


Figure 49: Counts and frequencies of innate cells in blood. (A). Cells counts of mDC, monocytes, and granulocytes. Counts were determined as the number of events within each gate. **(B).** Proportions of mDC, monocytes and granulocytes among all leukocytes. **(C).** Correlation between proportions of monocytes among total leukocyte population and frequencies of monocytes expressing cytokines in infants at 36 weeks of age. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range. **(D).** Correlation between proportion of cells among leukocytes and proportion of cells among GFP⁺ leukocytes in infants at 36 weeks of age. Statistical analyses were done using the Kruskal-Wallis test (overall effect over the first 9 months of life) followed by Mann-Whitney test, or the Spearman correlation test.

3.5 Discussion

We investigated changes in innate immune responses over the first 9 months of life in response to live BCG, and mycobacteria-related TLR ligands. We observed the following: 1. Pro-inflammatory cytokine expression in response to mycobacteria and TLR agonists were generally lower in newborns, than infants; 2. The ability of monocytes to co-express multiple pro-inflammatory cytokines was lower in newborns, than infants; 3. Anti-inflammatory cytokine expression in response to mycobacteria was similar in newborns and infants; 4. Changes in expression of maturation markers of innate cells in response to BCG varied depending on the marker evaluated. 5. Higher frequencies of mDC and monocytes acquired BCG in newborns, compared with infants.

Pro-inflammatory cytokine responses were lower in newborns compared with infants. Upon recognition of mycobacteria, including *M.tb* and *M. bovis* BCG, innate cells respond by producing inflammatory mediators. Our observations are in accordance with our hypothesis and previous studies that have shown similar or low levels of IL-12, TNF- α and IL-6 in newborns compared with infants or adults in responses to TLR ligands (3, 178, 248, 304, 306). High amounts of soluble factors such as adenosine present in whole blood in newborns have been shown to influence the production TNF- α , but not IL-6 (310). It should also be noted that even though the trends observed with soluble TNF- α were similar to those observed with frequencies of TNF- α^+ monocytes, this cytokine can also be produced in culture by other leukocytes such as T and NK cells (311). The reduced pro-inflammatory cytokine production in newborns indicates that this group of individuals may induce less inflammation during infection and may be more susceptible to infectious disease as was previously suggested (312). The higher production or expression of IL-12p40 and TNF- α in infants as well as lower CD40 expression in newborns in response to mycobacteria may underlie our previous observation that BCG vaccination at 10 weeks of age resulted in increased Th1 responses (143). Thus delaying BCG vaccination to a later stage in life or using adjuvants that increase pro-inflammatory cytokine responses in newborns, in combination with BCG may improve BCG-induced responses and possibly protection afforded by BCG.

The levels of IL-10 produced were mostly similar in newborns and infants and were dependent on the incubation condition. Mycobacterial stimulation resulted in similar production of IL-10, while stimulation with ligands for TLR2/1 and TLR4 resulted in generally higher production of IL-10 in infants compared with newborns. Previous studies have shown that newborns produced higher IL-10 compared with infants or adults in response to TLR agonists (3, 178, 248, 307), and one study showed higher secretion of IL-10 by monocyte-derived DCs in cord blood compared with adult peripheral blood in response to BCG (313). However, Belderbos *et al.* (305) observed different patterns of IL-10 production between newborns and infants, depending on which TLR agonist was used for incubation.

Some differences were noted between our results and those of others. For example, the patterns of IL-10 were different in our study, compared with others (3, 178, 248, 307). These differences could be due to variations in populations or exposure to environmental factors such as cigarette smoking and helminths (133, 302, 314-317). Most infants get exposed to helminth infection after 9 months of age, when they start crawling (318, 319). Some infants are sensitised with helminth antigens *in utero* during pregnancy, if mothers have been infected (320). However, in our field site where we recruited our participants, the prevalence of helminth infections in pregnant women was as low as 2.9%. This contrasts with the prevalence in East Africa, which has been estimated at 60.5% (318, 321). The differences in intracellular cytokine expression may also be due to differences in assay conditions. Previous studies comparing innate responses in newborns and infants or adults typically used purified TLR agonists or evaluated soluble cytokines rather than cell-associated cytokine expression (3, 178, 248, 305, 307). BCG is a whole organism that can be recognised by multiple receptors, which may result in different activation and functional responses by innate cells.

We, and others, have shown that monocytes and mDC in newborns are generally less polyfunctional, compared with those in infants and older children or adults (3, 178). The capacity of innate cells to co-express cytokines (polyfunctional cells) may reflect a qualitative difference in response to specific stimulation or a quantitative difference in which cells that are more responsive are more likely to produce multiple cytokines (3). We observed that monocytes in infants were more polyfunctional than those in newborns after stimulation with mycobacteria or LPS, while mDC in infants were more polyfunctional only upon stimulation with LPS. Other studies have also described

different degrees of polyfunctionality in monocytes, mDC and pDC in newborns compared with infants or adults (3, 178). The importance of polyfunctionality by innate immune cells in mycobacterial infection is not known. However, polyfunctionality of T cells has been correlated with better control of viral infections in humans and animal models (322-325). High multifunctional Ag85A-specific Th1 cells producing cytokines in the lungs have been correlated with protection against TB disease after *M.tb* aerosol challenge in mice (326). In humans, polyfunctionality of T cells has been associated with TB disease or probably antigen load where polyfunctional T cell responses were reported to decrease following TB treatment, or were higher in TB patients compared to latently infected healthy individuals (327-329). Data from our lab show that BCG-specific polyfunctional T cells increase with age in healthy infants with a peak at 6 weeks of age following BCG vaccination at birth (A Soares, K Cheong, T Scriba, W Hanekom, unpublished data).

mDC in newborns upregulate CD83 expression upon stimulation with mycobacteria or LPS. These data did not support our hypothesis and suggest that mDC in newborns can be activated by mycobacteria even though these cells produce less cytokines and are less capable of activating adaptive responses compared with older infants (299). Few studies have shown decreased expression of CD86 by DCs in newborns compared with those in adults (330, 331), and one study showed similar expression between newborns and adults in response to BCG (313). Liu *et al.* (313) also showed lower BCG-induced expression of CD83, and similar expression of CD40 and CD80 in newborns, compared with adults. The differences in patterns of CD83 expression in newborns and adults between our study and that of Liu *et al.* (313) may be due to differences in assays and cell types. We evaluated blood mDC after 18 hours of stimulation while Liu *et al.* (313) evaluated monocyte-derived DCs after 7 days of *in vitro* culture and 2 days of stimulation with BCG. To our knowledge, no studies have compared CD83 and CD86 expression between newborns and infants. Nguyen *et al.* (248) showed that monocytes, mDC and pDC in infants expressed more CD80 (which belongs to the same family of co-stimulatory molecules with CD86) than newborns after stimulation with LPS or CpG.

Several factors could account for the differences in cytokine responses and maturation of monocytes, mDC and granulocytes in response to mycobacteria, including differential recognition and processing of mycobacteria by innate cells, or differential expression of PRR on innate cells, frequencies of innate cells in blood or other concurrent infections

that were present during enrollment. The frequency of cell subsets acquiring mycobacteria did not associate with differences observed in the expression of cytokines and maturation markers. However, the trend for mycobacterial acquisition by mDC was similar to the upregulation of CD83 expression by mDC. More mDC, similar monocytes and less granulocytes acquired BCG-GFP in infants, compared with newborns. This correlated with the frequencies of these cells in blood, suggesting that the proportions of cells acquiring BCG among total GFP⁺ leukocytes were due to their relative numbers in blood. The patterns observed with the proportions of cells in blood were similar to those described previously (305). A limitation of this finding is that we did not use a leukocyte marker to identify leukocytes and depended on the FSC and SSC properties to identify the total leukocyte population. However, frequencies of innate cell subsets in blood and frequencies of cell subsets that acquired mycobacteria did not associate with differences in cytokine production and expression of maturation markers. Changes in expression of PRR that recognize BCG in infants could also influence the results. We did not evaluate the expression of PRR on innate cells and changes over the first 9 months of life. This will be explored in the future. Infants enrolled in the study were screened for *M.tb* or HIV infections and only healthy infants who were not on medication for any other ailments were enrolled. It is possible that at enrollment, some infants may have been exposed to other mycobacteria causing systemic infections that could influence our results and these infections were not detected.

Infants at 10 weeks of age in our study had an age range of 8 to 12 weeks and some infants would have received their EPI vaccines at 10 weeks of age. Similarly, infants at 36 weeks of age also had an age range of 34 to 38 weeks and some of them would have received their measles vaccines at 9 months of age. Unfortunately, we did not document the vaccination schedule of all infants, and we are not able to conclude whether those who had already received their vaccines at 10 weeks would exhibit differential innate responses to mycobacteria, compared with those who had not yet received their vaccines. To our knowledge, no data exist that evaluate the influence of EPI vaccines on innate responses in short-term whole blood assays.

Taken together, our results show that innate responses in newborns are different from those in infants, pro-inflammatory and Th1 polarising responses increased over the first 9 months of life, supporting our hypothesis that innate immune responses to mycobacteria are more mature in infants compared with newborns. The immaturity in

newborns may underlie their particular susceptibility to infection. Even though the correlates of protection afforded by BCG are not yet defined, vaccination strategies that increase pro-inflammatory responses at birth or early in life might reduce the high susceptibility to disease usually observed in early life. The link between maturation of innate responses and enhanced BCG-specific Th1 responses should be studied further. Overall, our data supported our hypothesis of increase pro-inflammatory cytokine responses but did not support the hypotheses that anti-inflammatory responses would decrease with age, or that expression of maturation markers would increase with age. However, the hypothesis was only true for CD40 expression.

University of Cape Town

Chapter 4: Association of TLR6 single nucleotide polymorphisms (SNPs) and innate immune responses to lipopeptides and mycobacteria

Studies reported in this chapter were published in *Genes and Immunity* journal and contain additional information that was left out in the publication.

Title: Single nucleotide polymorphisms in toll-like receptor 6 are associated with altered lipopeptide- and mycobacteria-induced interleukin-6 secretion.

Authors: Muki S. Shey, April K. Randhawa, Mark Bowmaker, Elizabeth Smith, Thomas J. Scriba, Marwou de Kock, Hassan Mahomed, Gregory Hussey, Thomas R. Hawn, and Willem A. Hanekom. *Genes Immun.* 2010; 11(7): 561-72.

4.1 Contributions

I did most of the laboratory assays and analyses and interpretation of results in this Chapter with supervision from Prof W.A. Hanekom and Dr T. J. Scriba. Dr T. Hawn did the linkage disequilibrium analysis and I combined and interpreted the data. Dr A. K. Randhawa did the luciferase experiments included. Richard Wells did the immunoblotting. I cloned the plasmids used in the luciferase and immunoblotting experiments.

4.2 Introduction

Host genetic factors may play a major role in the pathogenesis of mycobacterial infection and immune responses to vaccines in humans (332, 333). Genetic variations of human TLRs regulate signaling and inflammatory responses and are associated with susceptibility to various infections (174, 334, 335).

Human TLR6 is a 2 391 base pair gene that encodes a 796-amino acid (aa) type I transmembrane protein with a 630aa extracellular LRR region (including a 31aa signal peptide), a 21aa transmembrane domain and a 145aa intracellular TIR signaling domain. As mentioned previously, TLR6 forms a heterodimer with TLR2 in a complex incorporating CD14, which preferentially recognises di-acylated lipopeptides like PAM2

(PAM2CSKKKK, S-[2,3-bis(palmitoyloxy)-propyl]-(R)-cysteinyl-(lysyl)3-lysine). This contrasts with TLR1, which also heterodimerises with TLR2, in a complex with CD14, but which specifically recognises tri-acylated lipopeptides like PAM3 (Pam3CSKKKK, N-palmitoyl-S-[2,3-bis- (palmitoyloxy)-propyl]-(R)-cysteinyl-(lysyl)3-lysine) (336-338). Some studies suggest that ligand preference based on acyl number is partial, and that other structural features may also influence ligand recognition (339, 340). These include length of the fatty acid chain, chirality of the diacyloxypropyl carbon, position of the acyl group, and amino acid composition of the terminal peptides, which have also been shown to affect receptor specificity. The specific role of human TLR6 in recognition of different pathogens is less well understood than that of TLR1 and TLR2. For example, several studies suggest that TLR1 or TLR2 recognise *M.tb* and this initiates immune responses (196, 341, 342), whereas a role of TLR6 in response to this pathogen has only been suggested in a single study (343).

We and others reported variations in human cellular responses to di-acylated lipopeptides in studies that were principally focused on TLR1 polymorphisms (199, 344). Since TLR1 polymorphism did not completely account for the observed variation, we postulated that this might be due to variation in the TLR6 gene, which mediates differential signaling and cytokine responses; although no specific polymorphism has been defined. Other studies have suggested an association between TLR6 polymorphism and disease susceptibility. For example, the SNP T1932G (A644A) was associated with altered IFN- γ secretion in response to measles virus stimulation of peripheral blood mononuclear cells (PBMC)(345), while C745T was associated with asthma (346, 347) and with invasive aspergillosis (348) after allogeneic stem cell transplantation. This polymorphism has also recently been associated with inflammatory responses in hypertensive women (349). Other studies have evaluated associations between polymorphisms in the TLR1-TLR6-TLR10 gene cluster and diseases such as cancer (350, 351). A pooled analysis of these studies showed that polymorphisms in these gene clusters did not associate with risk of prostate cancer (352). We do not know if these polymorphisms alter TLR6 function. In this chapter, we focused on the polymorphisms occurring in the TLR6 coding region and their effect on innate immune responses to mycobacteria and mycobacterial lipopeptides.

Our aim was to investigate the role of TLR6 polymorphism in recognition and signaling of *M.tb*. Our hypothesis was that TLR6 polymorphisms are associated with differential immune responses to mycobacteria. Chronic inflammation is a hallmark of *M.tb* infection and substantial efforts have been made to identify the responsible bacterial components. We hypothesised that TLR6 polymorphisms contribute to differential immune responses, and ultimately differential protection against infectious diseases including TB. We examined whether TLR6 polymorphisms are associated with altered cytokine responses in response to mycobacteria and TLR agonists.

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4.3 Material and methods

4.3.1 Participants

Healthy adults were recruited at the South African TB Vaccine Initiative clinical site, near Cape Town in South Africa. This study was approved by the Research Ethics Committee of the University of Cape Town. Written informed consent was obtained from all participants. Exclusion criteria included HIV and other chronic infections, pregnancy, and active TB. The study population comprised individuals from different backgrounds, including Black African, Caucasian and South African mixed ethnicity.

4.3.2 Blood collection, peripheral blood mononuclear cell (PBMC) Isolation and cryopreservation

Heparinised blood was collected, for whole blood incubations and for PBMC isolation. PBMC were isolated from freshly collected whole blood using Ficoll density gradient centrifugation (Sigma). After isolation viable recovery was assessed using trypan blue (Sigma) exclusion staining. Multiple vials from each participants were step-frozen in fetal calf serum containing 10% DMSO. This involved placing vials each containing 1×10^7 cells in "Mr Frosty" (Nargene/Merck) containing isopropanol, which allows step-wise freezing to -80°C overnight. The vials were then transferred to liquid nitrogen for long-term storage.

4.3.3 Toll-like receptor ligands and antigens

Ultrapure lipopolysaccharide (LPS, TLR4 ligand) isolated from *Salmonella minnesota* R595 was obtained from List Biological Labs, Inc. The lipopeptides PAM2 (PAM2CSK KKK, S-[2,3-bis(palmitoyloxy)-propyl]-(R)-cysteinyl-(lysyl)3-lysine, TLR2/6 ligand, 100ng/mL), fibroblast stimulating lipopeptide 1 (FSL-1, TLR2/6 ligand, 300ng/mL) and PAM3 (Pam3CSK KKK, N-palmitoyl-S-[2,3-bis- (palmitoyloxy)-propyl]-(R)-cysteinyl-(lysyl)3-lysine, TLR2/1 ligand, 300ng/mL) were synthetic lipopeptides obtained from EMC Microcollections. Lysate from *M.tb* strain H37Rv (25 $\mu\text{g/mL}$) was

obtained from J. Belisle (NIAID reagent contract). Lyophilised live BCG (1.6×10^6 CFU/mL) was obtained from Statens Serum Institute.

4.3.4 Whole blood incubation

Whole blood diluted 1:5 in RPMI medium 1640 (BioWhittaker), was incubated with TLR ligands, BCG or *M.tb* lysate for 20 hours. Polymixin B (BioChemika, 10 μ g/mL) was added to the antigen wells containing the lipopeptides (except to the LPS condition), to minimise any possible effects of LPS contamination. IL-6 secreted into the supernatant was measured by ELISA (Human IL-6 OptEIA, BD Biosciences), according to the manufacturer's instructions.

4.3.5 DNA isolation and sequencing

Genomic DNA was isolated from 5ml blood using the Qiagen Blood Maxi Preparation Kit. TLR6 was PCR-amplified using Pfu polymerase (Promega) in two fragments (1600 and 900 base pairs, respectively) and nucleotide sequencing was performed (commercially by Macrogen). The following primers were used to amplify the two fragments:

TLR6-2 (5'-GTGGAGGTTTGAGAGTAACCATCCG-3'),

TLR6-15 (5'-GTGGGCTTCCTCTATAACTTTCTGGG-3'),

TLR6-5 (5'-GAGGTCAATAAAAGCAGGGGACAATCC-3') and

TLR6-16 (5'-GGCTAACCTCACCGCCTAGCTCAGTTCCCC - 3'). The sequencing reactions were performed with the following forward primers: TLR6-3 (5'-CACATGCTGTGTCCTCATGCACCAAGC-3');

TLR6-4 (5'-CACCCAAGTATTTATTCGCTATCC-3');

TLR6-6 (5'-CCTGCCATCCTATTGTGAGTTTCAGGC-3') and

TLR6-7 (5'-GAGGAACTTTGTCCCTGGCAAGAGC-3'). The sequences were aligned and analysed using the programs Phred/Phrap and Consed (University of Washington; Seattle, WA, USA (353, 354).

4.3.6 DNA expression vectors and plasmids

For functional studies, the single exon coding region of TLR6 was amplified from genomic DNA using Pfu Turbo polymerase (Promega) and primers hTLR6-start (5'-ATGACCAAAGACAAAGAACCTATTG-3') and hTLR6-nostop (3'-CAGTGACTTTTGTACTACACTTTAGA-5'). The amplified fragment was then cloned into the pEF6/V5-His-TOPO expression vector with a V5 epitope tag (Invitrogen, Carlsbad, CA, USA) (**Fig. 50**). The different polymorphic variants were generated with a whole plasmid PCR mutation strategy with techniques previously described (355). The following primers were used to generate the 745T (249S) variant, hTLR6-745T-F 5'-ACCAGAGGGTCAACCTTACTGAATTTTACC-3', and hTLR6-745T-R 5'-ATTCAGTAAGGTTGACCCTCTGGTGAGTTCTG-3'. For the dominant-negative control mutant, 2039A (680H), the primer set of hTLR6 2039A-F 5'-GAGGAACTTTGTCCATGGCAAGAGCATTGTGG-3' and hTLR6 2039A-R 5'-CCACAATGCTCTTGCCATGGACAAAGTTCCTCTCATG-3' was used. Subsequent digestion with the restriction enzyme *DpnI* removed the methylated DNA before transformation into JM109 competent *E. coli* cells (Invitrogen). Human TLR2 was cloned into pEF6/V5-His-TOPO vector after fragment amplification with the following primers, hTLR2-F 5'-ATGCCACATACTTTGTGG-3' and hTLR2-R 5'-GGACTTTATCGCAGCTCTCAG-3'. All plasmid constructs were verified by DNA sequencing.

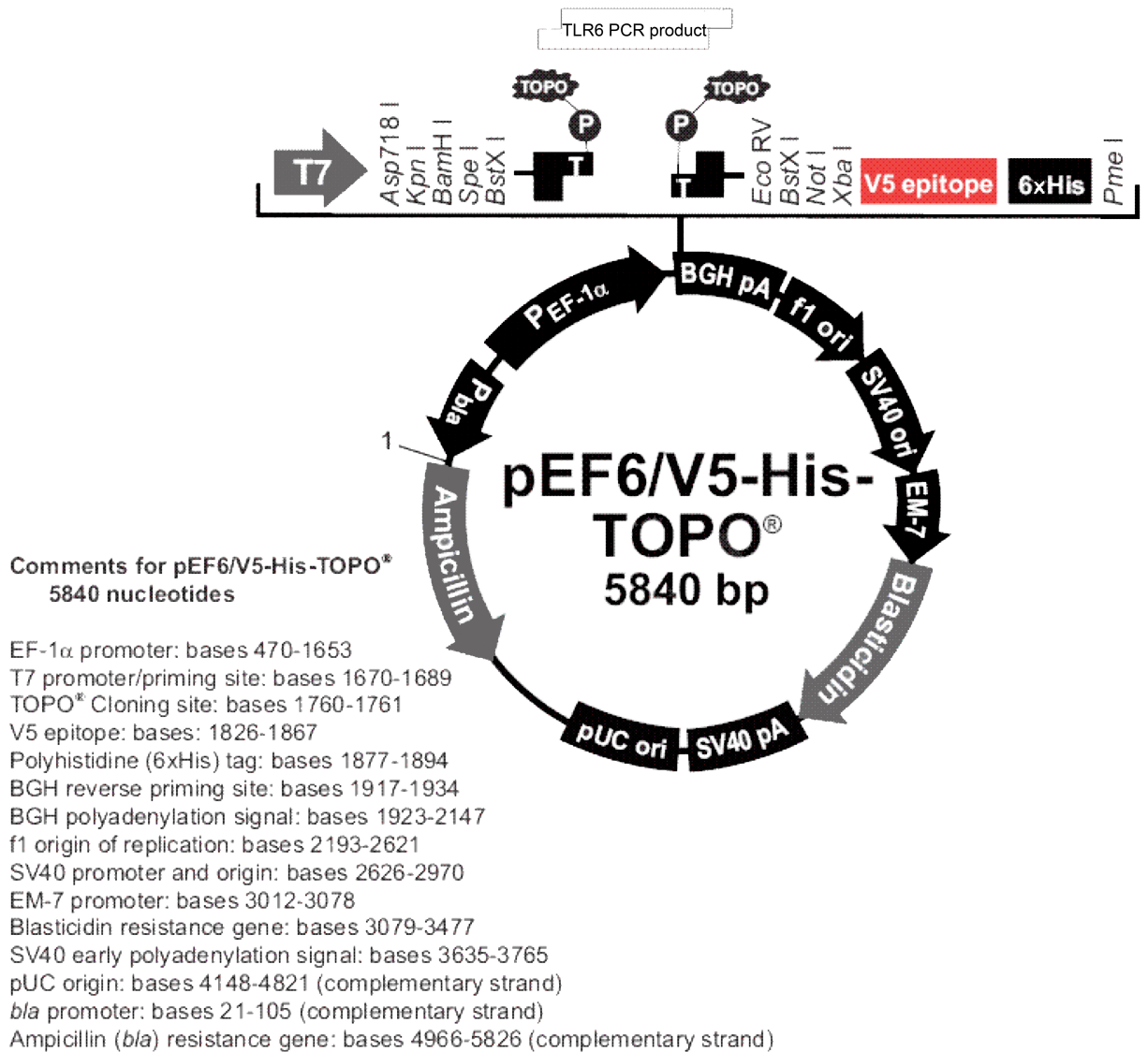


Figure 50: Cloning vector (Image adapted from www.invitrogen.com)

4.3.7 HEK293 cell transfections

HEK 293 cells (ATCC #CRL-1573) were grown according to ATCC recommendations. HEK293 cells were cultured in Dulbecco's modified Eagle medium (DMEM) (BD Biosciences), supplemented with 10% heat inactivated fetal bovine serum, 1% L-Glutamine (Life Technologies) and maintained at 37°C in 5% CO₂. HEK293 cells were transfected for 16-20 hours using Polyfect Transfection Reagent (Qiagen) at a cellular concentration of 2 x 10⁴ cells per well in a 96 well plate. Cells were transfected with the following plasmids (Promega) as previously described (199): 100ng ELAM-luciferase (NF- κ B reporter with ELAM promoter and Firefly luciferase), 10 ng pRL-TK luciferase

(Thymidine kinase promoter with Renilla luciferase reporter used as a control for transfection efficiency), 40ng CD14 as co-receptor to enhance signaling, 10ng TLR6 or TLR6 variants, and 50ng TLR2. The final concentration of transfected DNA was normalised with empty vector, pEF6. After overnight incubation, the transfected cells were stimulated for 4hours with PAM2 (250ng/mL), *M.tb* lysate (25µg/mL) or LPS (100ng/mL). The cells were lysed with cell lysis buffer (Promega) according to the manufacturer's instructions and luciferase activity was measured with a dual luciferase system (Promega). To control for transfection efficiency, Firefly luciferase activity was normalised to Renilla luciferase activity.

4.3.8 Immunoblotting and immunofluorescence

For immunoblotting, cells were transfected for 16-20 hours and then permeabilised with 1% Triton X-100 buffer containing a cocktail of protease inhibitors (Sigma Aldrich). The lysates were blotted on a membrane and probed with a mouse anti-V5 epitope antibody (Serotec) that recognises the V5-His tag on the TOPO cloning vector expressing the protein, followed by HRP-conjugated rabbit anti-mouse IgG (Zymed) and then developed with luminol chemiluminescent reagents (Roche).

For immunofluorescence, HEK293 cells were transfected for 16-20 hours with TLR6 constructs and plated in a 24 well plate on coverslips. After fixing with 10% neutral-buffered formalin solution (Sigma-Aldrich), the cells were lysed with 0.25% Triton X-100 in PBS, probed with the mouse anti-V5 epitope antibody and then with a FITC-conjugated rabbit anti-mouse conjugated secondary antibody. The coverslips were then washed and mounted for microscopy with mounting medium containing 25% glycerol, 10% polyvinyl alcohol (Sigma-Aldrich), 0.1M Tris-Cl pH 8.5 and 2.5% 1,4-diazabicyclo-[2.2.2]-Octane [DABCO].

4.3.9 mRNA expression

Cryopreserved PBMC were rapidly thawed at 37°C until a small ice crystal remained, RPMI containing 5% AB serum (Biowhittaker) was added dropwise with gentle shaking of the cryo vial. The addition continued until the cryovial was full, the content was then

transferred to a 15mL conical tube, which was then filled dropwise with medium. PBMC were washed twice before re-suspension in 5% AB serum. Viability was then determined by trypan blue exclusion staining. Five x 10⁵ PBMCs were incubated with PAM2 and LPS or left unstimulated, for 20 hours. RNA was isolated from the stimulated PBMCs using RNeasy Mini Kit (Qiagen) and complementary DNA (cDNA) was synthesised from the extracted RNA using Omniscript RT Kit (Qiagen) with Oligo dT₁₂₋₁₈ primers (Invitrogen). Recombinant RNaseOUT Ribonuclease inhibitor (Invitrogen) was used to inhibit ribonuclease activity. Quantitative real time PCR (qRT-PCR) was performed using SensiMix dT (Quantace; Watford, UK) containing 2X SensiMix dT, 50X SYBR Green solution and 50mM MgCl₂ Solution with the following primers for

TLR6-F: 5'-CTGTGTCCTCATGCACCAAG-3' and

TLR6-R: 5'-TCAACCCAAGTGCAGTTTC-3' and Glycerinaldehyde 3-Phosphate Dehydrogenase (GAPDH):

GAPDH-F: 5'-TTCACCACCATGGAGAGAGGC-3' and

GAPDH-R: 5'-GGCATGGACTGTGGTCATGA-3'. A standard curve was generated and used to quantify expression of TLR6 for each run. Results were normalised to relative expression of GAPDH.

4.3.10 Genotyping and linkage disequilibrium

Genotyping was done using Sequenom's MassARRAY™ technique to identify polymorphisms in the promoter or coding region of TLR6 or in TLRs 2, 1 and 10, as previously described (356). This technique uses allele-specific primer extension reactions to discriminate genotypes. We identified haplotype tagging SNPs from the YRI (Yorubans in Nigeria) and CEU (Utah residents with European ancestry) populations from the International HapMap Project (<http://www.hapmap.org>) and other public databases with the Genome Variation Server (<http://www.ncbi.nlm.nih.gov/SNP/> and www.innateimmunity.net). We searched a region on chromosome 4, 50 kilobases upstream and downstream of genes for TLR1, TLR6, TLR10, as well as TLR2 for tagged SNPs using an R² cutoff of 0.8 for linkage disequilibrium and a minor allele frequency cut-off of 5%.

Stata/Intercooled v10.0 software program PWLD (StataCorp LP; College Station, TX, USA) was used to calculate R^2 and D' as measurements of linkage disequilibrium between the polymorphisms.

We also genotyped the G-174C polymorphism in the IL-6 promoter region in our population using previously described methods (357, 358). Briefly, the region flanking position 174 was PCR amplified and the PCR product digested with the restriction enzyme, Hsp92 II (Promega), which cleaves at the C allele, but not the G allele.

4.3.11 Statistical analysis

GraphPad Prism v5 was used for data presentation and statistical analysis. The Mann-Whitney or Wilcoxon matched pairs tests were used to compare data sets. P values <0.05 were considered significant.

The observed allelic frequencies for the SNPs were determined by Hardy-Weinberg equilibrium equation for Black African and mixed ethnicity groups. A Mann-Whitney U test was used for statistical analysis of IL-6 secretion in whole blood while Student's t-test was used for the luciferase signaling assays. The differences were considered significant if the p value was less than 0.05 using a two-tailed test.

4.4 Results

One hundred healthy adults were enrolled, including 56 women and 44 men with an age range 18 to 57 years. The ethnic distribution comprised 24 Black Africans, 64 of mixed race (Coloureds) and 12 Caucasians.

4.4.1 SNP discovery

In order to identify common polymorphisms, we sequenced the coding region of TLR6 gene in 100 healthy adult volunteers and analysed the results using the PhredPhrap and Consed software. **Figure 51** shows a sequence analysis page that identifies the different genotypes for a T to G polymorphism (T1932G). We found 10 polymorphisms, which included 7 non-synonymous and 3 synonymous base pair changes (**Table 6**). The observed allelic frequencies were consistent with Hardy-Weinberg equilibrium. Nine of these polymorphisms have been reported before in public databases, including HapMap, NCBI and II-PGA (**Table 6**). One polymorphism (T34A) had not previously been described. All of the remaining nine TLR6 coding region polymorphisms were present in at least one HapMap population.

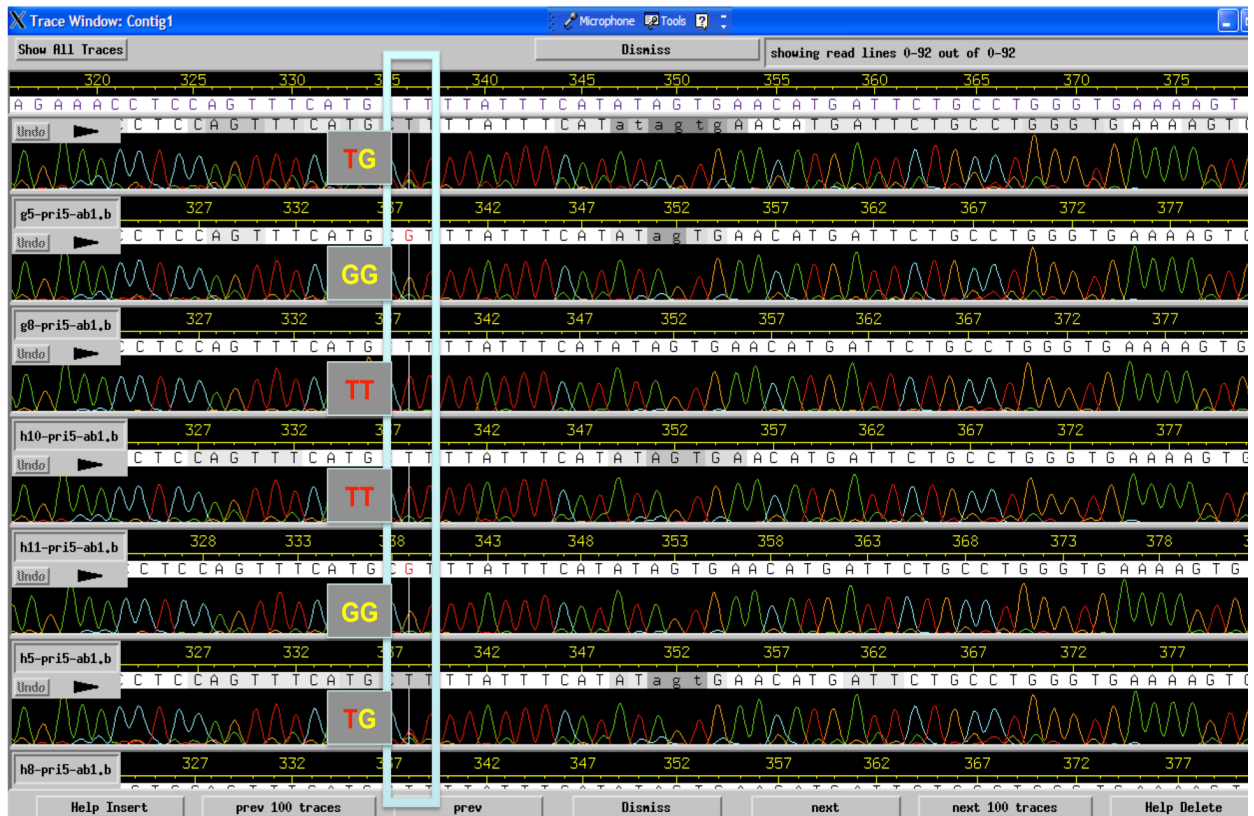


Figure 51: Sequence Analysis by PhredPhrap and Consed. The three different genotypes along the white line in the box correspond to the T1932G SNP are shown. TT, red; GG, yellow; and TG, red/yellow. The topmost sequence is the consensus (wild type) sequence while the other sequences are those of individual participants. The primers amplifying this TLR6 region bind at position 1596 (336 base pairs upstream of position 1932) of the wild type sequence.

SNP details		Current study					MAF data in previously described populations			
SNP	NCBI reference	Population	AA n(%)	Aa n(%)	aa n(%)	MA F (%)	CEU	YRI	LWK	MKK
							(%)	(%)	(%)	(%)
T34A (F12I)	ss161110012	Black	11(50)	11(50)	0(0)	25	na	na	Na	na
		Mixed	39(64)	22(36)	0(0)	18				
		Caucasian	9(75)	3(14)	0(0)	12.5				
T359C (I120T)	rs5743808	Black	18(86)	3(14)	0(0)	7.1	0	8.3	1.1	9.4
		Mixed	49(82)	11(18)	0(0)	9.1				
		Caucasian	12(100)	0(0)	0(0)	0				
T581C (L194P)	rs5743809	Black	21(100)	0(0)	0(0)	0	0	3.3	Na	na
		Mixed	53(87)	8(13)	0(0)	6.5				
		Caucasian	10(100)	0(0)	0(0)	0				
G740A (R247K)	rs35220466	Black	19(86)	3(14)	0(0)	6.8	0.8	2.5	Na	na
		Mixed	59(95)	3(5)	0(0)	2.4				
		Caucasian	12(100)	0(0)	0(0)	0				
C745T (P249S)	rs5743810	Black	22(92)	2(8)	0(0)	4.1	46.4	0	1.1	7.0
		Mixed	52(81)	11(17)	1(2)	10.1				
		Caucasian	3(25)	7(58)	2(17)	45.8				
G979A (V327M)	rs3796508	Black	19(95)	1(5)	0(0)	2.5	0	0.9	2.8	2.4
		Mixed	57(93)	4(7)	0(0)	3.2				
		Caucasian	11(100)	0(0)	0(0)	0				

Table 6 cont.

SNP details		Current study					MAF data in previously described populations			
SNP	NCBI reference	Population	AA n(%)	Aa n(%)	aa n(%)	MAF (%)	CEU	YRI	LWK	MKK
							(%)	(%)	(%)	(%)
G1083C (T361T)	rs3821985	Black	15(65)	7(30)	1(4)	19.5	72.4	31.4	Na	na
		Mixed	27(42)	25(39)	12(19)	38.2				
		Caucasian	2(17)	2(17)	8(66)	75				
A1263G (K421K)	rs3775073	Black	15(65)	7(30)	1(4)	15	29.2	70.8	15.7	32.5
		Mixed	29(48)	25(41)	7(11)	31.9				
		Caucasian	1(9)	1(9)	9(82)	86.3				
T1280C (V427A)	rs5743815	Black	21(100)	0(0)	0(0)	0	1.3	0	Na	na
		Mixed	61(100)	0(0)	0(0)	0				
		Caucasian	10(91)	1(9)	0(0)	4.5				
T1932G (A644A)	rs5743818	Black	13(62)	7(33)	1(5)	20	27	31	2.2	3.1
		Mixed	42(71)	16(27)	1(2)	15.2				
		Caucasian	9(75)	2(17)	1(8)	13.6				

Table 6: TLR6 coding region polymorphisms in South Africans, compared with those described in the Hapmap database. Genotype and allele frequencies of the ten polymorphisms for South African are listed, along with MAF described in the Hapmap database, for multiple other populations. CEU—Utah residents with European Ancestry; YRI –Yoruba in Nigeria); LWK – Luhya in Kenya; MKK – Maasai in Kenya. A, common allele, a, minor allele, MAF, minor allele frequency, na, not available. The HWE P-values are calculated for Black and mixed ethnicity populations. There were very few Caucasians and the HWE P-value was not calculated for this population.

4.4.2 Association of G1083C polymorphism with IL-6 secretion

To examine whether any of these polymorphisms were associated with altered downstream effects of TLR6 signaling, we stimulated whole blood from 70 of the 100 participants with di-acylated lipopeptides and several other TLR ligands. We have previously shown that measurement of IL-6 is ideal for assessing innate responses in whole blood stimulated with lipopeptides: IL-6 was secreted at readily detectable levels,

whereas IL-12, TNF- α , IL-1 β , and IL-10 were detected at low levels (199). We determined the association of polymorphisms we identified with levels of IL-6 secreted. Only two polymorphisms, C745T and G1083C, were associated with altered IL-6 production.

We examined G1083C first: the 1083CC (361T) genotype was associated with lower IL-6 production, compared with 1083GG (361T), in response to FSL-1, PAM2 and PAM3 (**Fig. 52**). Stimulation with LPS, as a control, was not associated with significant differences in the level of IL-6 produced.

We next examined whether TLR6 SNPs were associated with altered IL-6 response to whole mycobacteria, which have a complex repertoire of lipopeptides and other ligands. SNP G1083C was associated with a decrease in IL-6 secretion after *M.tb* lysate and BCG stimulation.

Together, these results suggest that allele 1083C is associated with decreased IL-6 levels in response to lipopeptide and mycobacterial stimulation.

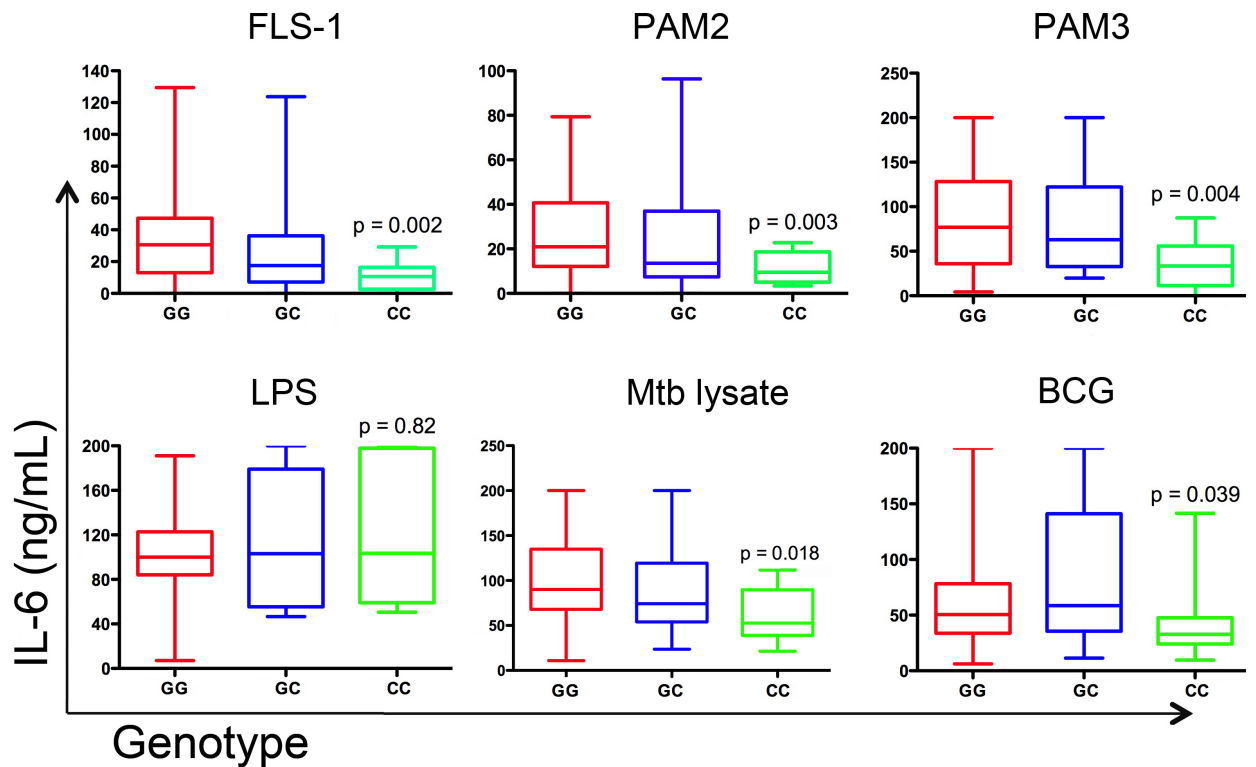


Figure 52: Effect of TLR6 G1083C polymorphism on IL-6 secretion: Whole blood was stimulated for 20 hours with TLR ligands or whole mycobacteria, followed by measurement of IL-6 levels in plasma, by ELISA. IL-6 levels were stratified by genotypes of G1083C polymorphisms. These figures show results from the following numbers of participants with specific genotypes: GG n=36; GC n=21; and CC n=12. Data presented as box and whiskers. Horizontal lines represent medians, boxes represent interquartile ranges and whiskers represent the range. P values represent comparisons between the CC and GG genotypes with a Mann-Whitney test.

4.4.3 Association of C745T polymorphism with IL-6 secretion

Polymorphism C745T is non-synonymous and encodes a proline to serine (P249S) change at amino acid 249 in the extracellular domain of TLR6. Due to small numbers of the 745TT genotype (n=2), we were unable to assess its homozygous effect independently. When CC and CT/TT genotypes were compared, individuals with the CT/TT genotype (n=15) had reduced levels of IL-6 upon stimulation with FSL-1 and PAM3, compared with individuals with the 745CC (n=55) genotypes (**Fig. 53**). There were no differences when PAM2, the control LPS, or the mycobacteria, *M.tb* and BCG were used as stimulants.

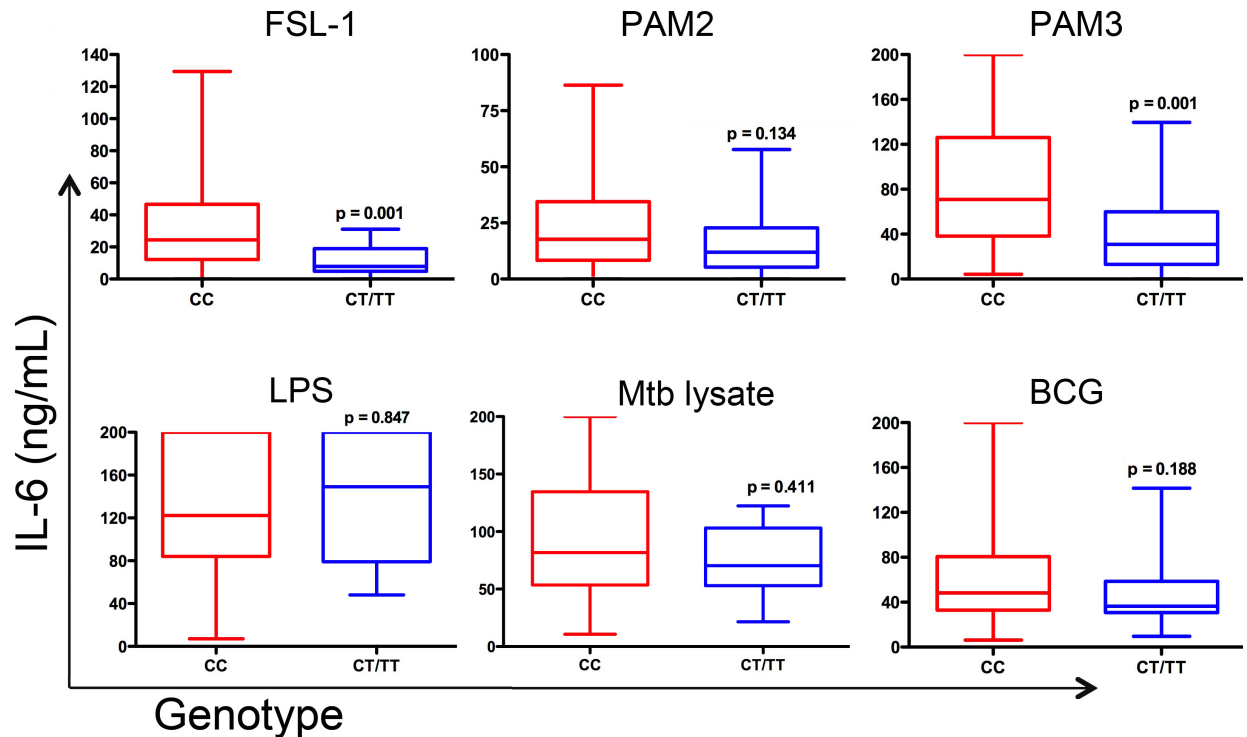


Figure 53 TLR6 C745T and Whole Blood IL-6 Secretion stratified by genotype. Whole blood IL-6 levels were measured by ELISA and stratified by polymorphism C745T genotypes. P values represent comparisons between the CC and CT/TT genotypes with a Mann-Whitney test. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range.

4.4.4 Reproducibility of our assay

To assess the reproducibility of these results, we repeated the experiment in 26 of the 100 individuals, and found the same results for G1083C in response to FSL-1 and PAM2, but not PAM3 (**Fig. 54A**). For the 745T SNP we compared the CC (n=18) and the CT/TT (n=8) genotypes, and observed similar trends (**Fig. 54B**). *M.tb* lysate and BCG were not used during the repeat experiment.

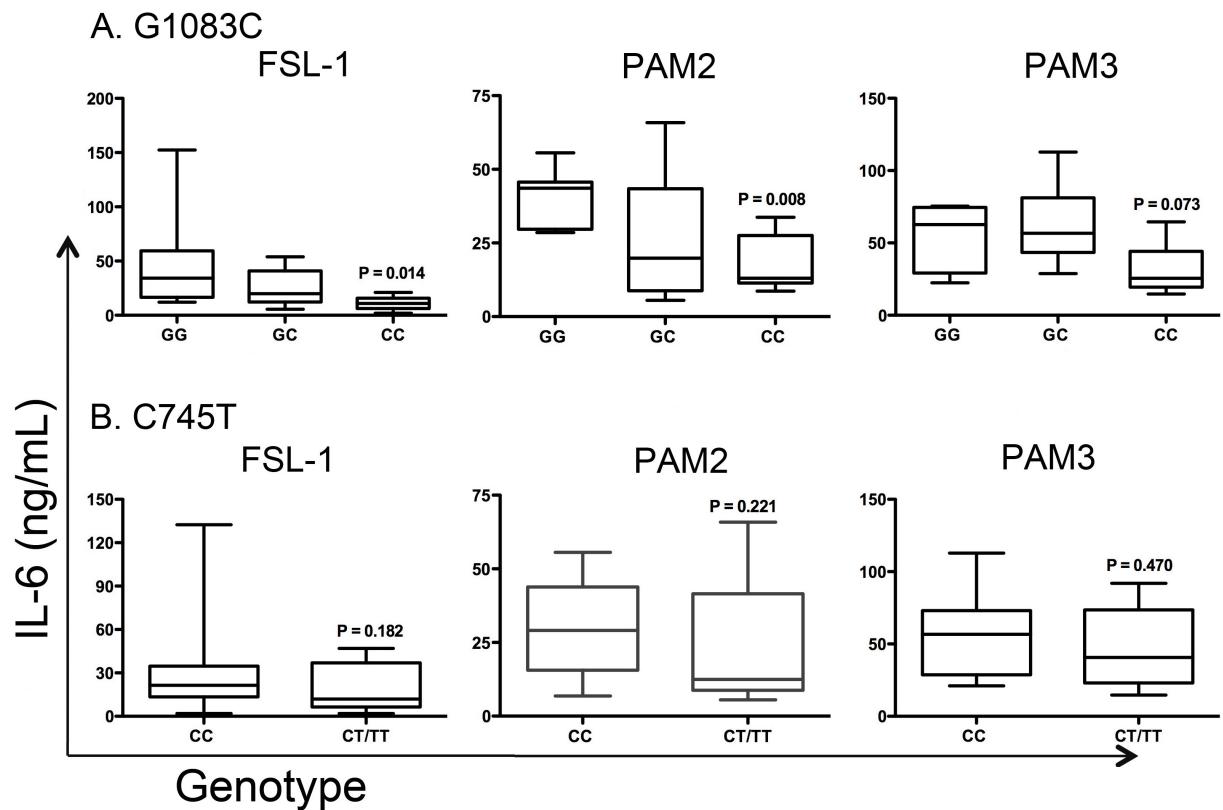


Figure 54: Validation of association between TLR6 genotypes and IL-6 production. To validate our data, blood from another 26 participants was examined with different genotypes for G1083C (**A**) and C745T (**B**) polymorphisms. P values representing assessment of differences between the GG and CC (for G1083C) or CC and CT/TT (for C745T) genotypes, using a Mann-Whitney test. The GG and GC genotypes were also compared, but no differences were found. GG n=8; GC n=12; and CC n=6. CC and CC n=18; CT n=6, TT n= 2. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range.

We also examined the intra- and inter-individual variation of the assay over time by comparing IL-6 levels in blood taken from individuals at different times. In a paired analysis, we found consistent IL-6 levels within individuals and a similar rank order between individuals, suggesting that IL-6 responsiveness is a stable biologic phenotype (**Fig. 55**).

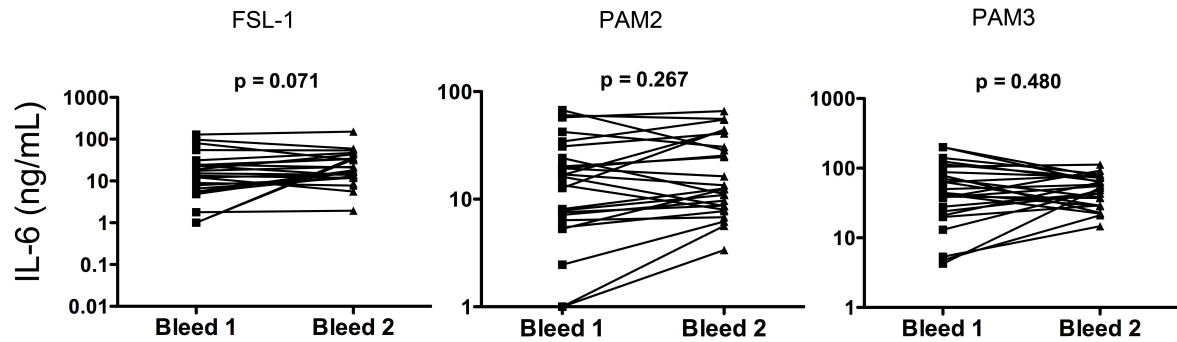


Figure 55: Reproducible levels of IL-6 in whole blood. Whole blood was stimulated for 20 hours with TLR ligands. Levels of IL-6 are depicted from 26 individuals, whose blood was collected at two different time points. To accommodate values of 0 on the log scale, these values were replaced with 1. P values represent comparison two bleeds using Wilcoxon matched pairs test.

We further sought to determine if the association observed with whole blood could be replicated in PBMC. We isolated PBMC from whole blood from the same individuals, as above, and stimulated with FSL-1, PAM2 and PAM3 for 20 hours. We then determined the levels of IL-6 secreted in supernatant collected from stimulated PBMC. We found a borderline association of IL-6 secretion with genotypes of TLR6 G1083C SNP after stimulation with FSL-1 ($p=0.05$) (**Fig. 56A**). There was no association with the other ligands with the G1083C genotypes. The TLR6 C745T genotypes were not associated with IL-6 levels in response to any of the ligands examined (**Fig. 56B**).

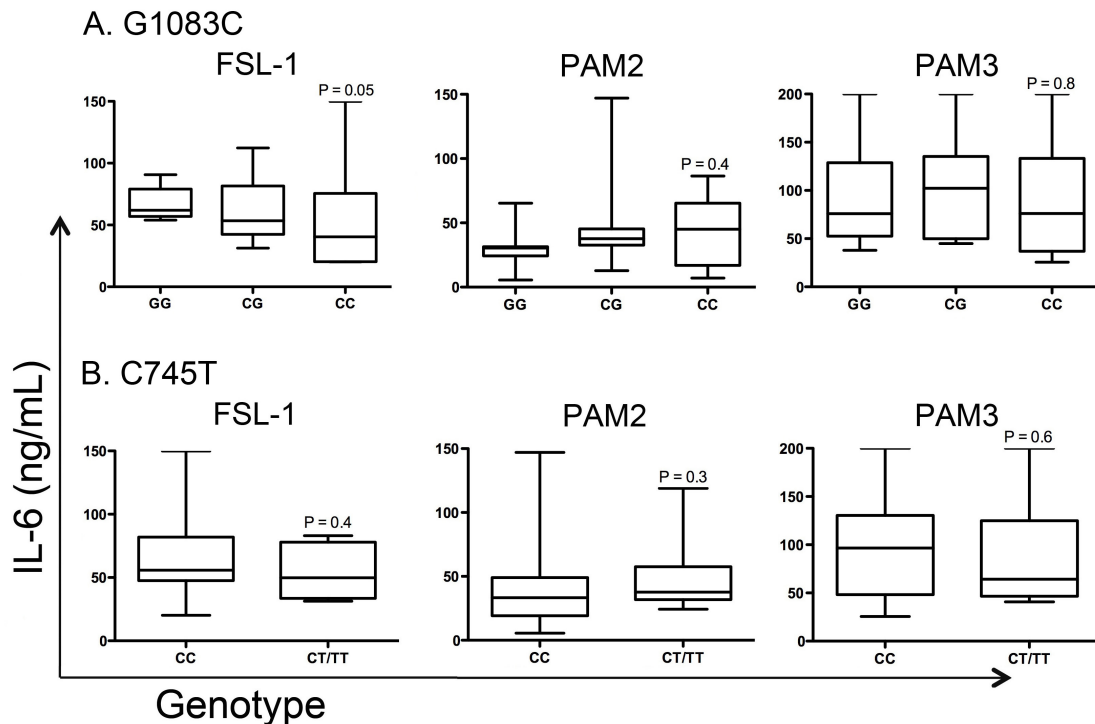


Figure 56: IL-6 secretion from PBMCs. PBMCs isolated from whole blood were stimulated and levels of IL-6 determined by ELISA. P values represent assessment of differences between the GG and CC for G1083C (A) or CC and CT/TT for C745T (B) genotypes, using a Mann-Whitney test. The GG and GC genotypes were also compared, but no differences were found. G1083C: GG n=8, GC n=12, and CC n=6. C745T: CC n=18; CT n=6, TT n= 2.

4.4.5 Mechanisms of altered IL-6 secretion TLR6 polymorphisms

4.4.5.1 Linkage disequilibrium

As a synonymous polymorphism, G1083C may directly regulate cytokine secretion in response to mycobacteria or lipopeptides through effects on transcriptional regulation of TLR6. Alternatively, this SNP may be in linkage disequilibrium with a polymorphism that directly regulates function. To address the latter possibility, we examined whether polymorphisms associated with altered IL-6 levels in response to FSL-1 or PAM2 were in linkage disequilibrium with G1083C. We first examined the other coding region polymorphisms in TLR6. The highest correlation was observed between G1083C and the A1263G polymorphisms ($R^2=0.75$, $D'=0.97$, Fig. 57). Although this suggests some LD correlation between 1083 and 1263, the latter SNP was not associated with altered levels of IL-6. Furthermore, A1263 is synonymous and has no known association with function. The

remaining TLR6 coding region SNPs had low levels of linkage disequilibrium with 1083 ($R^2 < 0.12$ for all SNPs except 745).

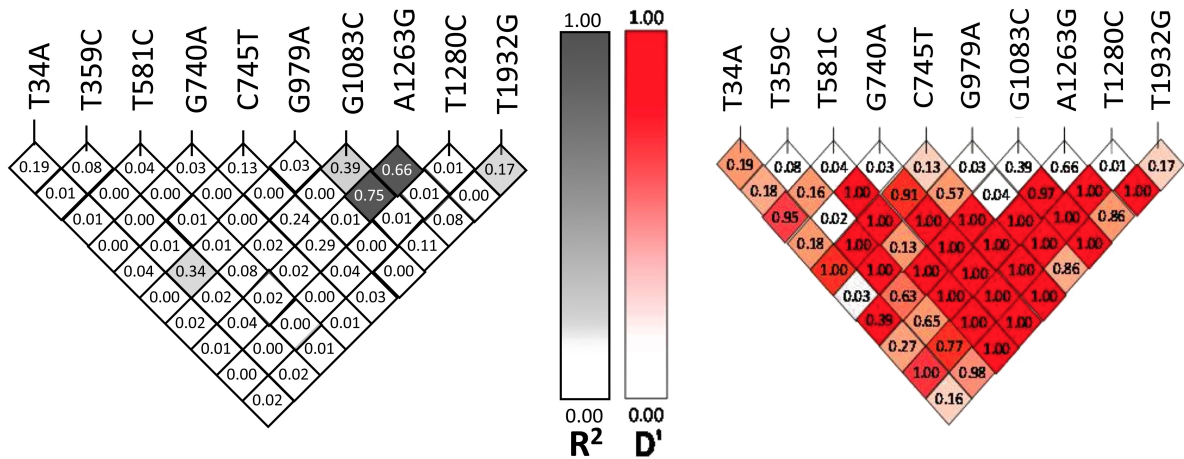


Figure 57: Linkage disequilibrium analyses between TLR6 SNPs. R-squared (R^2) (left) and D' (right) values for each SNP combination are shown numerically and by shading, based upon the legend in the middle. TLR6 and TLR1 SNPs were genotyped and analysed for level of LD with G1083C and C745T polymorphisms. The figure shows R^2 values for pairwise comparison of the different polymorphisms. D' or $R^2 = 0$ represents no LD correlation; D' or $R^2 = 1$ represents high (maximum) LD correlation. The program pwld in Stata was used to calculate the values. The MAF is shown adjacent to each corresponding SNP.

We next examined whether non-coding TLR6 SNPs or nearby polymorphisms were associated with IL-6 levels. TLR6 is located on chromosome 4p14 in a region adjacent to TLR1 and TLR10 that spans approximately 54 kb (359). We examined additional non-coding region haplotype-tagging TLR6 polymorphisms (rs1039559, rs7673348, rs7665774) as well as haplotype-tagging polymorphisms (in the CEU and YRI populations) in TLR1 (rs17616434, rs3923647, rs3924112, rs4833095, rs5743618) and TLR10 (rs4321646, rs10856837, rs7694115). We also examined seven haplotype-tagging SNPs in TLR2 on chromosome 4q32 (rs3804090, rs5743708, rs11935252, rs1337, rs1339, rs1439166, rs6535946).

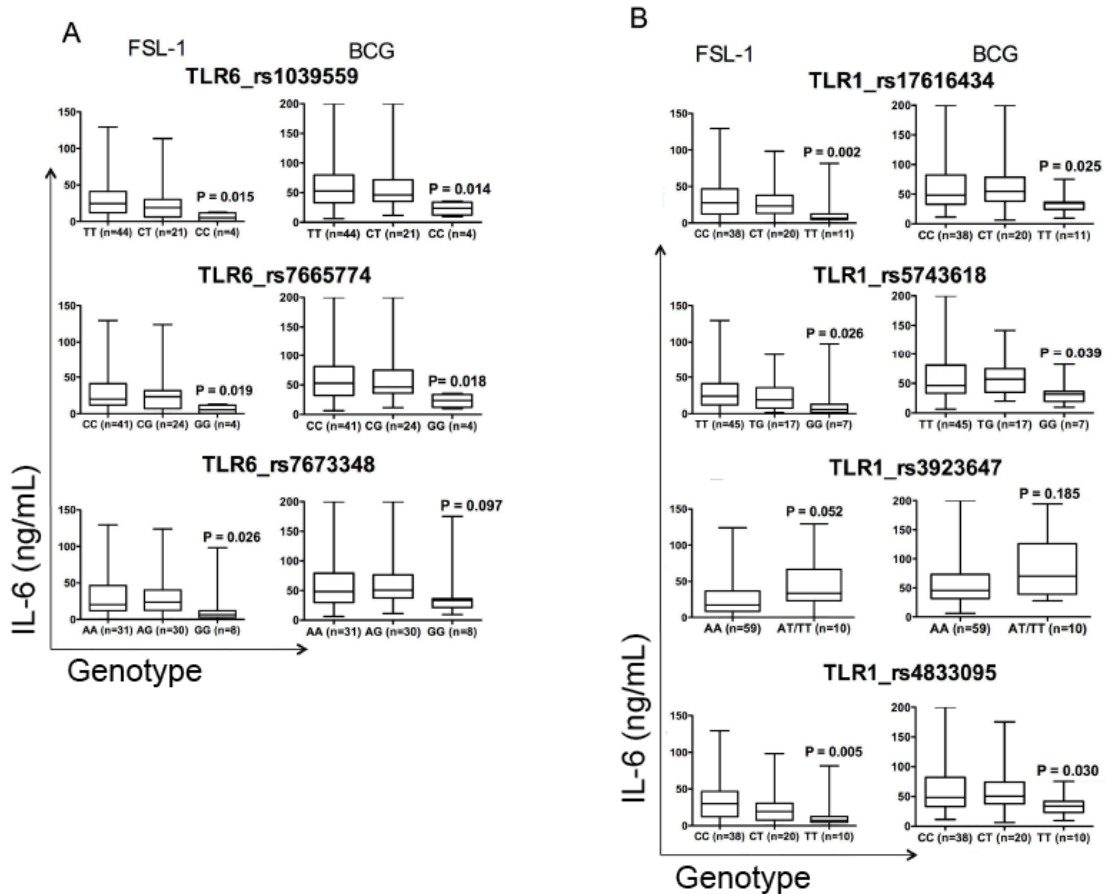


Figure 58: Association of IL-6 secretion in response to FSL-1 and BCG with polymorphisms in the non-coding region of TLR6, and polymorphisms in TLR1. Polymorphisms in TLR6 and TLR1 were identified from HapMap and genotyped in our participants. **(A).** TLR6 tagged polymorphisms. **(B).** TLR1 tagged polymorphisms. P values represent differences between the homozygous genotypes with Mann-Whitney test. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range.

Gene	SNP	NCBI reference	Frequency			P value for comparison of IL-6 levels between AA and aa genotypes				
			AA n (%)	Aa n (%)	aa n (%)	FSL- 1	PAM2	PAM3	BCG	<i>M.tb</i> lysate
TLR1	T/G	Rs5743618	77(77)	14 (14)	9 (9)	0.02	0.5	0.001	0.039	0.7
	A/T*	Rs3923647	85(85)	13 (13)	2 (2)	0.05	0.2	0.001	0.185	0.003
	C/T	Rs4833095	57(58)	29 (29)	13 (13)	0.005	0.5	0.0004	0.030	0.9
	C/T	Rs1761643 4	59(59)	26 (26)	15 (15)	0.002	0.3	0.0003	0.025	0.8
TLR6	C745T*	Rs5743810	76(77)	20 (20)	3 (3)	0.001	0.1	0.001	0.118	0.4
	G1083C	Rs3821985	45(45)	34 (34)	21 (21)	0.002	0.003	0.004	0.039	0.02
	T/C	Rs1039559	65(65)	28 (28)	7 (7)	0.015	0.2	0.002	0.014	0.6
	C/G	Rs7665774	60(60)	32 (32)	8 (8)	0.019	0.2	0.002	0.018	0.6
	A/G	Rs7673348	46(46)	42 (42)	12 (12)	0.026	0.9	0.002	0.097	0.7

Table 7: Polymorphisms in the TLR6 and TLR1 regions that are associated with altered IL-6 secretion. Genotypes were examined for association with IL-6 secretion after stimulation with TLR ligands, live BCG and *M.tb* lysate. The table lists SNPs with significant associations when comparing levels of IL-6 in individuals who were homozygous (AA compared with aa) for the indicated polymorphisms (assessed by a Mann-Whitney test). SNPs are listed in chromosomal order

* There were only two individuals who were homozygous for this minor allele. These individuals were grouped with those who were heterozygous and then compared with individuals who were homozygous for the major allele by Mann-Whitney test.

Seven polymorphisms in the TLR6 promoter region and TLR1 were significantly associated with altered IL-6 levels in response to FSL-1 (**Fig. 58A and B**). One polymorphism was associated with altered IL-6 in response to *M.tb*, and none was associated with altered IL-6 in response to PAM2. The levels of IL-6 secreted in response to FSL-1 and BCG are shown in **Figure 58**. For the other ligands only the P-values are represented since the trend of IL-6 secretion for these ligands was identical to FSL-1 and BCG (**Fig. 58 and Table 7**). TLR6 coding region polymorphisms and TLR1 polymorphisms that were significantly associated with altered levels of IL-6 secretion (**Fig. 59A**) were analysed for linkage disequilibrium (LD) with the G1083C and C745T polymorphisms. For the non-coding region polymorphisms, the most prominent correlation with the G1083C polymorphism was observed with the TLR6 promoter region polymorphisms rs1039559 ($R^2 = 0.42$, $D' = 1.0$) and rs7665774 ($R^2 = 0.35$, $D' = 0.84$), and with the C745T polymorphism ($R^2 = 0.23$, $D' = 1.0$) (**Fig. 59B**).

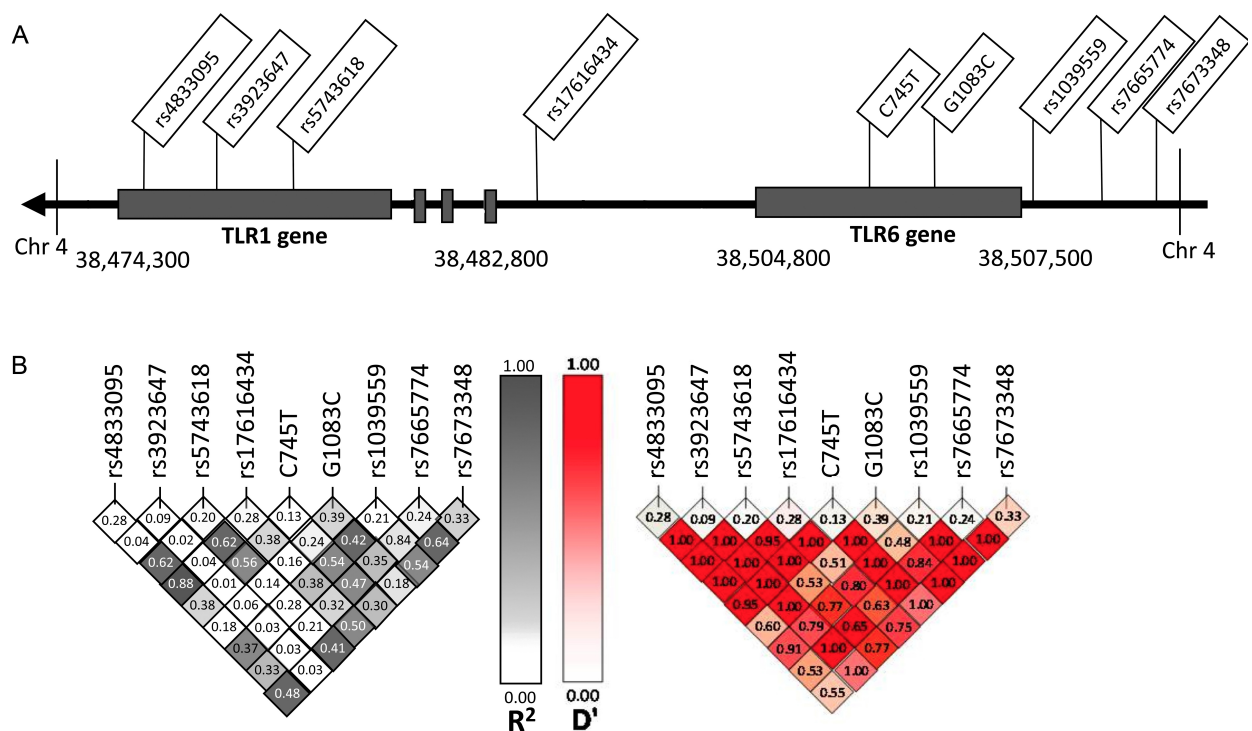


Figure 59: Linkage disequilibrium analyses between TLR6 and TLR1 SNPs. (A). TLR1 and TLR6 gene on chromosome 4. **(B).** R-squared (R^2) (left) and D' (right) values for each SNP combination are shown. The minor allele frequency is shown adjacent to each corresponding SNP. The program pwnd in Stata was used to calculate the values.

The G1083C polymorphism had very weak LD correlation with the TLR1 polymorphisms with $R^2 \leq 0.18$ (**Fig. 59B**). One of the polymorphisms associated with altered IL-6

secretion was TLR1_T1805G (rs5743618), which has been found to regulate signaling in response to PAM3 (199, 344, 360). The association of TLR1_T1805G with IL-6 signaling in our dataset was strongest for PAM3, weaker for FSL-1 and BCG, and absent for PAM2 (**Fig. 60**). The R^2 and D' values for TLR1_T1805G and TLR6_G1083C were 0.14 and 0.6, respectively, suggesting a low level of linkage disequilibrium. We also examined SNP TLR2_G2258A, which has previously been shown to regulate signaling (174, 361-364). We found that this polymorphism was not associated with IL-6 secretion in response to mycobacteria and lipopeptides even though the frequency of this SNP in our populations was very rare (97GG, 3GA and no AA). These results suggest that these two previously characterised SNPs in TLR1 and TLR2 do not explain the association of G1083C with IL-6 secretion in response to mycobacteria and lipopeptides. In summary, G1083C is in linkage disequilibrium with several SNPs that are also associated with altered IL-6 secretion in response to mycobacteria and lipopeptides. Based on these data, we are unable to determine which of these SNPs is most likely to directly regulate signaling in response to di-acylated lipopeptide stimulation.

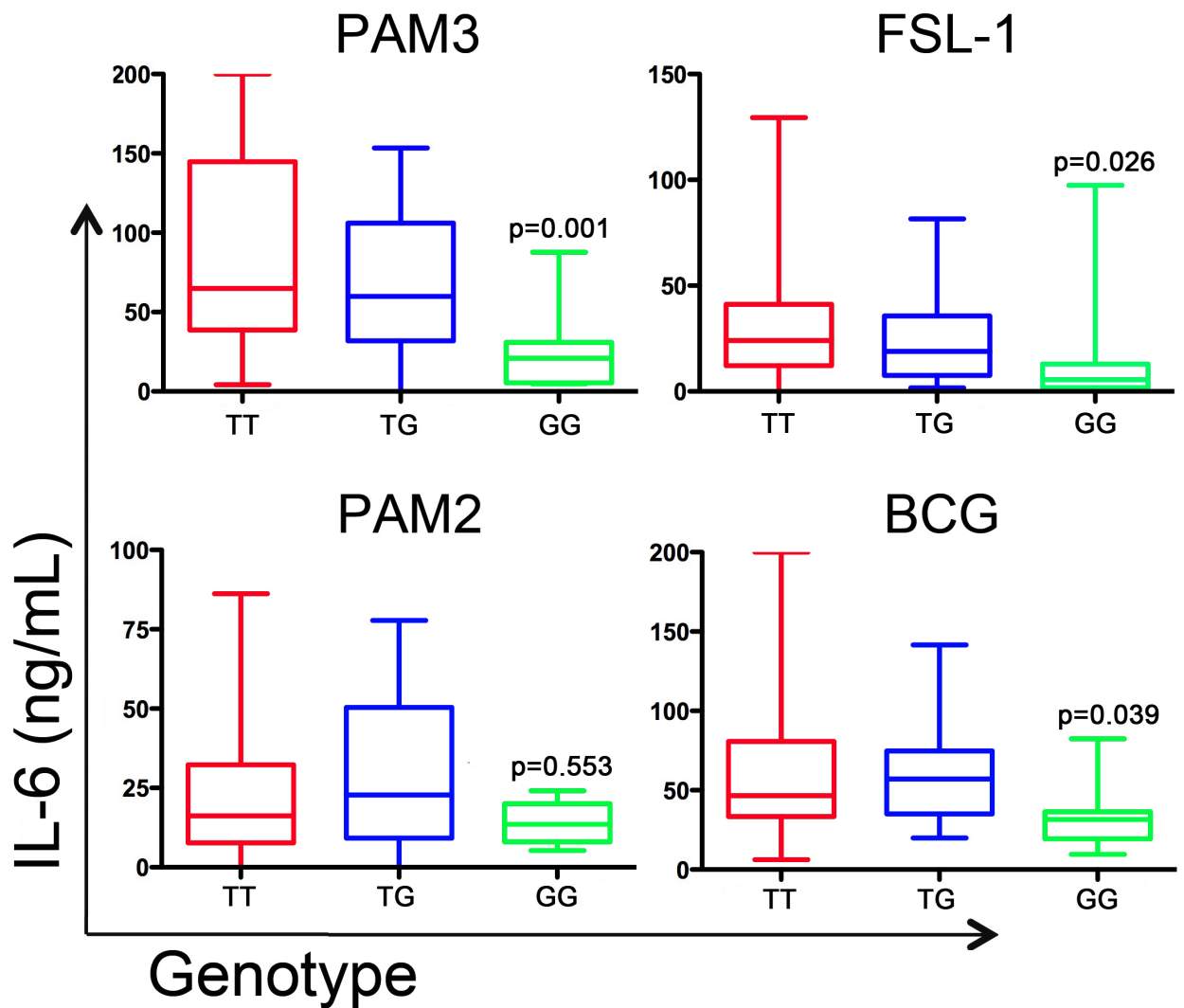


Figure 60: Effect of TLR1 T1805G polymorphism on IL-6 secretion. IL-6 levels were stratified by genotypes of T1805G polymorphisms. TT, n=45; TG n=17; and GG n=7. P values represent comparisons between the TT and GG genotypes with a Mann-Whitney test. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range.

4.4.5.2 TLR6 mRNA expression

We next examined other possible mechanisms that may explain the observed differences in IL-6 secretion. These factors may include effects of level of protein or mRNA transcript expression. Since the G1083C SNP is synonymous, we did not think this SNP would be associated with differential protein expression. By contrast, the C745T SNP could be possibly associated with altered protein expression since this results in a change in amino acid sequence. We then examined whether G1083C and C745T were associated with altered levels of TLR6 mRNA transcripts. We measured

TLR6 mRNA levels by qRT-PCR in PBMC from a subgroup of 26 individuals with different G1083C and C745T genotypes. No differences were observed in TLR6 mRNA levels when stratified according to genotypes in unstimulated PBMCs or in cells stimulated with PAM2 or LPS for both TLR6_G1083C and TLR6_C745T SNPs (**Fig. 61A and B**). Stimulation did not result in any significant increase in TLR6 expression. We also found that the other polymorphisms in the coding region (**Table 7**) were not associated with different TLR6 mRNA levels. Together, these results suggest that polymorphism TLR6_G1083C, C745T and other SNPs in this region were not associated with altered levels of TLR6 mRNA expression. TLR6 mRNA expression did not explain the observed differences in IL-6 secretion between different genotypes for the G1083C and C745T SNPs.

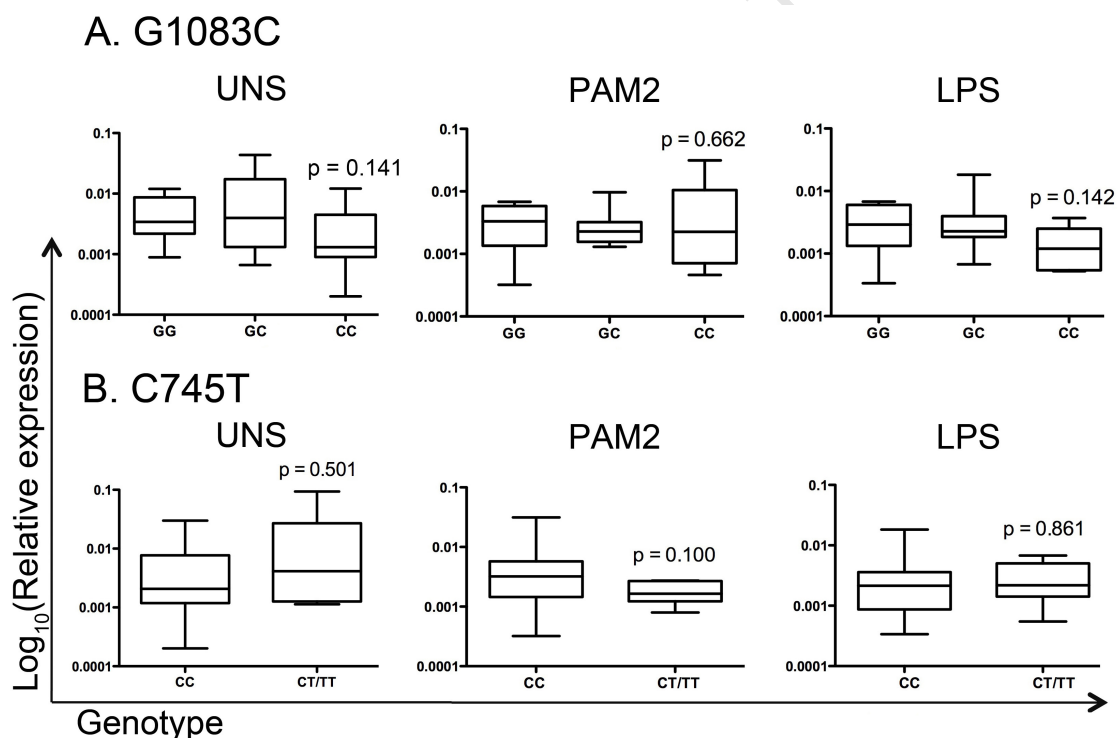
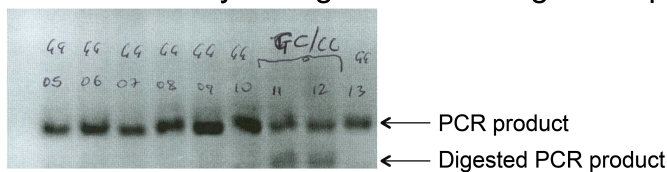


Figure 61: TLR6 mRNA expression. The expression levels of the TLR6 mRNA transcripts were measured by qRT-PCR in PBMC stimulated with PAM2, LPS or media and associated with genotypes for G1083C (**A**) and C745T (**B**) polymorphisms. GG (n=8), GC (n=12) and CC (n=6) for G1083C; CC (n=18) and CT/TT (n=8). P values represent assessment of differences between the GG and CC (for G1083C) or CC and CT/TT (for C745T) genotypes. The Mann-Whitney test was used for statistical analysis comparing GG and CC (for G1083C), or CC and CT/TT (for C745T) genotypes. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range.

4.4.5.3 IL-6 SNP genotyping

The G-174C promoter region polymorphism has been associated with reduced IL-6 levels (358, 365). To test whether this polymorphism was associated with altered IL-6 secretion, we genotyped this polymorphism in our study population by restriction digest (Fig. 62A). We found no association between IL-6 levels and genotype at position -174 of the IL-6 promoter (Fig. 62B) for all antigens except BCG. Overall, we concluded that this polymorphism does not alter IL-6 secretion.

A. Restriction enzyme digestion of IL-6 gene at position -174



B. Association of IL-6 G-174C genotypes with IL-6 secretion

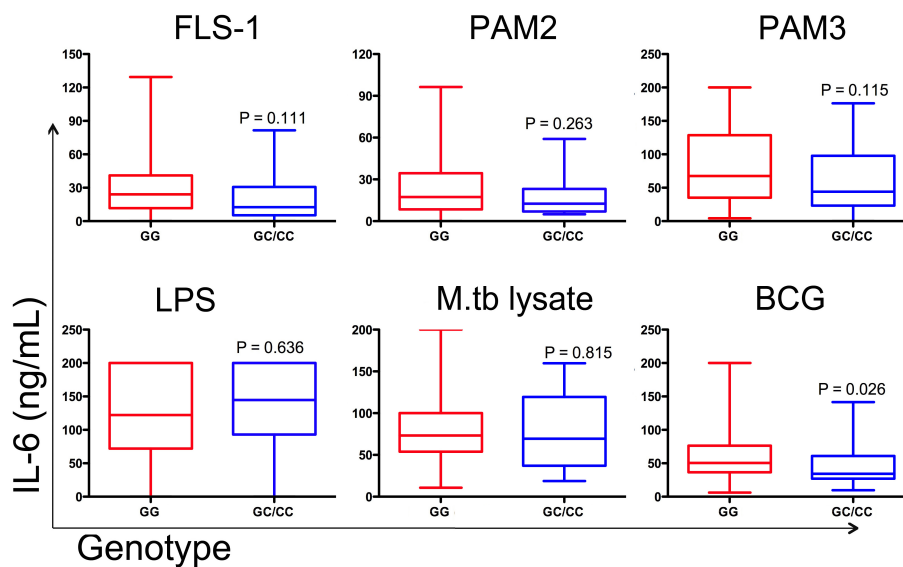


Figure 62: Genotyping IL-6 G-174C polymorphism. (A). This SNP was genotyped by restriction enzyme digestion with the enzyme cutting where there is a C allele (GC or CC) and no digestion when there is a homozygous G. (B). The different genotypes associated with levels of IL-6 secreted when whole blood was stimulated with FSL-1, PAM2, PAM3, LPS, M.tb lysate and BCG. GG (n=50) and GC/CC (n=18). The Mann-Whitney test was used for statistical analysis. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range.

4.4.5.4 Luciferase assay

To further investigate a possible regulatory effect of C745T on lipopeptide signaling, we cloned the 745C (wild type) and 745T variant into the EF6-V5-His-TOPO expression plasmid vector (pEF6). As a control, we also generated a dominant-negative TLR6 variant with a proline to histidine mutation at amino acid 680 (P680H, C2039A). This mutation is equivalent to the P712H substitution in TLR4, which abrogates LPS signaling in C3H/HeJ mice (366). We examined whether the TLR6 variants were able to mediate NF- κ B signalling in HEK293 cells. In response to PAM2 stimulation, allele 745T mediated significantly decreased NF- κ B signaling activity in comparison to allele 745C (**Fig. 63**). The dominant negative variant, 680H, was also associated with decreased NF- κ B signaling. As a control, we stimulated the cells with LPS and found no significant signaling above baseline levels in comparison to wells stimulated with media alone. We next examined whether variant 745T mediated signaling in response to a complex microbial ligand by stimulating with *M.tb* lysate. We found that allele 745T mediated decreased NF- κ B signaling activity, compared with 745C. As a control, the 2039A dominant negative variant completely abolished signaling to PAM2 and to *M.tb* lysate. Together, these results suggest that polymorphism C745T directly regulates NF- κ B signaling and IL-6 production in response to PAM2 stimulation and *M.tb* lysates.

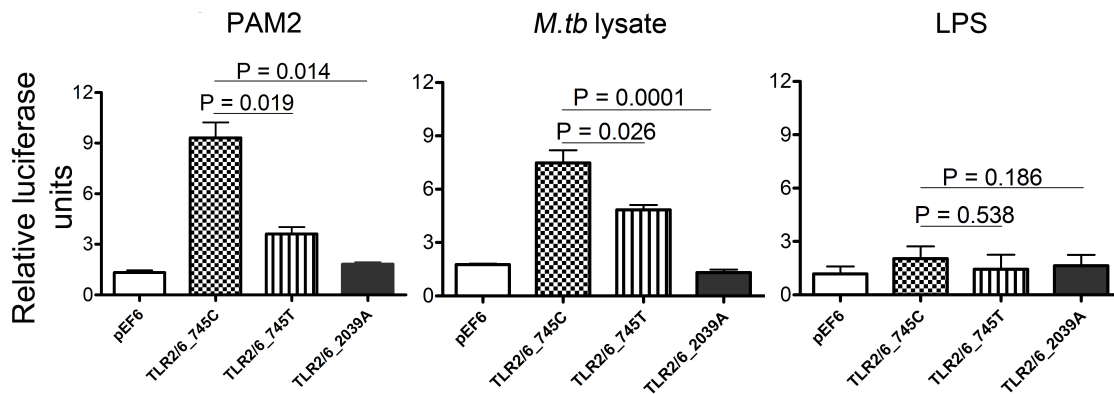


Figure 63: Regulation of NF- κ B signaling by a TLR6 polymorphism. HEK293 cells were transfected with CD14, an NF- κ B luciferase reporter, a *Renilla* luciferase construct to control for transfection efficiency (pRL-TK). Additional transfectants varied by condition and included an empty plasmid vector (pEF6), or, TLR2 with one of 3 TLR6 constructs, 745C, 745T, or 2039A. Polymorphism C2039A (P680H) is a TLR6 variant with a dominant-negative effect on NF- κ B signaling. Luciferase activity shown represents the ratio between basal activity (medium only) and that of transfected cells stimulated with PAM2, LPS, or *M.tb* Lysate. Mean values (+/- standard deviation) are depicted for three independent experiments, each performed in triplicate. P values calculated with Student's t-test (as mean values were calculated from the different experiments).

4.4.5.5 Immunofluorescence and Immunoblotting

We next examined whether differential localisation patterns or expression levels of the 2 variants could mediate observed signaling differences. The TLR6 variants had similar protein levels when assessed by immunoblot (**Fig. 64**). Further, TLR6 localisation was similar for the variants with expression at or near the plasma membrane. As a negative control, we transfected the empty backbone pEF6 vector, and did not observe any positive staining or expression. Together, these results suggest that TLR6 expression or localisation does not alter its function.

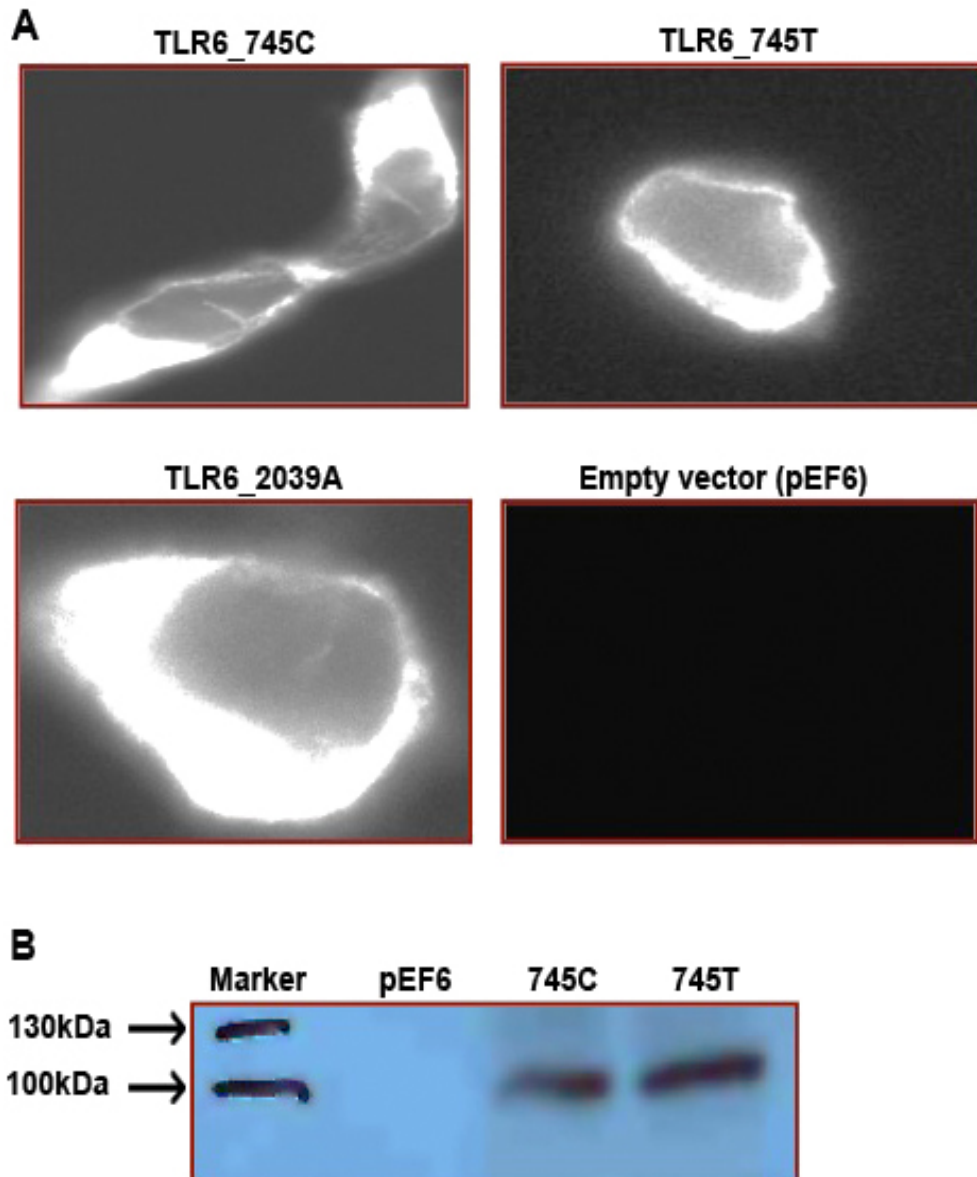


Figure 64: Expression of TLR6 protein. (A). Localisation of TLR6 745C (wild type), 745T (variant) and 2039A (control variant) on transfected but unstimulated HEK293 cells as determined by immunofluorescence using a fluorescent microscope. (B). Expression of the two TLR6 constructs as determined by immunoblotting in transfected but unstimulated HEK293 cells. The immunoblot and immunofluorescence are representative plots of two separate experiments.

4.5 Discussion

In this study, we aimed to identify TLR6 polymorphisms occurring in Cape Town, South Africa, and to examine whether TLR6 polymorphisms regulate innate immune responses to lipopeptides and mycobacteria including *M.tb* and *M. bovis* BCG. We identified a novel polymorphism, which was not associated with altered IL-6 secretion. We observed that altered IL-6 secretion from stimulated whole blood was associated with the G1083C (T381T) and C745T (P249S) polymorphisms.

The mechanism of altered IL-6 secretion associated with the G1083C polymorphism is not known. G1083C is synonymous and does therefore not alter the amino acid composition of TLR6. Further, individuals with different G1083C genotypes had similar levels of TLR6 mRNA expression, suggesting that transcriptional regulation is an unlikely mechanism. Possible alternative mechanisms include post-transcriptional regulatory mechanisms, or differential stability of the protein or mRNA within the cell. Several studies have shown that mRNA expression and protein levels are not always correlated (367, 368).

Alternatively, SNP G1083C may be in linkage disequilibrium with a polymorphism that acts through a post-transcriptional mechanism. Candidate LD regions include the promoter region of TLR6, or polymorphisms in TLR1 and TLR10, TLRs that are adjacent to TLR6. To examine this possibility, we genotyped haplotype-tagging polymorphisms in TLR1, TLR10, and non-coding regions of TLR6. Haplotypes are SNPs that occur in blocks and may be transmitted together (369). We found that overall there was a weak LD correlation between several polymorphisms and the G1083C and C745T polymorphisms. This suggests that the G1083C polymorphism may mediate differential IL-6 secretion through association with any of these polymorphisms, but we could not definitely identify these polymorphisms. The effects of two previously characterised polymorphisms, TLR2_G2258A (R753Q)(174, 361-363) and TLR1_T1805G (I602S)(174, 199, 344), could not explain our results. Together, our data suggest that G1083C is at least a useful genetic marker for regulation of IL-6 responses to di-acylated lipopeptides. Due to our strict selection criteria for the polymorphisms to be genotyped, we might have excluded some polymorphisms, which may be associated with mycobacterial and other diseases. Based on current data, we do not know the causative SNP underlying these observations. It is however possible that this

polymorphism might be in LD with a yet-to-be described polymorphism either in TLR6 or in genes adjacent to TLR6.

SNP C745T is non-synonymous and was associated with altered NF- κ B signaling in a transfected cell line and possibly with differences in IL-6 levels in response to lipopeptide stimulation of primary cells. Although the latter observation was not clearly reproducible, this may have been because we were unable to test sufficient numbers of persons with the rare homozygous 745TT genotype. Based on the NF- κ B signaling data in a reconstitution system, this non-synonymous SNP appears to directly alter TLR6 signaling. Given the location of C745T (P249S) in the extracellular domain, we speculate that this polymorphism alters ligand recognition. Alternatively, the substitution could result in a conformational change that affects the assembly of a TLR2/6 signaling complex. A recent study reported no differences in NF- κ B signaling between the two C745T variants after PAM2 stimulation of HEK293T cells (370). Compared with our experiments, these investigators used 10-fold more TLR6 DNA for transfection (100ng), and 25-fold less PAM2 (10ng/mL) as TLR6 ligand. These observations suggest that the effect of C745T on NF- κ B signaling in a reconstituted system may be partial and results may vary in different *in vitro* experimental conditions.

Interestingly, the C745T polymorphism was associated with altered IL-6 production in response to stimulation with FSL-1, but not PAM2. Although both of these lipopeptides are di-acylated, these ligands have different peptide moieties, which may affect TLR6 binding (371). Furthermore, PAM3 stimulation resulted in significantly different IL-6 levels in individuals with the different TLR6 genotypes, an unexpected finding given that PAM3 is predominantly a TLR2/1 ligand. These results may be due to our incomplete understanding of ligand specificity of these receptors. Previous studies indicate that the specificity of lipopeptides for TLR1 and TLR6 is not solely dependent on the number of acylation side groups. Other structural features such as length of the fatty acid chain, chirality of the diacyloxypropyl carbon, position of the acyl group, and amino acid composition of the terminal peptides have also been shown to affect receptor specificity (336, 337, 339, 340, 371, 372). The specificity of our findings was supported by the observation that IL-6 produced in response to LPS, a TLR4 ligand, was not associated with differences when comparing TLR6 genotypes.

Two previous studies have examined the functional role of TLR6 polymorphisms. A recent study demonstrated that rare SNPs in TLR6 were associated with altered NF- κ B signaling and an increased risk of TB disease in certain ethnic populations (359). We did not find any of these polymorphisms in our sample of 100 individuals. Recently, three SNPs in TLR6 (rs5743795, rs1039560, and rs3775073) were associated with differential responses to PAM3 (344). PAM2 and FSL-1 were not tested in this study, so we cannot directly compare our results. We also examined one of these SNPs, A1263G (rs3775073), but this was not associated with differential responses to PAM3. We did not identify the other 2 SNPs in our cohort. Possible explanations for apparent discrepancies in results may be due to protocol differences including PAM3 dose (10 ng/mL vs. 300 ng/mL), incubation time (6 hours vs. 20 hours), and different genetic backgrounds between the populations (Seattle, USA vs. Cape Town, South Africa). This suggests that other variables including dose of lipoproteins and genetic background may also influence TLR signaling responses.

Interestingly we observed that the TLR6 SNP association with IL-6 production was lost when PBMC cultures were used compared with whole blood cultures. The lack of association could be due to inadequate sample size or the contribution from other cell types in whole blood, such as neutrophils. Also, a flow based assay that identifies specific cell subsets producing IL-6 may be done in individuals with these TLR6 SNPs to identify associations between cell-specific IL-6 production and TLR SNPs.

Although the role of TLR1 in *M.tb* recognition is well established, the role of TLR6 has been less clear and has not been studied well (343). Our NF- κ B signaling data supports a role for TLR6 in *M.tb* recognition. In addition, we found that TLR6_G1083C genotypes were associated with different levels of IL-6 after stimulation with *M.tb* lysate and BCG. We were surprised that the response to a combination of ligands may be associated with TLR6 genotypes. Since TLR6 recognises di-acylated lipopeptides, this suggests the presence of these lipopeptides in *M.tb* and also that these lipopeptides may play a major role in immunity to *M.tb*. Previous studies on lipoproteins in TB show that the inactivation of the *M.tb* LspA by allelic replacement plays an important role in lipoprotein synthesis and the pathogenesis of *M.tb* (373). The cell wall associated lipoprotein LprA of *M.tb* was also shown to regulate innate immunity and inhibits antigen presentation in macrophages (374). In addition to *M.tb*, TLR6 is likely to mediate immune responses to a wide variety of pathogens (375, 376). TLR6 polymorphisms show some evidence of

association with IL-6 secretion upon stimulation with lipopeptides and mycobacteria. These results require further replication to confirm the findings. This work thus suggests a role for TLR6 in regulating mycobacterial signaling and offers a strong impetus to evaluate the relationship between TLR6 polymorphisms and susceptibility to mycobacterial diseases including TB.

University of Cape Town

Chapter 5: Conclusions

The following are novel contributions from this thesis to the field of developmental innate immunity, immunogenetics, and TB:

1. Optimization of new assay system to measure innate responses to live mycobacteria in small volumes of blood.
2. Changes in cytokine expression and production in response to live mycobacteria over the first 9 months of life.
3. Changes in maturation marker expression in response to live mycobacteria over the first 9 months of life.
4. Acquisition of mycobacteria by innate cell subsets and changes over the first 9 months of life.
5. Identification of a novel TLR6 polymorphism and association of TLR6 polymorphisms with altered immune responses to mycobacteria.

We fully optimised methods including stimulation, cell harvesting, lysis, cryopreservation, and flow cytometry antibody panels to identify monocytes, DCs, and granulocytes and measure the function of these cells in response to live mycobacteria or mycobacterial components using several short-term whole blood assays including flow cytometry, Milliplex MAP Multiplex Immunoassays, and ELISAs. The use of these whole blood assays is particularly useful for infants and small children, allowing comprehensive description of the host response. We were unable to identify all innate subsets in small volumes of blood and simultaneously measure their function due to complexities in developing higher flow cytometry panels.

We applied the optimised flow cytometry assays to measure maturational changes in innate responses to mycobacteria over the first 9 months of life, and compared these with responses in adults. We observed a general increase in pro-inflammatory responses and maturation with age, while expression of IL-10, the anti-inflammatory cytokine, did not change with age (**Fig. 65**). Our results also suggested that different innate cell subsets matured differently in response to mycobacteria. The increase in IL-12 production by monocytes and CD40 expression by monocytes and mDC may

underlie the greater ability for Th1 polarisation and increased Th1 responses reported in older infants compared with newborns (299). This supports our previous hypothesis that delaying BCG vaccination to an age where the innate immune response is more mature may lead to enhanced Th1 responses to BCG (143). We did not measure adaptive responses in these participants to directly correlate these with innate responses.

Even though BCG-induced protection against pulmonary TB is poor and highly variable, the vaccine can effectively protect against disseminated forms of TB (138, 139). Several factors have been suggested to underlie the poor protection offered by BCG against lung TB (as discussed in **Chapter 1**) and studies are under way in our laboratory to determine correlates of risk of TB disease or protection afforded by BCG. Other studies have investigated whether delaying BCG vaccination may lead to enhanced BCG-induced responses but different, sometime contrasting, results have been observed (143, 377). In South Africa, Kagina *et al.* (143) showed enhanced Th1 responses at 1 year of age when BCG vaccination was delayed by 10 weeks, while Burl *et al.* (377) in Gambia showed that Th1 and Th17 responses were reduced at 9 months of age when BCG vaccination was delayed to 4½ months. Another study shows that when BCG vaccination is delayed by 6 weeks, in a natural setting in Uganda, frequencies of IFN- γ expressing T cells are lower compared with vaccination at birth (F. Lutwama, H Mayanja-Kizza, W Hanekom *et al.*, unpublished data). Regardless of whether vaccination at birth or later in life may provide better or poorer protection against TB, the innate immune response induced by BCG is critical for inducing an appropriate adaptive response. In order to investigate the direct association between maturation of innate responses and enhanced adaptive responses, randomised controlled studies will need to be developed that assess delaying BCG vaccination to a later time point and measuring both innate and adaptive responses in the same samples. Also, a longitudinal study can be designed and executed where APCs from the same infants at birth and at later time points are co-cultured with T cells and their antigen presenting function evaluated. This may also provide information on the direct relationship between maturation of innate responses and enhanced T cell responses.

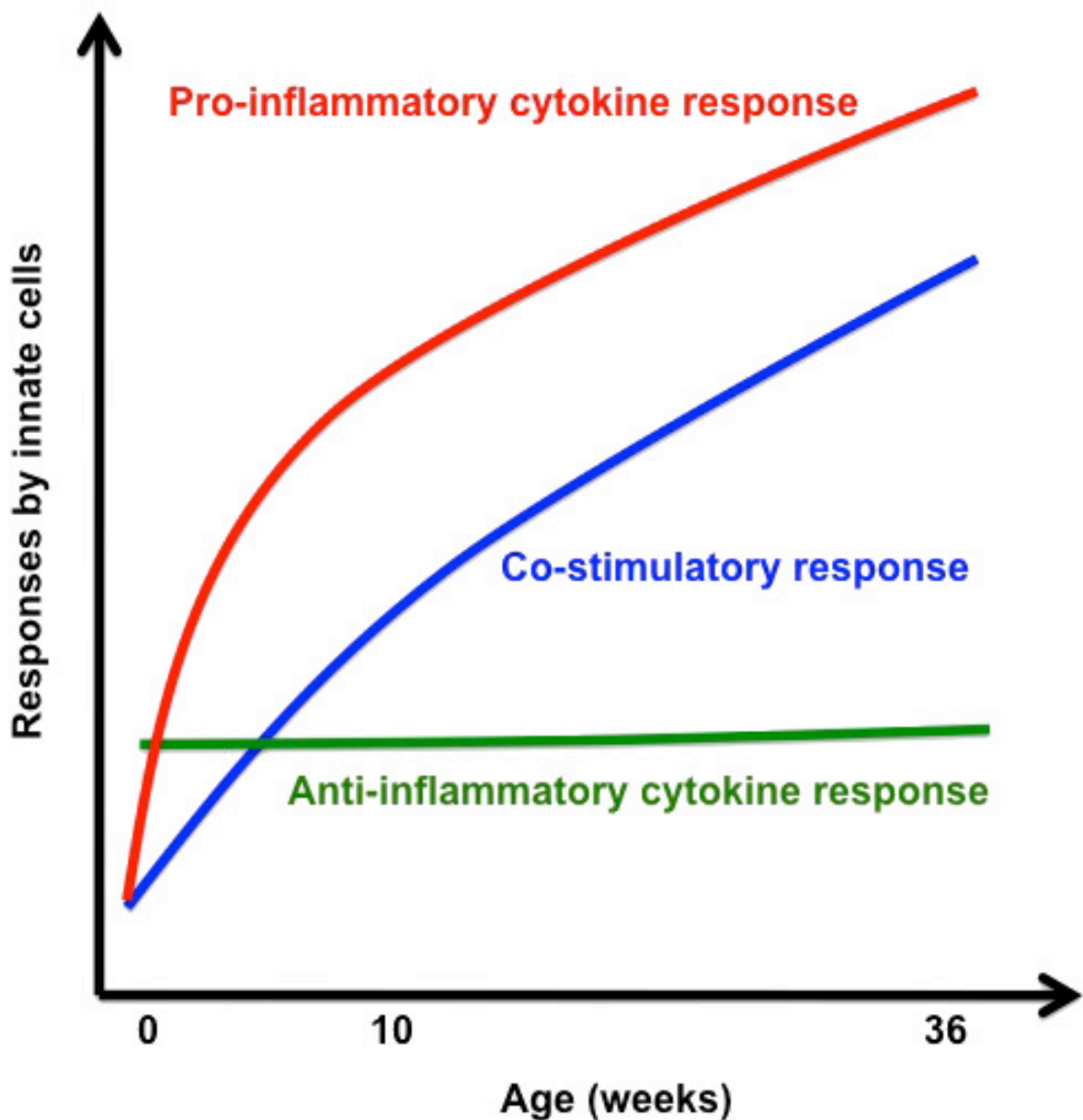


Figure 65: Summary model of response by innate cells to BCG. Red, pro-inflammatory cytokine responses; Blue, maturation of innate cells; Green, anti-inflammatory cytokine response.

Higher frequencies of mDC and monocytes, and similar frequencies of granulocytes, acquired BCG in newborns, compared with those in infants. On the contrary, the proportion of granulocytes that acquired BCG among leukocytes was higher in newborns compared with infants while the proportion mDC was lower in newborns. The lower numbers of mDC and acquisition of BCG in newborns may be directly or indirectly associated with low antigen presentation capacity and T cell activation in newborns

(299). Also, since adaptive responses are lower in newborns, these groups of individual may depend largely on innate responses such as phagocytosis and direct killing of pathogens by neutrophils to control infection. Changes in recognition of mycobacteria over the first 9 months of life have not previously been investigated. This knowledge is required to understand changes in immune responses after mycobacterial recognition. Here we evaluated GFP⁺ cells as those that have recognised and or phagocytosed BCG, which may be intracellular or extracellular. Also from our assays, we are unable to determine how many GFP-expressing bacilli should be inside a cell to be detected. Standard phagocytosis assays may be required to comprehensively evaluate uptake of mycobacteria by innate cells and the direct association between phagocytosis of mycobacteria and killing or antigen presentation.

Several factors may influence responses to mycobacteria including socio-economic factors, co-infections with helminths, exposure to environmental mycobacteria, and co-administration of other EPI vaccines (186, 187, 318, 378, 379). One study reported that co-administration of BCG and oral polio vaccine at birth was associated with downregulation of T cells responses to PPD at 6 weeks of age (379). Older infants are more likely to be exposed to helminth infections or environmental mycobacteria, compared with newborns, which may influence their responses to BCG (318, 321, 378). Socio-economic factors including smoking, and household income may influence immune responses to mycobacteria, as these factors have been associated with susceptibility to TB (186, 187).

The differences in responses between newborns and older infants suggest that different cell subsets contribute differently to immune responses at different ages. In newborns the capacity to induce adaptive responses is generally reduced, the adaptive immune system is less developed, and newborns may depend largely on innate responses for protection (299, 303). Our results and those of others that there are higher numbers of monocytes and granulocytes, and higher GFP⁺ monocytes and mDC in newborns, compared with infants, may support this hypothesis (**Chapter 3**) (305). Monocytes and granulocytes, and to some extent mDC, are effector cells that are involved in uptake and killing of pathogens, sometimes with help from mediators produced by cells of the adaptive immune arm. One of these mediators, IFN- γ is a classical T cell cytokine with important function in immune responses to mycobacteria. Among important functions of IFN- γ is classical activation of macrophages to kill mycobacteria (see **section 2.4.4**).

This cytokine is also produced by cells with innate functions such as NK and $\gamma\delta$ T cells (234, 303). Thus monocytes and granulocytes may kill pathogens and eliminate the infection without sensitization of the adaptive immune response. In children and adults, the adaptive response is more mature, and both innate and adaptive responses together can confer protection against pathogenic infections.

The possible mechanisms that may underlie differential innate responses in newborns and infants may include differential PRR expression or phosphorylation capacity of molecules involved in the signaling pathways. Some reports have suggested that immature innate immune responses, including incomplete DC maturation in newborns, compared with adults, result from reduced TLR signaling (299, 380). mRNA levels of TLR4 as well as molecules downstream of TLR4 signaling such as MAPKKK and NF- κ B were markedly decreased in LPS-stimulated cord blood DCs, compared with adult DCs (380). Differential capacity for p38 phosphorylation in activated monocytes from newborns, compared with adults has been reported and suggested to underlie differential cytokine expression at different ages (381-383). Levy *et al.* (381) showed that TLR8 ligand induced higher phosphorylation of p38 MAPK in blood monocytes of newborns compared with adults. Sohlberg *et al.* (382) reported that p38 phosphorylation in purified monocytes in response to peptidoglycan was higher in newborns, compared with adults. In contrast, Koch *et al.* (383) showed decreased phosphorylation of p38 in newborns compared with adults when blood was stimulated with LPS. Changes in p38 phosphorylation over the first year of life have not been investigated and this could be a possible mechanism underlying differential cytokine expression over the first 9 months of life, and should be investigated.

Finally, we identified a new TLR6 polymorphism and reported that two other TLR6 polymorphisms altered innate responses to mycobacteria and mycobacterial components. We extended these findings to also investigate whether polymorphisms in TLR6 and other TLRs could alter adaptive immune responses induced by BCG. These investigations provided very interesting results. Whereas the TLR6 G1083C and C745T, and TLR1_T1805G were associated with decreased innate responses (IL-6 and IL-10 secretion), these polymorphisms were associated with increased Th1 adaptive responses such as IFN- γ and IL-2 secretion (255). These polymorphisms were also associated with increased expression of cytotoxic molecules (granulysin, granzyme B and perforin) by CD4⁺ and CD8⁺ T cells. Th2 responses (IL-13 secretion) were not

affected by these polymorphisms (255). These findings suggest that genetic variations between individuals may have to be considered when designing vaccines or therapeutic interventions to prevent infections or treat mycobacterial diseases. IFN- γ is used as immunogenicity outcome in most trial assays for new vaccines. Furthermore, IFN- γ release assays (IGRA) to diagnose *M.tb* infection rely on the levels of IFN- γ produced (384). Since individuals without the TLR polymorphisms mentioned above may produce less IFN- γ , we hypothesise that they may be latently infected with *M.tb* but their IFN- γ levels would be too low for detection by IGRA. Such individuals who could potentially benefit from INH preventive therapy would miss out on treatment. In contrast, individuals with these polymorphisms might also be protected from progression to active TB due to increased production of cytotoxic molecules that can kill *M.tb*. We are conducting studies to determine whether polymorphisms mentioned above, or others, are associated with risk of developing active TB in infants, and through transmission disequilibrium test (TDT) analyses, we will determine whether infants can preferentially inherit specific alleles from their parents predisposing them to developing disease.

In summary, we have shown that functional response of monocytes, mDC and granulocytes to mycobacteria differs between newborns, 10 week olds, 36 week olds and adults. We also described the role of TLR6 polymorphisms and their association with innate responses to mycobacteria. These studies are essential for understanding the role of innate immune responses to mycobacteria, and how maturation of the immune system influences these responses. These results are very relevant in the field of mycobacterial immunology and development of novel vaccine strategies against TB. Our data supports the use of TLR adjuvants in vaccines to prevent TB and suggest that other adjuvants that may co-stimulate TLR2/1 and TLR4 should be investigated and tested. Our data also provide information on how genetic variations between individuals influence their immune responses. These studies are expected to set the stage for more definitive clinical trials to assess differential immunity induced by vaccination at different ages and the underlying mechanisms for differential immune responses.

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Appendices

Whole blood assay and staining protocol

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1. PURPOSE

Innate cell whole blood assay and flow cytometry staining protocol for innate immunity studies.

2. STUDY STAFF

Trained SATVI Laboratory staff

3. PRINCIPLE:

This protocol describes the procedures for measuring functional and phenotypic characteristics of peripheral blood innate cells, such as monocytes, dendritic cells and granulocytes by flow cytometry. Innate cell expression of cytokines/chemokines and/or cell surface markers will be determined after stimulation of whole blood for 6 or 18 hours. Stock plates containing antigens are prepared at 10X the final concentration and stored at -80°C until required, when 200µl of blood is added. For blood stimulated for 18 hours, supernatants are

harvested and frozen until they can be tested in batch for soluble cytokines or chemokines by ELISA or Luminex assay. After stimulation red blood cells are lysed and while white blood cells are harvested and cryopreserved for antibody staining and acquisition on an LSR II flow cytometer at a later stage.

4. **REAGENTS:**

4.1 RPMI 1640 (with added Glutamine)

Supplier: Whitehead.

Cat No: 12-702F.

Stock: 500mL bottles.

Store: between 2°C to 8°C out of direct light.

Note: The medium normally has a deep orange colour. If the pH changes, the colour of the medium will change to pink.

Use fresh medium if:

- there is a colour change.
- use the contents of an opened bottle within a week.

Store at 4°C and discard after expiry date is reached.

4.2 Sterile Water

Supplier: SATVI Lab.

Stock: 1 liter sterile bottled water.

Store: at room temperature.

NOTE: Open bottles aseptically in biosafety cabinet. Replace cap after use and seal cap with parafilm before placing it in storage.

4.3 20% DMSO in FCS

Dimethyl sulfoxide (DMSO):

Supplier: E Merck.

Cat No: BB103234L.

Stock: Analar grade, 100mL bottles.

Store: Room temperature.

Fetal Calf Serum (FCS), Heat Inactivated:

Supplier: Scientific Group.

Cat No: 10500064.

Stock: 500mL bottles.

Store: At -20°C. For use, thaw 500mL bottle at 37°C, thereafter keep at 4°C in sterile 50mL centrifuge tubes.

For use: Thaw 500mL bottle at 37°C, aliquot into 50mL sterile polypropylene tubes and re-freeze at -20°C (if required).

Stock solution:

Final concentration required is 20% v/v DMSO in heat inactivated FCS.

Work in the laminar flow hood.

Using a sterile, disposable 25mL plastic pipette and pipette aid, transfer 40mL heat inactivated FCS into the top portion of the filtration unit (use a 0.2 micron filter) then add 10mL DMSO stock solution.

Note: Do not pour DMSO directly onto the filter because it will destroy the filter. Add the DMSO to the FCS in the filtration unit.

This working solution **MUST** be stored at 4°C in the dark (i.e. **wrapped in foil**).

4.4 Cryo-solution

20% DMSO in FCS.

Working solution:

The final concentration required is one part RPMI + one part 20% DMSO in FCS. eg. 500µL medium of choice + 500µL 20 % DMSO in FCS.

This makes a working solution of 10% of DMSO in FCS in medium of choice.

4.5 BD Perm/Wash Buffer

Supplier: Becton Dickenson (BD) Biosciences
Cat No: 554723
Stock: 100mL bottles.
Store: At 4°C. For use, make a 1:10 dilution in sterile water

4.6 BD FACS CompBeads (Anti-mouse Ig, κ/negative control (FBS*) Compensation Particles Set)

Supplier: Becton Dickenson (BD) Biosciences
Cat No: 552843
Stock: 6mL of each
Store: At 4°C

4.7 BD FACS Flow Solution

Supplier: Becton Dickenson (BD) Biosciences
Cat No: 342003
Stock: 100mL
Store: At room temperature

5. STIMULATING ANTIGENS/REAGENTS:

All handling and preparations of dilutions must be done under sterile conditions.

All stocks are stored at -80°C (long term) or -20°C (short term)

1. Polymyxin B (Sigma P-4932). Resuspend in PBS, stock 10 mg/ml, use at 10µg/ml, sonicate for about 1 minute before use. Incubate with stimulant at room temperature for 30 minutes prior to use.
2. Lipopolysaccharide (*S. minnesota* cat# tlr1-smlps from Invivogen). Stock 2.5mg/ml. Use 100ng/ml final concentration.
3. CpG DNA (cat# ODN 2216 from Invivogen). Stock 1mg/ml. Use 15µg/ml final concentration.
4. PAM₃CSK₄ (bacterial lipopeptide), (cat #L2000, from EMC Microcollections, <http://www.microcollections.de/>). Stock 5 mg/ml in PBS, use at 1000 ng/ml final concentration.

5. Bacille Calmette-Guerin (BCG) from SSI. Resuspend in 300 μ l of RPMI. Add 20 μ l to each well of the plate.
6. BCG-GFP is grown in the lab. Stock 3500000CFU/ml. Use 20 μ l per well.
7. Fibroblast-stimulating factor 1 (FSL-1, cat # L2026 from EMC Microcollections, <http://www.microcollections.de/>). Stock 2 mg/ml in PBS. Use at 1000 ng/ml final concentration.
8. Brefeldin A (cat# B7651-5MG, from Sigma-Aldrich). Stock 1mg/mL in DMSO. Make aliquots of 20 μ l. For use, dilute 1:10 in RPMI and add 20 μ l into each well (final concentration of 10 μ g/mL). This blocks the cytokines expressed from being transported out of the cells.

6. **HARDWARE:**

- 1 Minus 80°C freezers: Snijders Ultra Low chest freezer.
- 2 Racks for -80°C freezers.
- 3 Disposable pipettes, 5ml and 10ml.
(Whitehead Scientific; Cat# PN5E1 and PN10E1)
- 4 Na Heparin Vacutainer tubes (Scientific Group; Cat# BD368480)
- 5 5ml Falcon tubes (BD; Cat #: 352054)
- 6 96 well (round bottomed) microtitre plates with lids (Sterilin: Cat# 116U)
- 7 Microtitre plate sealers (AEC Amersham; Cat# 236366).
- 8 Eppendorf multipette plus (stepper) (MERCK; Cat # HK4981000019).
- 9 1ml Combi-tips plus (for use with stepper) (Merck; Cat # HK0030069234)
5ml Combi-tips can also be used.
- 10 P20, P200, P1000 Gilson Pipetman (LASEC).
- 11 Filtered micropipette tips: p20, 200 and 1000
(Whitehead Scientific; Cat #s FA113-N-20, FA 108-200 and FA 111-NXL-1000).
- 12 Non-filtered micropipette tips: p200 .
- 13 Finnpipette with 8 (or 12) x 300 μ l multichannel module.
- 14 Second Nature pipet-aid (Model Whisper 600).
- 15 Polypropylene 15ml and 50ml sterile screw cap tubes
(LASEC; Cat #s PGRE 188261 and PGRE 227261).
- 16 0.5ml and 1.5ml eppendorf tubes (Sigma; Cat # T8911and T9661).

- 17 Vortex (Snijders).
- 18 Water-bath (MEMMERT WB22).
- 19 CO₂ incubator ('Thermo Steri Cycle' LABOTECH; Model 371).
- 20 10x10 cryoboxes (for Eppendorf tubes) (LASEC; cat # HTBS 5026-1010).
- 21 Polycard boxes (for storage of plates) (Whitehead Scientific; Cat # K52-12).
- 22 Ice
- 23 Ice bucket
- 24 Temperature controlled centrifuge
- 25 Refrigerator (set to 4°C).
- 26 Flow Cytometer (BD LSR II).

7. WHOLE BLOOD STIMULATION - METHOD:

a. Preparation of frozen antigen plates

1. Prepare round bottom 96-well plates with antigens in duplicates (PAM3, FSL-1, CpG, BCG, BCG-GFP, LPS and RPMI; see table 1 and table 2) and freeze at -80°C.
2. Add polymixin B (to final concentration of 10µg/ml) to wells containing lipopeptides only. Polymyxin B neutralises the effect of LPS.

b. The whole blood assay

1. Draw blood into heparinised Vacutainer tube.
2. Add 180µl whole blood into well of 96-U plate containing the antigens (Plates are defrosted at room temperature at least 15 minutes before blood is added).
3. Incubate at 37°C, 5% CO₂ with humidity.
4. For the 6 hr short term ICS assay, add Brefeldin A into each well after 3 hrs and incubate for further 3 hrs.

Note: For the 18hr long term assay, no Brefeldin A is added.

c. HARVESTING CELLS

Note: For 18hr stimulation, transfer 100 μ l supernatants into a 96 well plate before harvesting the cells. The supernatants will be used to determine cytokine secretion by luminex.

NB: Cells are only harvested for three conditions: UNS, BCG-GFP, and LPS.

1. Add EDTA (final concentration of 2 mM) to the appropriate wells of the plate.
2. Incubate at room temperature for 10 minutes to detach adherent cells.
3. Transfer the mixture into 1 mL of 1X BD FACS Lysing solution in cryo tubes using 1000 μ l pipette. Make sure all cells are sucked up from the wells. Pool the duplicates into one cryo tube.
4. Incubate for 10 minutes.
5. Spin down (1800 rpm for 5 minutes) with breaks on.
6. Decant the supernatant.
7. Resuspend the cell pellet in 1mL of 1X BD FACS Lysing solution.
8. Incubate for 10 minutes.
9. Spin down (1800 rpm for 5 minutes) with breaks on.
10. Decant the supernatant and blot.
11. Resuspend the cells in 500 μ l RPMI in cryo tubes.
12. Add 500 μ l of 20% DMSO/FCS.
13. Transfer to -80 °C overnight in Mr Frosty.
14. Transfer to Liquid nitrogen (LN2) until needed.

8. STAINING

Note: Because the white blood cells used in this assay are fixed before cryo-preservation, the cells are relatively insensitive to DMSO and do not have to be thawed as carefully as live PBMC. The cell vials can be transferred from liquid N₂ to an ice bucket.

1. Remove the cells from LN2 and place the cryotubes in dry ice
2. Transfer the tubes to water bath at room temperature until completely thawed.

a. Intracellular cytokine staining (Table 3)

1. Transfer the cells into Falcon tubes containing 2 mL of 1X BD Perm/wash buffer.
2. Spin down (1800 rpm for 5 minutes).
3. Resuspend the cells in 1 mL 1X BD Perm/wash buffer.
4. Allow to permeabilise for 10 minutes at room temperature.
5. Spin down (1800 rpm for 5 minutes).
6. Decant the supernatant and blot.
7. Add antibody cocktail (Prepared with concentrations indicated in table 3. See preparing antibody cocktail below).
8. Incubate 4 °C (in the fridge) for 1 hour.
9. Add 1 mL 1X BD Perm/wash buffer.
10. Spin down (1800 rpm for 5 minutes).
11. Decant the supernatant.
12. Acquire on LSR II.

b. **Surface staining** (two-step staining; see Table 4)

1. Transfer the cells into Falcon tubes containing 2 mL of 1X PBS.
2. Spin down (1800 rpm for 5 minutes).
3. Resuspend the cells in 1 mL 1X PBS.
4. Spin down (1800 rpm for 5 minutes).
5. Decant the supernatant and blot.
6. Add antibody cocktail, excluding the two QDOT antibodies (Prepared in 1X PBS with concentrations indicated in table 4. See preparing antibody cocktail below).
7. Incubate 4 °C for 1 hour.
8. Add 1 mL of BD Perm/Wash
9. Spin down (1800 rpm for 5 minutes).
10. Decant the supernatant and blot
11. Add the second antibody cocktail containing the two QDOT antibodies only in Perm/Wash.
12. Incubate 4 °C for 1 hour.
13. Spin down (1800 rpm for 5 minutes).
14. Decant the supernatant
15. Acquire on LSR II.

Preparing antibody cocktail: Staining panels and volumes. Add the antibodies to an Eppendorf tube to make the staining antibody cocktail. Always calculate the number of stains required and add 1 extra volume of each antibody to allow for pipeting error (for example, if you are staining 10 tubes, prepare antibody cocktail for 11 tubes).

9. RESULTS AND RECORDS

File all results and records in the designated files.

10 TABLES

Table 1: Plate layout: stimulation

a. 18 hour Incubation

	1	2	3	4	5	6	7	8	9	10	11	12
A	UNS	FSL-1		PAM3		UNS		FSL-1		PAM3		
B	UNS	FSL-1		PAM3		UNS		FSL-1		PAM3		
C												
D	BCG-GFP	CpG		LPS		BCG-GFP		CpG		LPS		
E	BCG-GFP	CpG		LPS		BCG-GFP		CpG		LPS		
F												
H												
G												

b. 6hr Incubation

	Add BFA after 3 hours							Harvest cells after 6 hours				
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		UNS		BCG-GFP				UNS		BCG-GFP		
C		UNS		BCG-GFP				UNS		BCG-GFP		
D												
E												
F				LPS						LPS		
G				LPS						LPS		
H												

Table 2: Stimulants for Whole Blood Cytokine Assay

Stimulant	TLR1	TLR2	TLR3	TLR4	TLR5	TLR6	TLR7,8	TLR9	NOD2
LPS, <i>S.m</i>				+					
PAM ₃ CSK ₄	+	+							
BCG-GFP	+	++	-	++	-	+	-	-	-
CpG DNA								+	
FSL-1		+				++			

+ means that the TLR mediates the signal (++ indicates where a stronger signal is observed)

- means that the TLR is not involved

Table 3: Intracellular cytokine flow panel (6 hour incubation)

Fluorochrome	Marker	Titers (in μ L)
QDot 605	CD14	0.2
PerCP-Cy5.5	CD11c	0.5
AlexaFluor700	HLA-DR	0.5
QDot 565	CD66	1.25*
Pacific Blue	IL-12/23p40	2.5
PE	IL-10	2.5
APC	IL-6	1.0
PE-Cy7	TNF- α	5.0
N/A	GFP**	

*This antibody was conjugated in-house and titrated after each conjugation

** This was detected in the FITC channel.

Table 4: Surface flow panel (18 hour incubation)

Fluorochrome	Marker	Titers (in μ L)
QDot 605	CD14	0.2
Pacific Blue	CD11c	0.5
AlexaFluor700	HLA-DR	0.5
QDot 565	CD66	1.25*
PerCP-Cy5.5	CD40	0.5
PE	CD86	2.5
APC	CD83	0.3
N/A	GFP**	

*This antibody was conjugated in-house and titrated after each conjugation

** This was detected in the FITC channel

12. TRAINING

The SATVI Laboratory hereby acknowledge that the undersigned staff have been trained in the procedure outlined in this document and are competent to perform the procedure.

Date	Name	DOCUMENT		COMPETENCY	
		Sign Trainer	Sign Trainee	Sign Trainer	Sign Trainee

Publications



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Research paper

Optimization of a whole blood intracellular cytokine assay for measuring innate cell responses to mycobacteria

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Innate cytokines

ABSTRACT

Innate cells are essential for host defense against invading pathogens, and the induction and direction of adaptive immune responses to infection. We developed and optimized a flow cytometric assay that allows measurement of intracellular cytokine expression by monocytes, dendritic cells (DC) and granulocytes, as well as cellular uptake of green-fluorescent protein (GFP)-expressing mycobacteria, in very small volumes of peripheral blood.

We show that innate cell stimulation resulted in increased granularity of monocytes and mDC and decreased granulocyte granularity that precluded flow cytometric discernment of granulocytes from monocytes and myeloid DC by forward and side scatter gating. Anti-CD66a/c/e antibody staining allowed reliable identification and exclusion of granulocytes for subsequent delineation of monocytes and myeloid DC. Intracellular cytokine expression by granulocytes, monocytes and mDC was remarkably sensitive to the dose of mycobacterial inoculum. Moreover, activation of monocytes and mDC with live BCG reduced expression levels of CD14 and CD11c, respectively, necessitating optimization of staining conditions to reliably measure these lineage markers. Finally, we characterized expression of IL-12/23p40, TNF- α , IL-6, and IL-10, by GFP⁺ and GFP⁻ monocytes and mDC from 25 healthy adults.

This assay may be applied to the study of innate cell responses to any GFP-expressing pathogen, and can be performed on blood volumes as low as 200 μ L per condition, making the assay particularly suitable for pediatric studies.

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1. Introduction

Innate immunity is critical for host defense against invading pathogens, and the induction and direction of adaptive immune responses to infection (Kollmann et al., 2009). Innate phagocytic cells, such as macrophages and neutrophils, constitute the first line of defense. Upon recognition of pathogen associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs), these cells become activated and

may phagocytose, kill and eliminate the infecting microorganism (Blomgran and Ernst, 2011; Dorhoi et al., 2011; Eum et al., 2010; Silva, 2009). Activated cells also secrete cytokines and chemokines, which mediate recruitment of additional cells and orchestrate an inflammatory response (Dorhoi et al., 2011).

In addition, antigen-presenting cells that have internalized pathogen, such as dendritic cells (DC), traffic to draining lymph nodes where they present processed antigens to naïve T cells (Banchereau and Steinman, 1998; Mellman and Steinman, 2001). The quality and magnitude of the ensuing T cell response may depend on a number of factors, including the number of invading organisms, the type and combination of PRR(s) triggered, and the subsequent efficiency of phagocytosis, innate cell

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activation, pathogen killing and processing, and the release of inflammatory mediators (Dorhoi et al., 2011; Kapsenberg, 2003; Mazzone and Segal, 2004). Intracellular pathogens, such as mycobacteria, possess a number of escape pathways that arrest phagosome acidification and maturation to allow survival and replication within the phagocyte (Russell, 2011; Flynn and Chan, 2003). Optimal cytokine and chemokine-mediated cross-talk between innate and adaptive cells is required for optimal activation of macrophages to overcome this subversion and mediate pathogen killing (Flynn and Chan, 2003).

Several studies have described flow cytometric methods for *ex vivo* characterization of peripheral blood monocytes and DCs (Autissier et al., 2010; Fung et al., 2010; Ida et al., 2006; Wang et al., 2006, 2009). In these studies granulocytes are typically excluded based on their unique size and granularity, before identifying monocytes and mDC using lineage markers, such as CD14 and CD11c.

We developed and optimized a flow cytometric assay that measures intracellular expression of key pro- and anti-inflammatory cytokines by peripheral blood innate cells in response to live mycobacteria. We show that upon activation with viable mycobacteria or LPS, changes to several properties of innate cells have to be accounted for to accurately delineate peripheral blood innate cell subsets and measure intracellular cytokine expression. We describe multiple important factors for assay success and apply this intracellular cytokine staining assay to characterize the innate cell response to the live mycobacterium, *M. bovis* Bacille Calmette-Guerin (BCG), using 200 μ L of whole blood per condition.

2. Materials and methods

2.1. Participant recruitment and enrollment

Healthy adults, aged 18–50 years, were enrolled at the South African Tuberculosis Vaccine Initiative Field Site in the Cape Town region of South Africa. Exclusion criteria included pregnancy, HIV-1 infection, *M.tb* infection and any other acute or chronic infection. HIV-1 infection was diagnosed by rapid HIV antibody test (HIV Determine 1&2), while *M.tb* infection was defined as a positive interferon gamma (IFN- γ) response to ESAT6/CFP-10 protein, measured by ELISA, as described previously (Kagina et al., 2009). The study protocol was approved by the Research Ethics Committee of the University of Cape Town, and all participants provided written informed consent.

2.2. TLR ligands and bacteria, and antibodies

Ultrapure lipopolysaccharide (LPS, TLR4 ligand, 100 ng/mL final concentration), isolated from *Salmonella minnesota*, was obtained from InvivoGen. This LPS concentration was previously found to induce optimal cytokine expression by innate cells in whole blood (Jansen et al., 2008). Viable BCG expressing green fluorescent protein (BCG-GFP, Pasteur strain; 3.5×10^5 CFU/mL final concentration, unless otherwise stated, donated by Muazzam Jacobs, University of Cape Town) was cultured in our laboratory. Bacteria were harvested at log phase 3 weeks after start of culture (optical density of 0.8) and CFU counts determined by plating bacteria on agar

plates. LPS and bacteria were prepared at 10 times the final concentration in RPMI 1640, and 20 μ L was added into 96 round bottom well plates.

The following antibodies were used, CD14-QDot605 (clone Tuk4, Invitrogen), CD14-Pacific Blue (clone M5E2, BD Pharmingen), TNF- α -PECy7 (clone Mab11, BD Pharmingen), CD11c-PerCPy5.5 (clone Bu15), HLA DR-AlexaFluor700 (clone L243), IL-12/23p40-Pacific Blue (clone C11.5), IL-10-PE (clone JES3-19 F1) and IL-6-APC (clone MQ2-13A5), all from Biolegend. CD66a/c/e (ASL-32, Biolegend) was conjugated in-house to QDot565 (Invitrogen) using the manufacturer's protocol.

2.3. Blood collection and processing

Heparinized blood was collected from each participant and processed immediately (maximum delay between blood collection and incubation with ligands was 30 min). We previously investigated the effects of delayed processing of blood and showed that delay by 60 min or more affected cytokine expression by mDC (Mendelson et al., 2006). An undiluted blood volume of 180 μ L was added to wells of a 96-well plate containing LPS or bacteria. The cultures were incubated at 37 °C, 5% CO₂ in humidified conditions. RPMI 1640 was used as medium control. After 3 h of incubation, brefeldin A (BFA, 10 μ g/mL, Sigma-Aldrich) was added to each well and the plate was incubated for 3 additional hours as previously optimized (Jansen et al., 2008). After a total incubation of 6 h, EDTA was added (2 mM final concentration, Sigma-Aldrich) and blood incubated for 10 min at room temperature in order to detach adherent cells. To lyse red cells and fix white cells, FACS Lysing Solution (BD Biosciences) was added and cells were incubated at room temperature for 10 min. This lysing step was repeated to ensure complete red cell lysis. Fixed white cells were either stained immediately or cryopreserved in 10% DMSO in heat inactivated fetal calf serum (10% DMSO/FCS) or in FACS Lysing Solution.

2.4. Intracellular cytokine staining and flow cytometric acquisition

Cryopreserved, stimulated cells were thawed in batch, and cells were washed twice with either phosphate buffered saline (PBS, without calcium and magnesium) or BD Perm/Wash buffer (BD Biosciences). Cells were stained with fluorescent antibodies in a total volume of 30 μ L in either PBS or BD Perm/Wash, at 4 °C for 1 h. Stained cells were washed and 1 million cells or the total sample volume were acquired on a BD LSR II flow cytometer.

2.5. Data analysis

Flow cytometry data were analyzed using FlowJo v9.2. Results from single-stained and unstained mouse κ beads were used to calculate compensations, for each run. Cell doublets were excluded using forward scatter-area versus forward scatter-height parameters. Cytokine co-expression by innate cell subsets was assessed by boolean gating. Subtraction of background cytokine expression (unstimulated samples) was done using Pestle V1.6.2, while data sorting

and analysis were done with Spice V5.1 (Roederer et al., 2011; <http://exon.niaid.nih.gov/spice>, accessed February 25th, 2011). GraphPad Prism v5 was used for data presentation and statistical analysis. The Mann–Whitney or Wilcoxon signed rank tests were used to compare data sets. P values <0.05 were considered significant.

3. Results

3.1. CD66a/c/e allows identification and exclusion of granulocytes

The different size and granularity of granulocytes, compared with monocytes and mDC, allows identification of these cell subsets by *ex vivo* flow cytometric analysis (Autissier et al., 2010; Fung et al., 2010). Upon stimulation with live mycobacterium BCG-GFP, or LPS, we observed a decrease in side scatter fluorescence of granulocytes, while the side scatter fluorescence for mDC and monocytes increased (Fig. 1A). This precluded separation of mDC and monocytes from granulocytes using forward and side scatter parameters.

The CD66 isoforms a, c, d and e are members of the carcinoembryonic antigen (CEA) family of the Ig superfamily, and are exclusively expressed on granulocytes and epithelial cells (Gray-Owen and Blumberg, 2006). Staining with anti-CD66a/c/e antibody allowed identification of peripheral blood granulocytes (CD66a/c/e⁺, Fig. 1B). Since granulocytes express high levels of HLA DR and low levels of CD14 and CD11c, exclusion of granulocytes was required for accurate identification of CD14⁺HLA DR⁺CD11c⁺ mDC and HLA DR⁺CD14⁺ monocytes. Upon granulocyte exclusion the frequency of cells falling into the HLA DR⁺ gate decreased from 61% (IQR, 58–72%) to 9% (IQR, 7–13%, Fig. 1C–E). Similarly, the proportion of HLA DR⁺CD14⁺ cells expressing CD11c amongst all leucocytes decreased from 55% (IQR, 51–58%) to 1% (IQR, 0.7–1.5%) upon exclusion of CD66a/c/e⁺ cells (Fig. 1C–E).

3.2. BCG-activated monocytes downregulate CD14 expression

We investigated whether innate cell activation affects expression levels of innate lineage markers and flow cytometric delineation of monocytes, mDC and granulocytes. Lower frequencies of CD14⁺ monocytes were detected upon BCG stimulation, compared with unstimulated samples. This was observed when expression of CD14 was measured by flow cytometric staining with QDot605 or Pacific Blue conjugated anti-CD14 antibodies (Fig. 2A and B). A decrease in median fluorescence intensity of CD14 was also observed upon BCG stimulation, albeit only when QDot605-conjugated anti-CD14 was used. A recent study reported that the HLA DR⁺CD11c⁺CD14^{dim} cell population may also contain CD14⁺CD16⁺ monocytes (Cros et al., 2010). We could not delineate these monocyte sub-populations, as we did not measure CD16 expression in our analyses.

No difference in the frequency of CD66a/c/e⁺ granulocytes or CD11c⁺ mDC among HLA DR⁺CD14⁺ cells was observed upon BCG stimulation, and CD11c fluorescence was only moderately decreased ($p=0.03$, Fig. 2A and data not shown). Similar results were obtained when whole blood was incubated with LPS (data not shown).

The downregulation of CD14 and CD11c necessitated optimal blood processing and antibody staining conditions to

identify these key lineage markers after incubation of whole blood with BCG or LPS. Because monocytes are adhesive cells, we tested whether EDTA treatment after stimulation would increase the number of CD14⁺ monocytes. Although we observed a higher proportion of CD14⁺ cells in most donors after EDTA treatment, compared with untreated samples, this difference was not significant (Supplementary Fig. 2). Regardless, EDTA treatment was included for all subsequent experiments. Using this protocol, a median (IQR) of 8049 mDC (6152–11,432), 27,218 monocytes (19,447–33,437) and 368,000 granulocytes (273,500–412,500) were acquired for analysis.

3.3. QDot-conjugates are sensitive to staining buffer

Given the difference in staining performance between the QDot and Pacific Blue conjugated anti-CD14 antibodies, we further optimized staining conditions for QDot conjugates. QDots are fluorescent nanocrystals commonly used for imaging and flow cytometric analysis (Zarkowsky et al., 2011). Performance of these fluorochromes can be sensitive to components in staining buffers, such as heavy metals (Chen et al., 2002; Meallet-Renault et al., 2006). To optimize the antibody staining method for innate cell delineation, we tested the performance of fluorochrome-conjugated antibodies in different staining buffers. We observed low fluorescence of CD66a/c/e-QDot565 and CD14-QDot605 staining when cells were incubated with a single cocktail of all 8 antibodies in PBS (Fig. 2C). The low signal of these markers precluded reliable delineation of monocyte and DC subsets, especially after BCG or LPS stimulation. By contrast, cell staining with the single antibody cocktail in BD Perm/Wash buffer resulted in higher fluorescence of the QDot-conjugates, allowing more precise gating of cell subsets. When cells were first stained with non-QDot-conjugated antibodies in PBS, followed by a second staining step with anti-CD66a/c/e-QDot565 and anti-CD14-QDot605 in BD Perm/Wash buffer, the fluorescence of CD14-QDot605 was even brighter. However, this did not enhance CD66a/c/e-QDot565 fluorescence markedly. The fluorescence of non-QDot-conjugated antibodies did not change when PBS or BD Perm/Wash was used as staining buffer (data not shown).

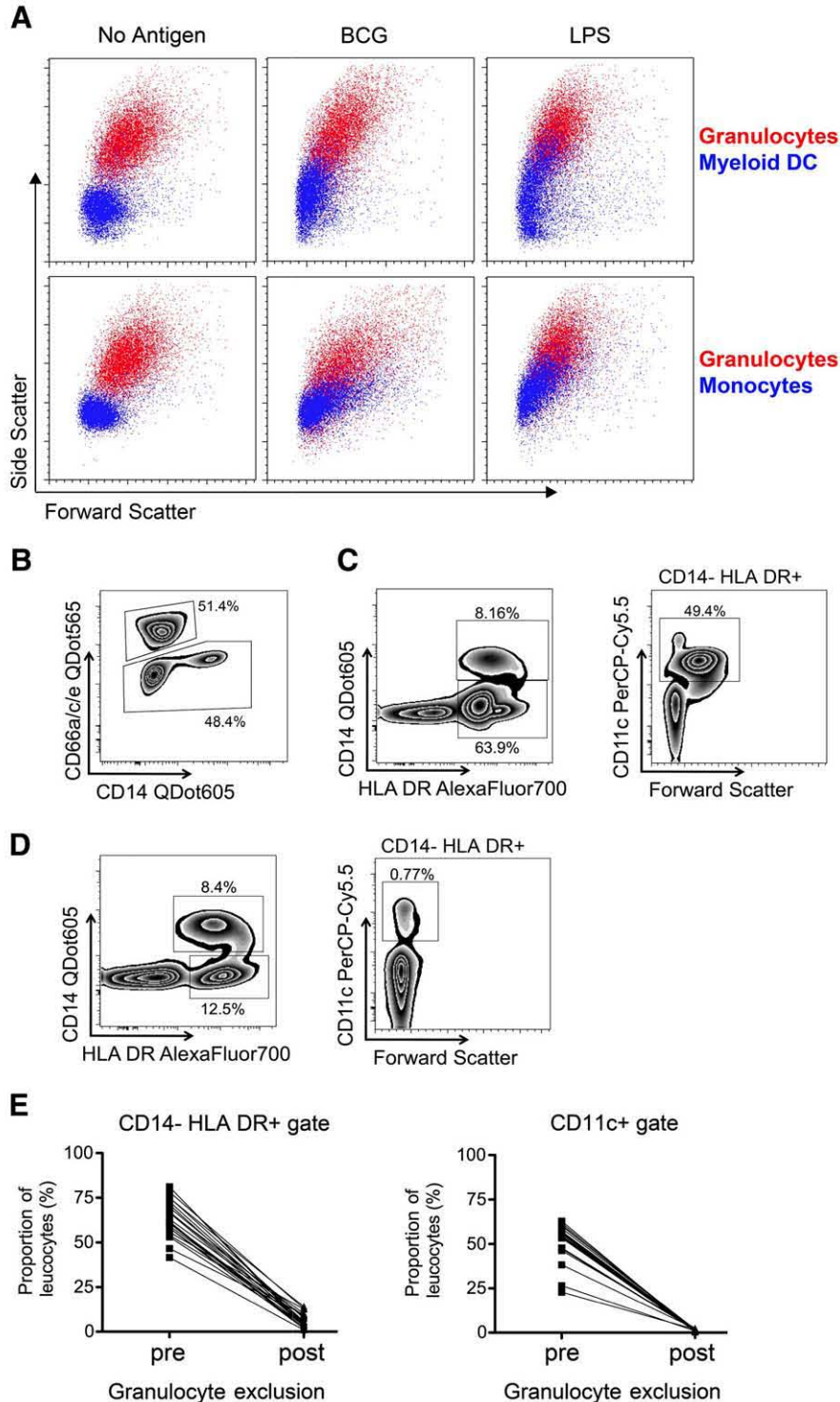
1 mM EDTA in staining buffer has been shown to improve QDot fluorescence (Zarkowsky et al., 2011). We did not observe any improvement in fluorescence of QDot-conjugated antibodies when cells were stained in PBS containing 1 mM EDTA (data not shown). We therefore proceeded with single-step staining in BD Perm/Wash buffer for all subsequent experiments.

3.4. Green fluorescent protein-expressing BCG (BCG-GFP) allows measurement of BCG binding and/or internalization by innate cells

Upon pathogen recognition, activated innate cells may bind to and/or phagocytose the microbe and express cytokines and chemokines (Gille et al., 2009). Alternatively, activation and cytokine expression may occur in the absence of pathogen binding or phagocytosis. We used a GFP-expressing BCG to allow detection of innate cells that have bound and/or internalized BCG, by flow cytometry (Fig. 3A). The concentration of BCG inoculum was an important

determinant of innate cell response to BCG. The proportion of GFP⁺ monocytes and granulocytes reached a plateau at an inoculum concentration of 3.5×10^5 CFU/mL of whole blood, whereas the proportion of GFP⁺ mDC appeared to reach a maximum at a lower inoculum dose of 1.7×10^5 CFU/mL (Fig. 3B, upper panel). Surprisingly, expression of the pro-

inflammatory cytokine IL-6 was remarkably sensitive to the mycobacterial inoculum concentration (Fig. 3A and B). IL-6 expression by monocytes, mDC and granulocytes peaked at a mycobacterial inoculum of 3.5×10^5 CFU/mL and markedly decreased at higher doses (Fig. 3B). The dose of 3.5×10^5 CFU/mL was chosen for subsequent experiments.



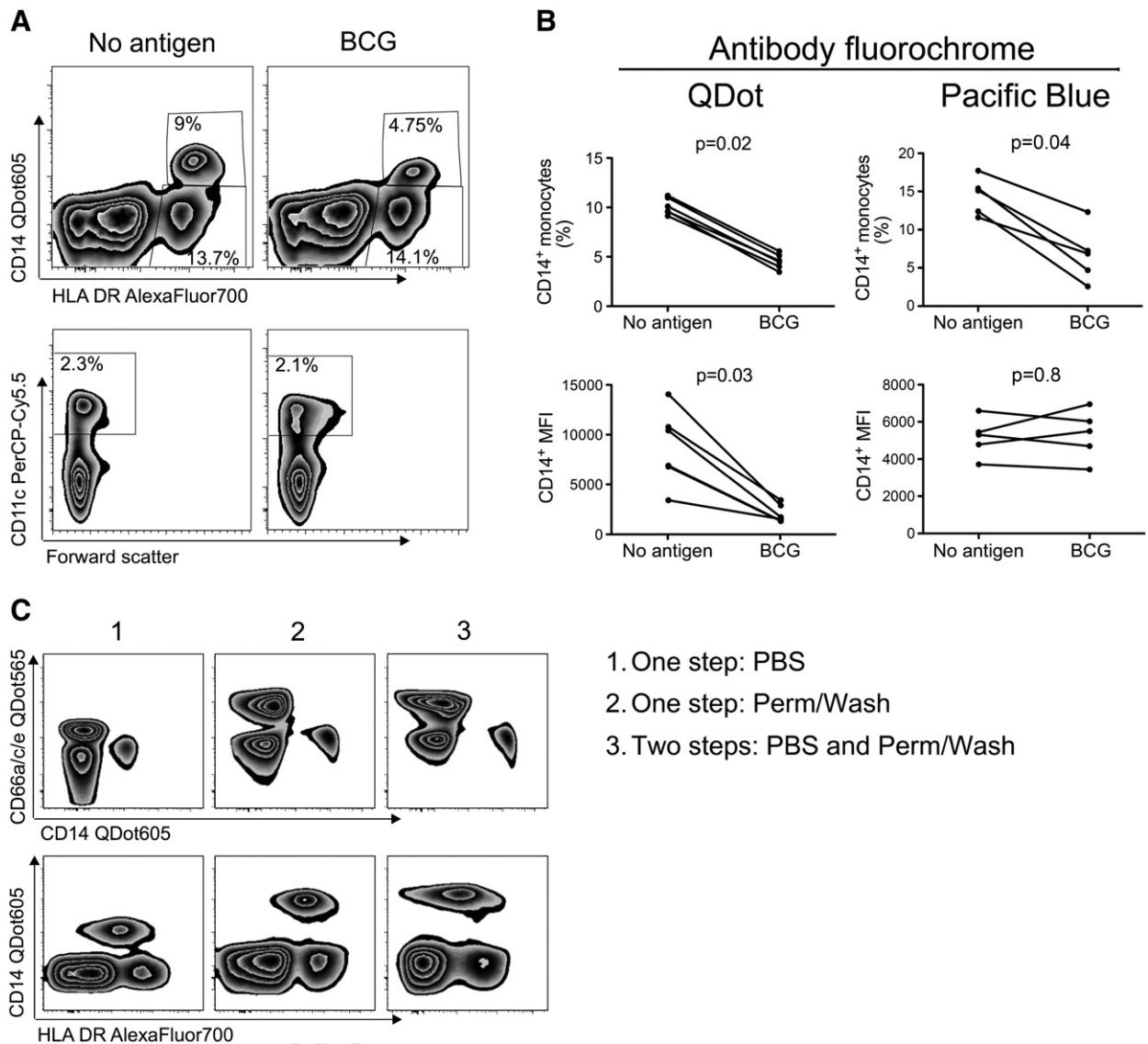


Fig. 2. Cell activation downregulates CD14. Whole blood from 5 donors was incubated with no antigen or 3.5×10^5 CFU/mL BCG-GFP for 6 h before flow cytometric analysis of cell subsets. (A) Representative plots showing flow cytometric detection of cells falling into the CD14⁺ and HLA DR⁺ (top plots) and CD11c⁺ (lower plots) gates. (B) Frequencies and CD14 median fluorescence intensities (MFI) of monocytes detected with anti-CD14 QDot (left panels) or anti-CD14 Pacific Blue (right panels). The Wilcoxon signed rank test was used for statistical analysis. (C) Comparison of QDot-conjugate antibody staining of whole blood from a single representative donor after single step staining in PBS or Perm/Wash or two-step staining in PBS (for non QDot-conjugated antibodies), then Perm/Wash (for QDot-conjugated antibodies).

3.5. Different frequencies of innate cells directly bind or internalize BCG

We next determined the proportion of GFP⁺ cells among each innate cell subset, as well as the proportion of each subset among GFP⁺ cells. A median proportion of 45% (IQR, 37–

50%) of granulocytes were GFP⁺ (Fig. 4A). A lower proportion of monocytes were GFP⁺ (Median, 36%; IQR, 31–38%) while mDC displayed the lowest proportion of GFP⁺ cells (median, 26%; IQR, 23–30%) (Fig. 4A). A similar, but markedly more pronounced picture was observed when we assessed the relative proportions of these innate cell subsets among

Fig. 1. Optimizing flow cytometric detection of innate cell subsets. (A) Forward scatter and side scatter properties of granulocytes and mDC (top row) or monocytes (bottom row), after incubation of whole blood with no antigen, BCG-GFP or LPS. Granulocytes were identified as CD66a/c/e⁺, monocytes as CD14⁺ and mDC as CD14⁺, HLA DR⁺ and CD11c⁺ (refer to Supplementary Fig. 1C for gating strategy). (B) Identification and exclusion of granulocytes by anti-CD66a/c/e staining (C and D). CD14 and HLA DR expression by peripheral blood innate cells (left plots), and CD11c expression by CD14⁺, HLA DR⁺ cells (right plots), before (C) or after (D) exclusion of CD66a/c/e⁺ granulocytes. (E) Frequencies of CD14⁺ HLA DR⁺ and CD11c⁺ mDC pre and post exclusion gating of CD66a/c/e⁺ granulocytes. In 25 adults. The frequencies of cell subsets among total leukocytes falling into the CD14⁺ HLA DR⁺ or CD11c⁺ gates are shown in each plot (refer to Supplementary Fig. 1C for gating strategy).

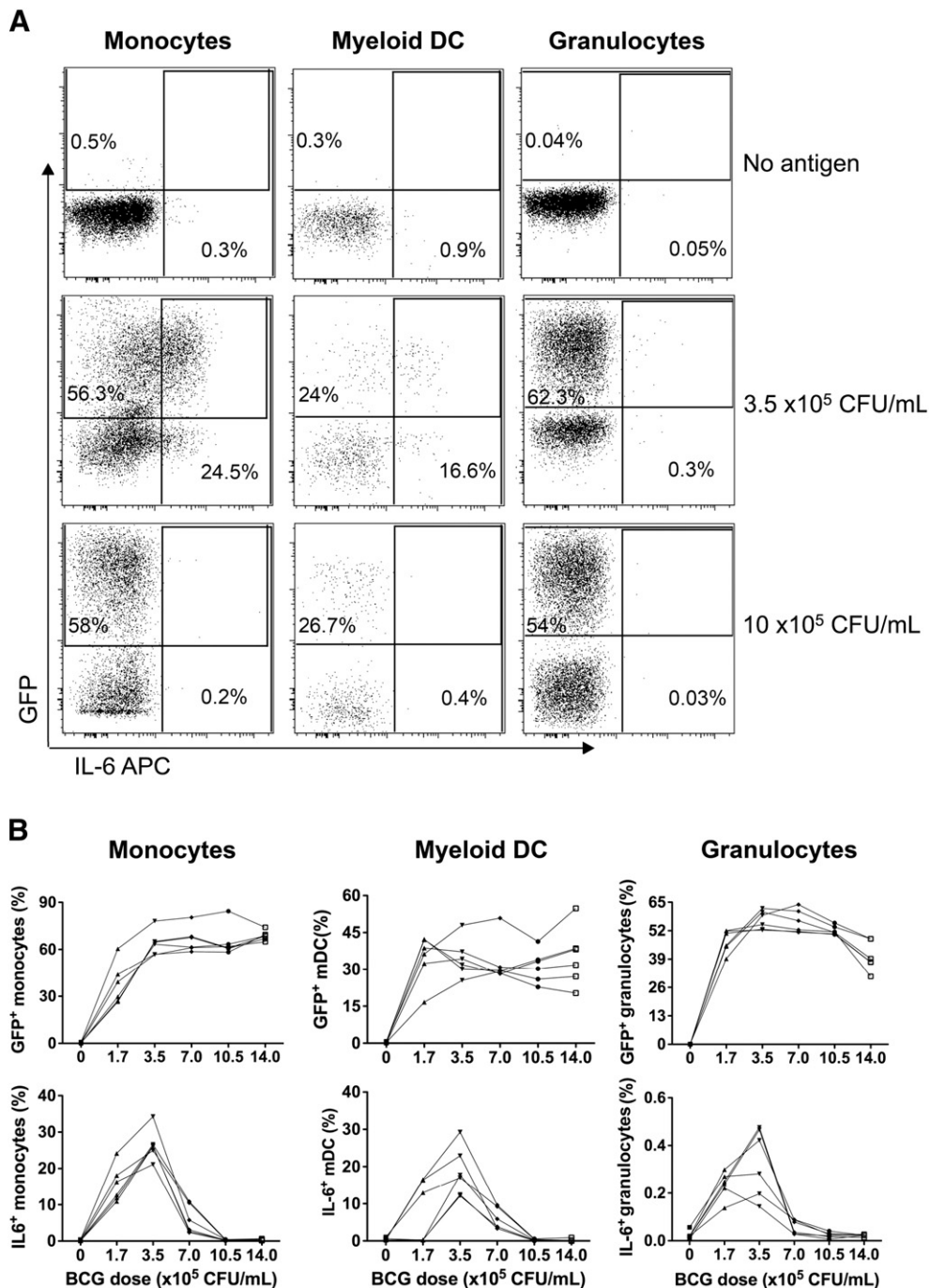


Fig. 3. Measurement of BCG uptake by innate cells. (A) Representative dot plots of IL-6 expression by GFP⁺ or GFP⁻ monocytes, mDC or granulocytes, after incubation without BCG-GFP (upper row), or with 3.5×10^5 CFU/mL (middle row), or 10×10^5 CFU/mL of BCG-GFP (bottom row). The frequencies of cells falling into each of the 2 boolean gates are shown. (B) Frequencies of GFP⁺ monocytes, mDC, or granulocytes (upper row) after titration of BCG-GFP into whole blood from 5 donors. (Bottom row) Frequencies of total IL-6 expressing monocytes, mDC or granulocytes at different BCG-GFP inocula.

GFP⁺ cells. Granulocytes comprised 87% (IQR, 82–89), monocytes comprised 7% (IQR, 5–9%), while mDC contributed 1% (IQR, 0.8–1.4%) of all GFP⁺ innate cells (Fig. 4B). Granulocytes were thus the peripheral blood innate cell subset with the highest capacity for binding and/or internalization of BCG.

3.6. Freezing medium is not a critical determinant of assay performance

Batch thawing of samples cryopreserved after stimulation and fixation allows more standardized antibody staining for flow cytometry. To determine the effect of cryopreservation

on assay performance, we compared the frequencies of monocytes and mDC when cells were stained immediately after culture without cryopreservation, with cells that were cryopreserved. Prior cryopreservation did not significantly affect the frequencies of mDC or monocyte subsets in unstimulated or BCG-stimulated blood (Supplementary Fig. 3A, right). Choice of cryopreservation medium may also affect cellular proteins and thus antibody staining. We evaluated the use of either FACS Lysing Solution (FLS) or 10% DMSO in FCS as cryopreservation media. Frequencies of CD14⁺ and CD11c⁺ cells in unstimulated or BCG-stimulated whole blood were also not significantly affected by the choice of cryopreservation medium (Supplementary Fig. 3B). Although cryopreservation did appear to result in lower frequencies of IL-6 expressing monocytes and mDC in 4 donors, this difference was not significant (Supplementary Fig. 3C). Similarly, frequencies of IL-6-expressing monocytes appeared to be higher when FACS Lysing solution was used, compared with DMSO in FCS, but this was not significant (Supplementary Fig. 3C). Cryopreservation medium did not influence frequencies of IL-6-expressing mDC after incubation of whole blood with BCG. Subsequent experiments were performed with 10% DMSO as freezing medium.

3.7. Cytokine expression by BCG-GFP-negative cells

Finally, we applied the optimized whole blood innate ICS assay to compare cytokine expression between innate cells

that bound and/or internalized BCG (GFP⁺) and GFP-negative cells. A substantial proportion of GFP⁻ monocytes and mDC expressed the pro-inflammatory cytokines IL-6, IL-12/23p40 and/or TNF- α , in various combinations, albeit at lower frequencies than GFP⁺ monocytes and mDC (Fig. 5A). Notably, monocytes expressing IL-12/23p40 alone were observed at a higher frequency in GFP⁻, compared with GFP⁺ cells (Fig. 5A). Monocytes expressing IL-6 alone and mDC co-expressing IL-6 and TNF- α were the dominant cytokine-expressing subsets in both GFP⁺ and GFP⁻ cells (Fig. 5A and B).

Upon LPS stimulation, monocytes expressing IL-6 alone also comprised the dominant subset, while cells co-expressing IL-6 and TNF- α were prominent (Fig. 5C). By contrast, similar frequencies mDC expressed IL-6 alone or co-expressed IL-6 and TNF- α (Fig. 5D).

IL-10-expressing cells were detected at very low frequencies compared with IL-6, TNF- α and IL-12/23p40, and IL-10 was not co-expressed with these pro-inflammatory cytokines (data not shown). Again, IL-10-expressing monocytes and mDC were observed at higher proportions amongst GFP⁺ cells, compared with GFP⁻ cells (Fig. 5E and F). LPS stimulation induced higher frequencies of IL-10-expressing monocytes, compared with BCG (Fig. 5E). BCG binding or internalization induced a higher frequency of cells expressing cytokines.

4. Discussion

We developed and optimized an assay for measuring intracellular cytokine expression by peripheral blood innate cells in response to viable mycobacteria using very small volumes of blood. Our method presents a number of important variables for optimal performance of this innate cell ICS assay: 1. Exclusion of granulocytes is required for reliable flow cytometric delineation of myeloid DC and monocytes in whole blood; 2. Anti-CD66a/c/e antibody staining allows flow cytometric identification and analysis of granulocytes among activated innate cells; this was not possible using forward and side scatter parameters; 3. Innate cells that have not bound or phagocytosed mycobacteria express cytokines, and this cytokine expression is exquisitely sensitive to the dose of mycobacterial inoculum; 4. Fluorescent antibody staining buffer and cell activation are important determinants of performance and outcomes of the ICS assay.

We show that the increase in monocyte and mDC granularity, and decrease in granulocyte granularity, after whole blood stimulation with BCG or LPS precludes discernment of granulocytes from monocytes and mDC. Further, granulocyte expression of the mDC lineage marker, CD11c, necessitated exclusion of granulocytes to identify peripheral blood mDC. We show that anti-CD66a/c/e antibody allows identification and exclusion of granulocytes, and subsequent identification of monocytes and mDC using key lineage markers. Our results highlight that innate assays should take into account the marked changes that occur upon innate cell activation. In our hands, the changes in granularity ruled out identification of granulocytes by side and forward scatter parameters, which is the most common method for phenotyping innate cells *ex vivo* (Autissier et al., 2010; Fung et al., 2010).

Our results also underscore an important consideration when identifying DC subsets in whole blood, since no single

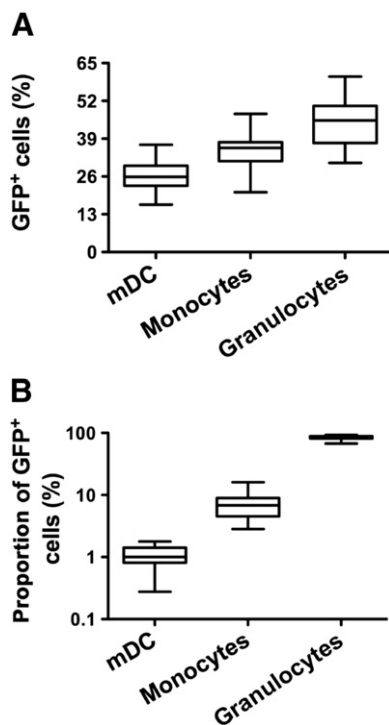


Fig. 4. Comparison of mDC, monocyte or granulocyte phagocytosis of mycobacteria. Whole blood from 25 donors was incubated with 3.5×10^5 CFU/mL BCG-GFP for 6 h before flow cytometric analysis of cell subsets. (A) Proportion of GFP⁺ cells among each innate cell subset. (B) Proportion of each innate cell subset among GFP⁺ cells. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range.

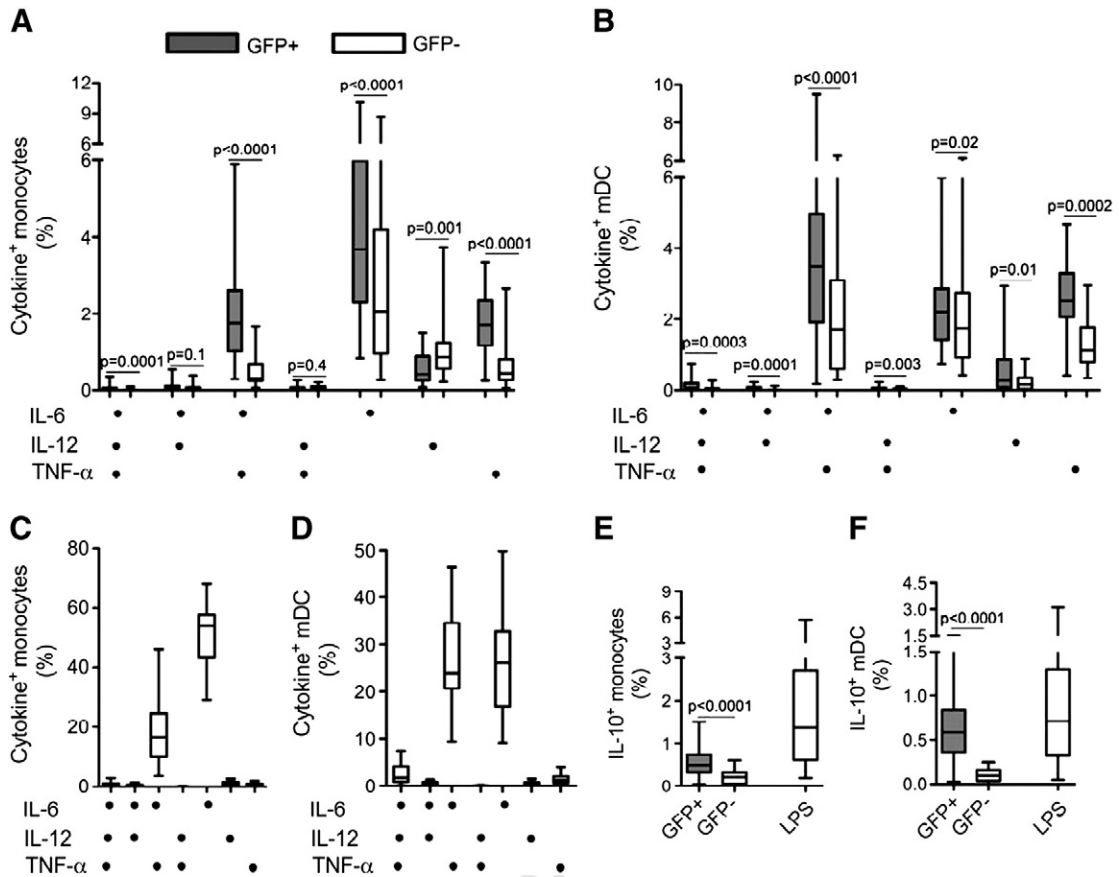


Fig. 5. Differential cytokine expression by GFP⁺ and GFP⁻ innate cells. Whole blood from 25 donors was incubated with 3.5×10^5 CFU/mL BCG-GFP for 6 h before flow cytometric analysis of cell subsets. Cytokine co-expression patterns in monocytes (A) or mDC (B) that have phagocytosed BCG (GFP⁺) or not (GFP⁻). Cytokine co-expression patterns in monocytes (C) or mDC (D) after LPS stimulation. Frequencies of IL-10-expressing monocytes (E) or mDC (F) after whole blood incubation with BCG-GFP or LPS. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range. Differences between GFP⁺ and GFP⁻ cells were calculated with the Wilcoxon signed rank test.

marker is expressed exclusively by all DC subsets. The most common DC identification methods enumerate HLA DR⁺ and CD11c⁺ DC after excluding T cells (CD3), B cells (CD19 or CD20), NK cells (CD56 or CD16), and monocytes (CD14) using lineage markers (Autissier et al., 2010; Ida et al., 2006; Wang et al., 2006, 2009). However, exclusion gating of lineage marker-positive cells may lead to exclusion of immature DC, which express low levels of CD14 and CD16 (Wang et al., 2006). We propose a combination of markers that allows identification of unstimulated or activated granulocytes, monocytes and mDC without these confounders, while allowing simultaneous analysis of cytokine expression patterns of these cells using a single antibody cocktail. Wang et al. (2006) also showed that CD66a/c/d/e antibody-containing lineage cocktails allowed detection of higher frequencies of DC, compared with cocktails containing anti-CD14 antibodies (Wang et al., 2006).

Incubation of whole blood with BCG or LPS led to markedly lower frequencies of CD14⁺ monocytes. Our results that EDTA treatment did not change CD14⁺ cell frequencies significantly suggest that greater adherence of monocytes upon activation was an unlikely contributor to this finding.

Downregulation of CD14 was previously reported upon stimulation with high doses of TLR7/8 or TLR4 ligands (Jansen et al., 2008). CD14 downregulation is also described in response to histamines in monocytes (Takahashi et al., 2003), LPS and *E. coli* in rabbit alveolar macrophages (Lin et al., 2004). However, *M.tb* infection has been shown to upregulate the expression of CD14 on monocytes (Shams et al., 2003). Given the role of CD14 as the TLR4 co-receptor for LPS binding, the downregulation is likely due to macropinocytosis-mediated internalization upon TLR4 stimulation (Mollen et al., 2008; Poussin et al., 1998). Lower frequencies of CD14⁺ cells after stimulation may also be due to shedding of CD14 or monocyte death. A spontaneous decrease in CD14 without stimulation has been reported, which could be due to internalization of membrane-bound CD14 followed by processing and secretion of soluble CD14, or the rapid recycling of CD14-TLR4-MD2 complexes between the plasma membrane and the Golgi apparatus (Bossart and Heinzelmann, 2011). Incubation of PBMC with *E. coli* or Group B streptococcus also leads to a reduction in viable monocytes (Gille et al., 2009). Similarly, monocytes are known to rapidly die through either classical apoptosis or alternative cell death processes after

phagocytosis of mycobacteria (Webster et al., 2010). Notably, HLA DR⁺CD11c⁺CD14^{-dim} cell population may also contain CD14⁺CD16⁺ monocytes (Cros et al., 2010). Peripheral blood frequencies of CD16⁺ monocytes were reported to increase during *M.tb* infection, but these cells were more susceptible to apoptosis, and, unlike CD14⁺ monocytes, did not differentiate *in vitro* into monocyte-derived-macrophages (Castano et al., 2011). We could not delineate these monocyte sub-populations, as we did not measure CD16 expression in our analyses.

The reduction in CD14⁺ frequency and fluorescence necessitated optimization of staining conditions for detection of monocytes. QDot-conjugated antibodies (CD14 and CD66a/c/e) performed best when antibody staining was performed in BD Perm/Wash buffer. Fluorescence of QDot nanocrystals is sensitive to staining buffers, and depends on concentrations of heavy metals (Chen and Rosenzweig, 2002; Meallet-Renault et al., 2006). Low concentrations of cupric ions were recently shown to eliminate QDot fluorescence (Zarkowsky et al., 2011). The latter study showed that 1 mM EDTA completely protected the fluorescent properties of these nanocrystals. However, in our hands staining in 1 mM EDTA/PBS did not result in enhanced QDot fluorescence.

Infection of innate cells with GFP-expressing BCG permits evaluation of the proportion of cells that have phagocytosed BCG, and allows comparison of cytokine expression by BCG-containing cells with those that have not internalized BCG. Importantly, our GFP-based assay system did not allow discrimination between cells that have phagocytosed BCG and cells with surface-bound mycobacteria. Although not investigated here, we anticipate that the proportion of cells with internalized BCG markedly exceeds those with surface-bound BCG, as was previously shown for human epithelial cells (de Boer et al., 1996). Interestingly, while the proportion of GFP⁺ cells increased with greater bacterial inocula, the proportion of functional, IL-6 expressing cells peaked at an inoculum of 3.5×10^5 CFU/mL. IL-6 expression of these cells dropped rapidly at higher bacterial loads. Our data highlight that titrating the mycobacterial inoculum when measuring innate cell cytokine expression is an important optimization step. The exact mechanism for the lower cytokine expression at high bacterial inocula is not clear, but may be related to the known inhibitory effect of polar lipids, such as phenolic glycolipids (PGL), found in the cell wall of BCG (Reed et al., 2004; Vergne and Daffe, 1998). PGL derived from *M.tb* H37Rv or BCG was shown to inhibit the production of TNF- α and IL-6 by murine bone marrow derived macrophages (BMM) in a dose-dependent manner (Reed et al., 2004). It is unknown whether this sensitivity of *in vitro* innate cell cytokine expression to mycobacterial dose applies to innate cell behavior *in vivo*. Such sensitivity would imply that infection with high doses of pathogen might lead to suboptimal inflammatory responses.

We observed cytokine expression by a considerable proportion of GFP⁻ cells. A higher frequency of GFP⁻ monocytes expressed IL-12/23p40 alone, compared with GFP⁺ monocytes. The significance of this observation is unknown. Cytokine expression by GFP⁻ innate cells is likely due to bystander activation by cytokines secreted by phagocytic cells, or other cells that can directly recognize mycobacteria, such as NK or $\gamma\delta$ T cells.

Granulocytes were the major peripheral blood phagocytes of BCG, although little or no cytokine response was detected in these cells. This finding is consistent with a recent study showing that neutrophils were the predominant *M.tb*-infected cell subset in sputum and bronchoalveolar lavage from patients with multidrug resistant TB (Eum et al., 2010). However, these data do not accord with several murine studies that invariably showed alveolar macrophages and DC to be the predominant populations of BCG-infected lung cells *in vivo* (Humphreys et al., 2006; Pecora et al., 2009). Despite being present at high numbers in lungs of infected mice, granulocytes were not infected to the same extent (Humphreys et al., 2006). These differences may simply be due to distinct immune characteristics or presentation of TB infection/disease in mice and humans (Eum et al., 2010). The different markers and methods for identification of cell subsets may also underlie the discrepant outcomes; for example, we identified granulocytes as CD66a/c/e⁺ while Humphreys et al. (2006) identified these cells as CD11b^{+/Hi}CD11c⁻.

A limitation of the flow cytometric ICS assay described here was the absence of a viability marker to exclude dead cells. Stimulation with BCG or LPS was performed on fresh whole blood, which reduced the likelihood of cell death typically observed when culturing thawed cells. In line with this, we observed no or only very minor evidence of artifactual antibody staining and/or autofluorescence. Although not tested here, a staining step with a fixation-resistant viability dye may be incorporated after red cell lysis, but before cell fixation, to allow exclusion of dead cells, as we recently described for another whole blood assay (Soares et al., 2010). We also could not assess whether BCG in GFP⁺ cells was intracellular or on the surface.

To summarize, we developed an innate cell ICS assay that measures cytokine expression by flow cytometry to mycobacteria. This assay may be applicable to studying innate cell responses to any fluorescent pathogen, and can be performed on blood volumes as low as 200 μ L per condition, making it particularly suitable for pediatric studies.

Conflict of interest

The authors declare that they have no commercial or financial conflicts of interest.

Supplementary materials related to this article can be found online at doi:10.1016/j.jim.2011.11.011.

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ORIGINAL ARTICLE

Single nucleotide polymorphisms in toll-like receptor 6 are associated with altered lipopeptide- and mycobacteria-induced interleukin-6 secretion

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Toll-like receptors (TLRs) are critical mediators of the immune response to pathogens. The influence of human TLR6 polymorphisms on susceptibility to infection is only partially understood. Most microbes contain lipopeptides recognized by TLR2/1 or TLR2/6 heterodimers. Our aim was to determine whether single nucleotide polymorphisms in TLR6 are associated with altered immune responses to lipopeptides and whole mycobacteria. We sequenced the TLR6 coding region in 100 healthy South African adults to assess genetic variation and determined associations between polymorphisms and lipopeptide- and mycobacteria-induced interleukin (IL)-6 production in whole blood. We found two polymorphisms, C745T and G1083C, that were associated with altered IL-6 secretion. G1083C was associated with altered IL-6 levels in response to lipopeptides, *Mycobacterium tuberculosis* lysate (Mtb lysate, $P = 0.018$) and Bacille Calmette-Guerin (BCG $P = 0.039$). The 745T allele was also associated with lower NF- κ B signaling in response to di-acylated lipopeptide, PAM2 ($P = 0.019$) or Mtb ($P = 0.026$) in an HEK293 cell line reconstitution assay, compared with the 745C allele. We conclude that TLR6 polymorphisms may be associated with altered lipopeptide-induced cytokine responses and recognition of Mtb. These studies provide new insight into the role of TLR6 variation and the innate immune response to human infection.

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Keywords: toll-like receptor 6; polymorphism; interleukin-6; tuberculosis; immune response

Introduction

The innate immune system enables the host to recognize invading microbes as foreign. Toll-like receptors (TLRs) are central in this process. These innate immune cell receptors recognize pathogen-associated molecular patterns present in a wide variety of microbes, through a transmembrane receptor with a leucine-rich repeat motif.¹ On recognition of the ligand by a TLR, a signaling cascade is initiated through activation of a cytoplasmic Toll/interleukin (IL)-1R homology domain. This results in activation of transcription factors, including NF- κ B, which translocate into the nucleus for induction of pro-inflammatory molecules.^{2–4} The resulting inflammation is a critical early step in immune responses aimed at controlling the invading microbe.

Genetic variation of human TLRs regulates signaling and inflammatory responses and is associated with susceptibility to infection.^{5–7} We and others have shown

that a polymorphism in TLR1, T1805G, regulates the immune response to tri-acylated lipopeptides, present in cell walls of many microbes.^{8–10} In contrast, this single nucleotide polymorphism (SNP) was not associated with altered responses to di-acylated ligands.⁸

This study's focus was TLR6. Human TLR6 is a 2391 base pair gene that encodes a 796-amino acid (aa) type I transmembrane protein with a 630aa extracellular leucine-rich repeat region (including a 31aa signal peptide), a 21aa transmembrane domain and a 145aa intra-cellular Toll/IL-1R signaling domain. TLR6 forms a heterodimer with TLR2 in a complex incorporating CD14, which preferentially recognizes di-acylated lipopeptides like PAM2 (PAM2 CSK K K K, S-[2,3-bis(palmitoyloxy)-propyl]-(R)-cysteinyll-lysyl)3-lysine). This contrasts with TLR1, which also heterodimerizes with TLR2, in a complex with CD14, but which specifically recognizes tri-acylated lipopeptides like PAM3 (Pam3CSK K K K, N-palmitoyl-S-[2,3-bis-(palmitoyloxy)-propyl]-(R)-cysteinyll-lysyl)3-lysine).^{11–13} Recent studies suggest that ligand preference based on acyl number is partial, and that other structural features may also influence ligand recognition.^{14,15} These include length of the fatty acid chain, chirality of the diacyloxypropyl carbon, position of the acyl group and aa composition of the terminal peptides, which have also been shown to affect receptor specificity. The specific role of human TLR6 in

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recognition of different pathogens is less well understood than that of TLR1 and TLR2. For example, several studies suggest that TLR1 or TLR2 mediate responses to *Mycobacterium tuberculosis* (Mtb),^{16–18} whereas a role of TLR6 in response to this pathogen has only been suggested in a single study.¹⁹

Variation in human cellular responses to di-acylated lipopeptides has been shown in studies that have principally focused on TLR1 polymorphisms.^{8,10} As TLR1 polymorphism did not completely account for the observed variation, it was postulated that this might be due to variation in the TLR6 gene, which mediates differential signaling and cytokine responses; although no specific polymorphism has been defined. Other studies have suggested an association between TLR6 polymorphism and disease susceptibility. For example, the SNP T1932G (A644A) was associated with altered IFN- γ secretion in response to measles virus stimulation of peripheral blood mononuclear cells (PBMC),²⁰ whereas C745T was associated with asthma^{21,22} and with invasive aspergillosis²³ after allogeneic stem cell transplantation. It is not known if these polymorphisms alter TLR6 function.

Our aim was to learn about the role of TLR6 polymorphism in recognition and signaling of *M. tuberculosis*. The current global epidemic of tuberculosis

is responsible for 1.7 million deaths annually.²⁴ Chronic inflammation is a hallmark of tuberculosis infection and substantial efforts have been made to identify bacterial components responsible. *M. tuberculosis* (Mtb) is recognized by several TLRs including TLR 1, 2, 4 and 9.^{12,16–18,25} We hypothesized that TLR6 polymorphisms contribute to differential immune responses, and ultimately differential protection against this disease. We examined whether TLR6 polymorphisms are associated with altered di-acylated lipopeptide- and mycobacteria-induced cytokine responses in humans.

Results

One hundred healthy adults were enrolled, including 56 women and 44 men with an age range from 18 to 57 years, and from a mixture of ethnic backgrounds: 24 Black Africans, 64 participants of mixed ethnicity and 12 Caucasians.

To identify common polymorphisms in the TLR6 gene, we sequenced the coding region in 100 healthy adult volunteers. We found 10 polymorphisms, which included 7 non-synonymous and 3 synonymous base pair changes (Table 1). The observed allelic frequencies were consistent with Hardy–Weinberg equilibrium. Nine of

Table 1 TLR6 coding region polymorphisms in South Africans, compared with those described in the Hapmap database

SNP detail		Current study				MAF data in previously described populations					
SNP	NCBI reference	Population	AA n (%)	Aa n (%)	aa n (%)	MAF (%)	HWE (%)	CEU (%)	YRI (%)	LWK (%)	MKK (%)
T34A (F12I)	ss161110012	Black	11 (50)	11 (50)	0 (0)	25	0.12	NA	NA	NA	NA
		Mixed	39 (64)	22 (36)	0 (0)	18	0.08				
		Caucasian	9 (75)	3 (14)	0 (0)	12.5					
T359C (I120T)	rs5743808	Black	18 (86)	3 (14)	0 (0)	7.1	0.72	0	8.3	1.1	9.4
		Mixed	49 (82)	11 (18)	0 (0)	9.1	0.43				
		Caucasian	12 (100)	0 (0)	0 (0)	0					
T581C (L194P)	rs5743809	Black	21 (100)	0 (0)	0 (0)	0		0	3.3	NA	NA
		Mixed	53 (87)	8 (13)	0 (0)	6.5	0.58				
		Caucasian	10 (100)	0 (0)	0 (0)	0					
G740A (R247K)	rs35220466	Black	19 (86)	3 (14)	0 (0)	6.8	0.73	0.8	2.5	NA	NA
		Mixed	59 (95)	3 (5)	0 (0)	2.4	0.84				
		Caucasian	12 (100)	0 (0)	0 (0)	0					
C745T (P249S)	rs5743810	Black	22 (92)	2 (8)	0 (0)	4.1	0.83	46.4	0	1.1	7.0
		Mixed	52 (81)	11 (17)	1 (2)	10.1	0.64				
		Caucasian	3 (25)	7 (58)	2 (17)	45.8					
G979A (V327M)	rs3796508	Black	19 (95)	1 (5)	0 (0)	2.5	0.91	0	0.9	2.8	2.4
		Mixed	57 (93)	4 (7)	0 (0)	3.2	0.79				
		Caucasian	11(100)	0 (0)	0 (0)	0					
G1083C (T361T)	rs3821985	Black	15 (65)	7 (30)	1 (4)	19.5	0.87	72.4	31.4	NA	NA
		Mixed	27 (42)	25 (39)	12 (19)	38.2	0.16				
		Caucasian	2 (17)	2 (17)	8 (66)	75					
A1263G (K421K)	rs3775073	Black	15 (65)	7 (30)	1 (4)	15	0.87	29.2	70.8	15.7	32.5
		Mixed	29 (48)	25 (41)	7 (11)	31.9	0.65				
		Caucasian	1 (9)	1 (9)	9 (82)	86.3					
T1280C (V427A)	rs5743815	Black	21 (100)	0 (0)	0 (0)	0		1.3	0	NA	NA
		Mixed	61 (100)	0 (0)	0 (0)	0					
		Caucasian	10 (91)	1 (9)	0 (0)	4.5					
T1932G (A644A)	rs5743818	Black	13 (62)	7 (33)	1 (5)	20	0.96	27	31	2.2	3.1
		Mixed	42 (71)	16 (27)	1 (2)	15.2	0.71				
		Caucasian	9 (75)	2 (17)	1 (8)	13.6					

Abbreviations: A, common allele; a, minor allele; NA, not available.

Genotype and allele frequencies of the 10 polymorphisms for South African are listed, along with minor allele frequencies (MAF) described in the Hapmap database, for multiple other populations. CEU, Utah residents with European Ancestry; LWK, Luhya in Kenya; MKK, Maasai in Kenya; YRI, Yoruba in Nigeria. The HWE *P*-values are calculated for Black and mixed ethnicity populations. There were very few Caucasians and the HWE *P*-value was not calculated for this population.

these polymorphisms have been reported before in public databases, including HapMap, NCBI and IL-PGA (Table 1). One polymorphism (T34A) had not been described earlier. All of the remaining nine TLR6 coding region polymorphisms were present in at least one HapMap population.

To examine whether any of these polymorphisms were associated with altered cytokine production, we stimulated whole blood from 70 of the 100 participants with di-acylated lipopeptides and several other TLR ligands. We have shown earlier that measurement of IL-6 is ideal for assessing innate responses in whole blood stimulated with lipopeptides: IL-6 was secreted at readily detectable levels, whereas IL-12, TNF- α , IL-1 β and IL-10 levels were low.⁸ Two polymorphisms, C745T and G1083C, were associated with altered IL-6 production. We examined G1083C first: the 1083CC (361T) genotype was associated with lower IL-6 production, compared with 1083GG

(361T), in response to fibroblast-stimulating lipopeptide 1 (FSL-1) (Figure 1a), PAM2 (Figure 1b) and PAM3 (Figure 1c). As a control, stimulation with lipopolysaccharide (LPS) was not associated with significant differences in the level of IL-6 produced (Figure 1d).

We next examined whether TLR6 SNPs were associated with altered IL-6 response to whole mycobacteria, which have a complex repertoire of lipopeptides and other ligands. SNP G1083C was associated with a decrease in IL-6 secretion after Mtb lysate and Bacille Calmette-Guerin (BCG) stimulation (Figures 1e and f).

To assess the reproducibility of these results, we repeated the experiment in 26 of the 100 individuals, and found the same results for G1083C in response to FSL-1 (Figure 1g) and to PAM2 (Figure 1h), but not to PAM3 (Figure 1i). We also examined the intra- and inter-individual variation of the assay over time by comparing IL-6 levels of individuals drawn at different times. In a

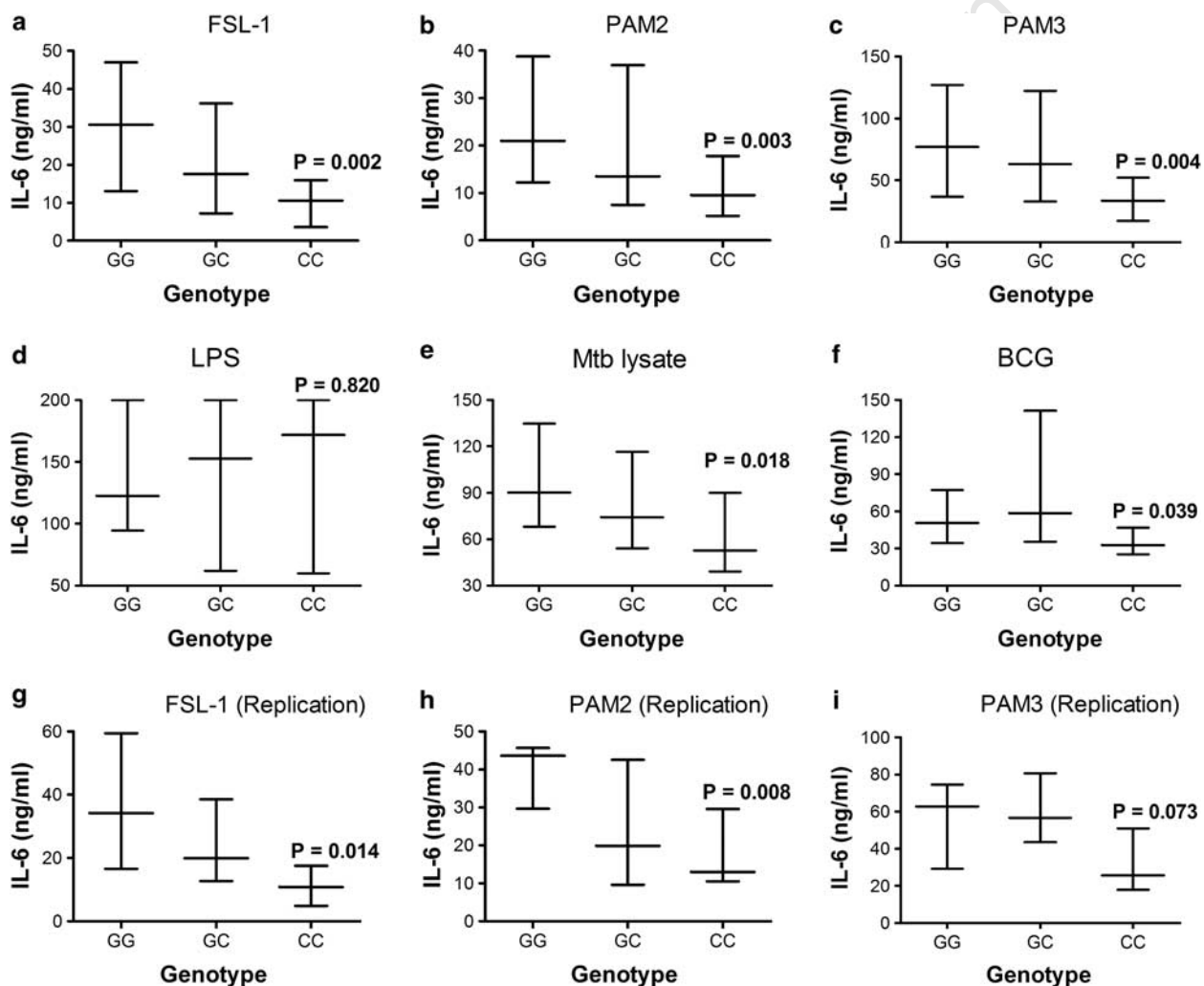


Figure 1 Effect of TLR6 G1083C polymorphism on IL-6 secretion: Whole blood was stimulated for 20 h with TLR ligands and whole mycobacteria, followed by measurement of IL-6 levels in plasma, by ELISA. IL-6 levels were stratified by genotypes of G1083C polymorphisms. Ligands used included (a) FSL-1, a TLR2/6 ligand; (b) PAM2, a TLR2/6 ligand; (c) PAM3, a TLR2/1 ligand; (d) LPS, a TLR4 ligand; (e) H37Rv Mtb lysate; and (f) whole lyophilized live BCG. These figures show results from the following numbers of participants with specific genotypes: GG $n = 36$; GC $n = 21$; and CC $n = 12$. To validate our data, blood from another 26 participants (GG $n = 8$; GC $n = 12$; and CC $n = 6$) was examined: IL-6 levels were determined after stimulation of whole blood with (g) FSL-1, (h) PAM2 and (i) PAM3. Data are shown as medians with interquartile ranges, and a P -value representing assessment of differences between the GG and CC genotypes, using a Mann-Whitney test. The GG and GC genotypes were also compared, but no differences were found.

paired analysis, we found consistent IL-6 levels within individuals and a similar rank order between individuals suggesting that IL-6 responsiveness is a stable biologic phenotype ($P=0.071$ for FSL-1; $P=0.267$ for PAM2; and $P=0.480$ for PAM3) (Supplementary Figure 1). Mtb lysate and BCG were not used during the repeat experiment.

Together, these results suggest that allele 1083C is associated with decreased IL-6 levels in response to lipopeptide and mycobacteria stimulation.

As a synonymous polymorphism, G1083C may directly regulate lipopeptide- or mycobacteria-induced cytokine secretion through effects on transcriptional regulation of TLR6 or be in linkage disequilibrium (LD) with a polymorphism that directly regulates function. To address the latter possibility, we examined whether polymorphisms associated with altered IL-6 levels induced by FSL-1 or PAM-2 were in LD with G1083C. We first examined the other coding region polymorphisms in TLR6. The highest correlation was observed between the G1083C and the A1263G polymorphisms ($R^2=0.75$, Figure 2, left panel). Although this suggests some LD correlation between G1083C and A1263G, the latter SNP was not associated with altered levels of IL-6. Furthermore, A1263G is synonymous and has no known association with function. The remaining TLR6 coding region SNPs had low levels of LD with 1083 ($R^2 < 0.12$ for all SNPs except 745). We next examined whether non-coding TLR6 SNPs or nearby polymorphisms were associated with IL-6 levels. TLR6 is located on chromosome 4p14 in a region adjacent to TLR1 and TLR10 that spans ~54 kb.²⁶ We examined additional non-coding region haplotype-tagging TLR6 polymorphisms (rs1039559, rs7673348, rs7665774) as well as haplotype-tagging polymorphisms (in the Utah residents with European ancestry (CEU) and Yorubans in Nigeria (YRI) populations) in TLR1 (rs17616434, rs3923647, rs3924112, rs4833095, rs5743618) and TLR10 (rs4321646, rs10856837, rs7694115). We also examined seven

haplotype-tagging SNPs in TLR2 on chromosome 4q32 (rs3804090, rs5743708, rs11935252, rs1337, rs1339, rs1439166, rs6535946).

We found that seven polymorphisms in the TLR6 promoter region and TLR1 were significantly associated with altered IL-6 levels induced by FSL-1, one was associated with altered IL-6 induced by Mtb and none were associated with altered IL-6 induced by PAM-2. The levels of IL-6 secreted in response to FSL-1 and BCG are shown in Figure 3. For the other ligands only the P -values are represented because the trend of IL-6 secretion for these ligands was identical to FSL-1 and BCG (Figure 3; Supplementary Table 1; data not shown). TLR6 coding region polymorphisms and polymorphisms that were significantly associated with altered levels of IL-6 secretion (Figure 3; Supplementary Table 1) were analyzed for LD with the G1083C and C745T polymorphisms for all participants in our study. For the non-coding region polymorphisms, the most prominent correlation with the G1083C polymorphism was observed with the TLR6 promoter region polymorphisms rs1039559 ($R^2=0.42$, $D'=1.0$) and rs7665774 ($R^2=0.35$, $D'=0.84$), and with the C745T polymorphism ($R^2=0.23$, $D'=1.0$) (Figure 2; Supplementary Figure 2). None of the other TLR6 coding region polymorphisms were associated with altered IL-6 levels (data not shown).

The G1083C polymorphism had very weak LD correlation with the TLR1 polymorphisms with $R^2 \leq 0.18$ (Figure 2; Supplementary Figure 2, right panels). One of the associated polymorphisms was TLR1_T1805G (rs5743618), which we and others have previously found to regulate PAM3-induced signaling.⁸⁻¹⁰ The association of TLR1_T1805G with IL-6 signaling in our dataset was strongest for PAM3 (TT median = 64 920 pg ml⁻¹ ($n=45$), GT = 59 980 pg ml⁻¹ ($n=17$), GG = 20 970 pg ml⁻¹ ($n=7$), $P=0.001$ for TT vs GG, Mann-Whitney test). The association was weaker for FSL-1 (TT = 24 118 pg ml⁻¹ ($n=45$), GT = 18 968 pg ml⁻¹ ($n=17$), GG = 5545 pg ml⁻¹

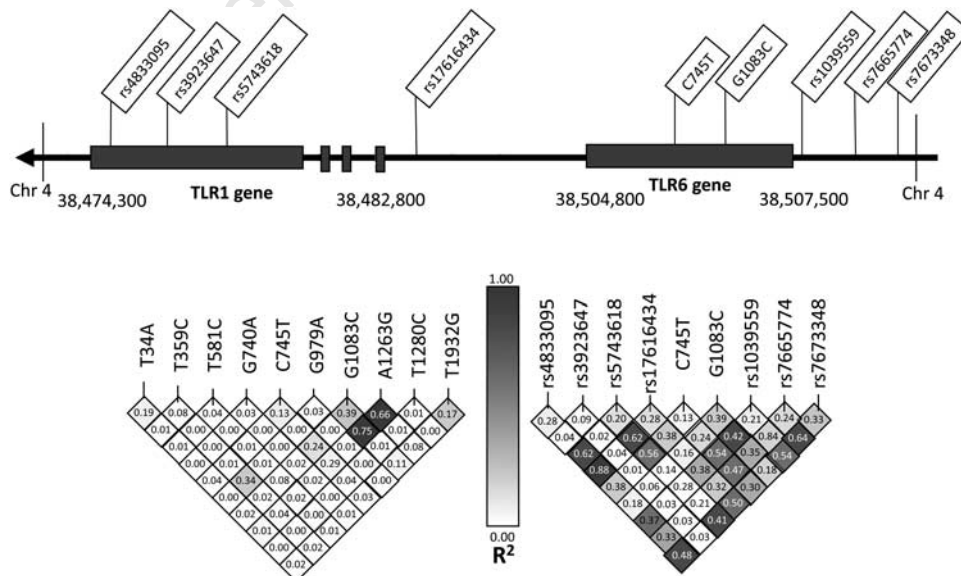


Figure 2 Linkage disequilibrium analyses between TLR6 and TLR1 SNPs. R -squared (R^2) values for each SNP combination are shown numerically and by shading, based on the legend in the middle. TLR6 and TLR1 SNPs were genotyped and analyzed for level of LD with G1083C and C745T polymorphisms. The figure shows R^2 values for pairwise comparison of the different polymorphisms. $R^2=0$ represents no LD correlation; $R^2=1$ represents high (maximum) LD correlation. The program *plink* in Stata was used to calculate the values. The minor allele frequency is shown adjacent to each corresponding SNP.

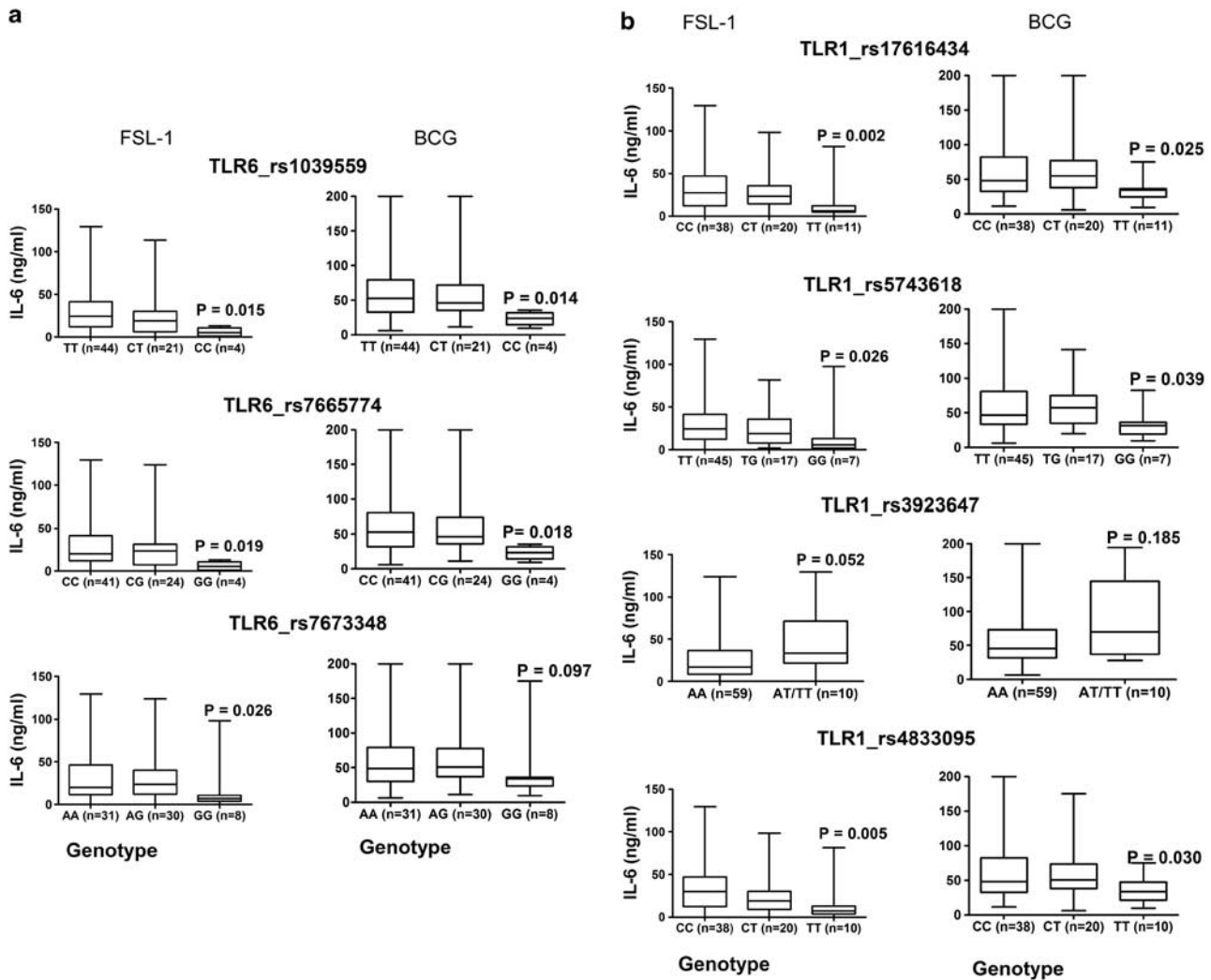


Figure 3 Association of IL-6 secretion in response to FSL-1 and BCG with polymorphisms in the non-coding region of TLR6, and polymorphisms in TLR1. Polymorphisms in TLR6 and TLR1 were identified from HapMap and genotyped in our participants. Whole blood was stimulated for 20 h with the TLR ligands and whole mycobacteria. IL-6 levels in plasma were measured by ELISA and responses stratified by genotype of the polymorphism. Data for FSL-1 and BCG are shown as box and whisker plots with medians and interquartile ranges. *P*-values represent differences between the homozygous genotypes with Mann–Whitney test. (a) TLR6-tagged polymorphisms, and (b) TLR1-tagged polymorphisms. Data for the rest of the ligands are shown in Supplementary Table 1.

($n=7$), $P=0.026$ for TT vs GG) and absent for PAM2 (TT = 16 182 pg ml⁻¹ ($n=45$), GT = 22 778 pg ml⁻¹ ($n=17$), GG = 13 520 pg ml⁻¹ ($n=7$), $P=0.553$ for TT vs GG). The R^2 and D' values for TLR1_T1805G and TLR6_G1083C were 0.14 and 0.6, respectively, suggesting a low level of LD. To adjust for a possible effect from this SNP, we stratified the analysis and only examined individuals with the high responding TLR1 genotypes (1805TT/TG). We found the trends for TLR6 G1083C polymorphism were preserved, but the statistical significance was decreased, possibly because of the decreased sample size (FSL-1: GG = 24 750 pg ml⁻¹ ($n=8$), CC = 12 061 pg ml⁻¹ ($n=6$), $P=0.066$; PAM2: GG = 17 010 pg ml⁻¹, CC = 11 512 pg ml⁻¹, $P=0.074$; PAM3: GG = 68 760 pg ml⁻¹, CC = 43 756 pg ml⁻¹, $P=0.08$; LPS: GG = 113 508 pg ml⁻¹, CC = 145 040 pg ml⁻¹, $P=0.784$).

We also examined SNP TLR2_G2258A, which has been shown earlier to regulate signaling.^{6,27–30} We found that it was not associated with lipopeptide and

mycobacteria-induced IL-6 secretion (data not shown) even though the frequency of this SNP in our populations was very rare (97GG, 3GA and no AA) to make any conclusions. These results suggest that these two previously characterized SNPs in TLR1 and TLR2 do not explain the association of G1083C with lipopeptide- and mycobacteria-induced IL-6 secretion.

We next examined possible mechanisms that may explain the observed differences in IL-6 secretion. These factors may include protein and mRNA transcript expression. As this SNP is synonymous, we did not think it would be associated with differential protein expression. We then examined whether G1083C was associated with altered levels of TLR6 mRNA transcripts. We measured TLR6 mRNA levels in PBMCs from a subgroup of 26 individuals with different G1083C genotypes. No differences were observed in TLR6 mRNA levels when stratified according to genotypes in unstimulated PBMCs or in cells stimulated with PAM2

or LPS (data not shown). We also found that the polymorphisms in Supplementary Table 1 were not associated with different TLR6 mRNA levels. Together, these results suggest that polymorphism TLR6_G1083C and other SNPs in this region were not associated with altered levels of TLR6 mRNA expression. In summary, G1083C is in LD with several SNPs that are also associated with altered lipopeptide- or mycobacteria-induced IL-6 secretion. On the basis of these data, we are unable to determine which of these SNPs is most likely to directly regulate signaling in response to di-acylated lipopeptide stimulation.

Polymorphism C745T is non-synonymous and encodes a proline to serine (P249S) change at aa 249 in the extracellular domain. Owing to small numbers of the 745TT genotype ($n=2$), we were unable to assess its homozygous effect independently. When CC and CT/TT

genotypes were compared, individuals with the 745CC genotype ($n=55$) had increased levels of IL-6 on stimulation with FSL-1 and PAM3, compared with individuals with the 745CT/TT ($n=15$) genotypes (Figures 4a and c, respectively). There were no differences when PAM2, the control LPS or the mycobacterial antigens Mtb and BCG were used as stimulants (Figures 4b and d–f, respectively). We again tested reproducibility of the results by repeating the assay in 26 additional participants (CC, $n=18$; CT, $n=6$; TT, $n=2$). When comparing the CC ($n=18$) and the CT/TT ($n=8$) genotypes, similar trends were observed, but the differences were not significant in response to stimulation with FSL-1 (Figure 4g), PAM2 (Figure 4h) or PAM3 (Figure 4i). The lack of a significant difference may have been due to an inadequate sample size. We also observed that this polymorphism had a relatively high LD

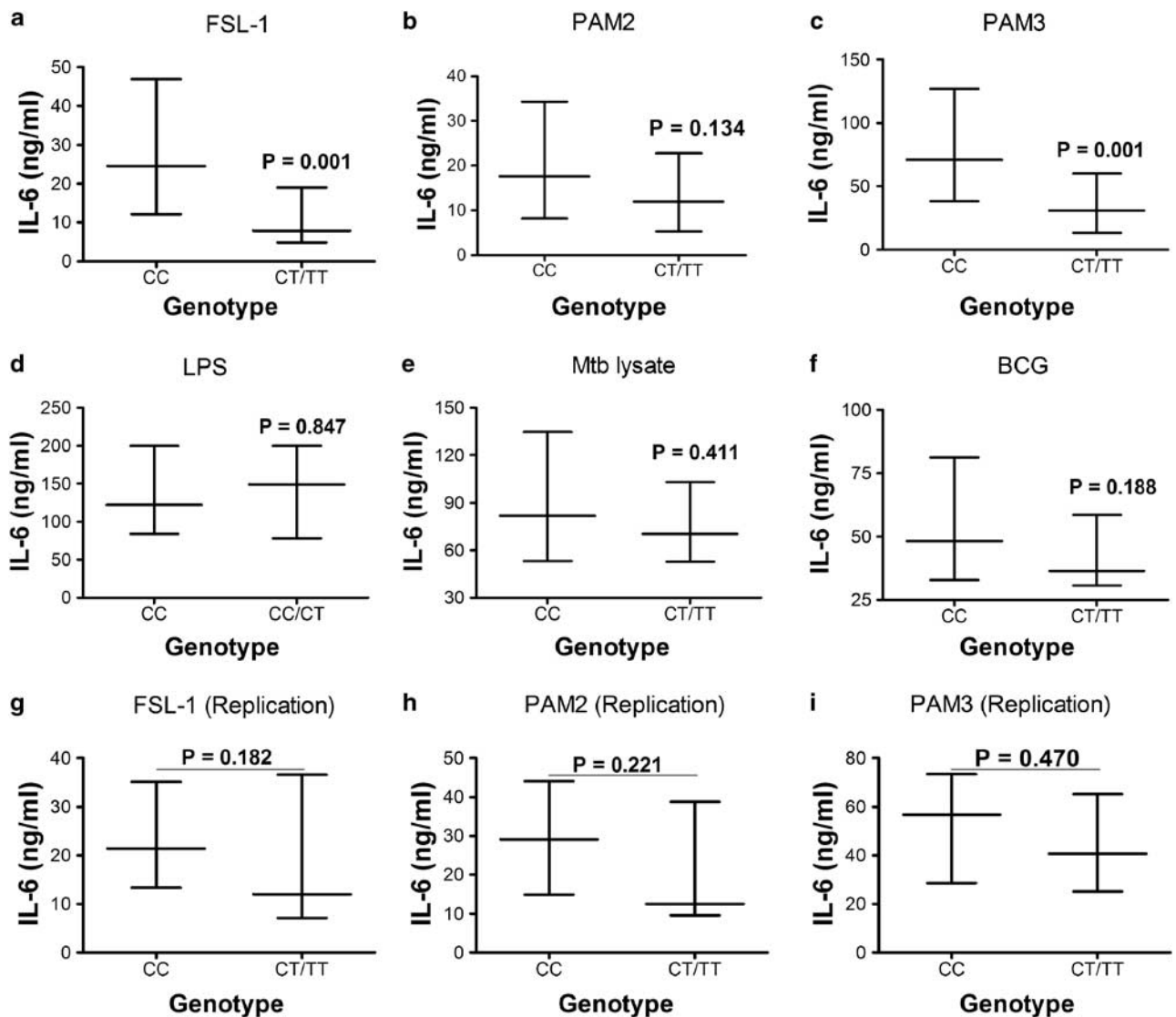


Figure 4 TLR6 C745T and whole blood IL-6 secretion stratified by genotype. Whole blood IL-6 levels were measured by ELISA and stratified by polymorphism C745T genotypes: Stimuli were (a) di-acylated lipopeptide FSL-1, a TLR2/6 ligand; (b) di-acylated lipopeptide PAM2, a TLR2/6 ligand; (c) tri-acylated lipopeptide PAM3, a TLR2/1 ligand; (d) LPS, a TLR4 ligand; (e) H37Rv Mtb lysate; and (f) whole lyophilized live BCG. To validate our data, blood was further collected from a subset of 26 participants and IL-6 levels determined after stimulation of whole blood with (g) FSL-1, (h) PAM2 and (i) PAM3. Data depicted as medians and interquartile ranges with P-values representing a comparison between the CC and CT/TT genotypes with a Mann-Whitney test. CC $n=55$; CT $n=13$; TT $n=2$ for (a–f), and CC $n=18$; CT $n=6$; TT $n=2$ for (g–i).

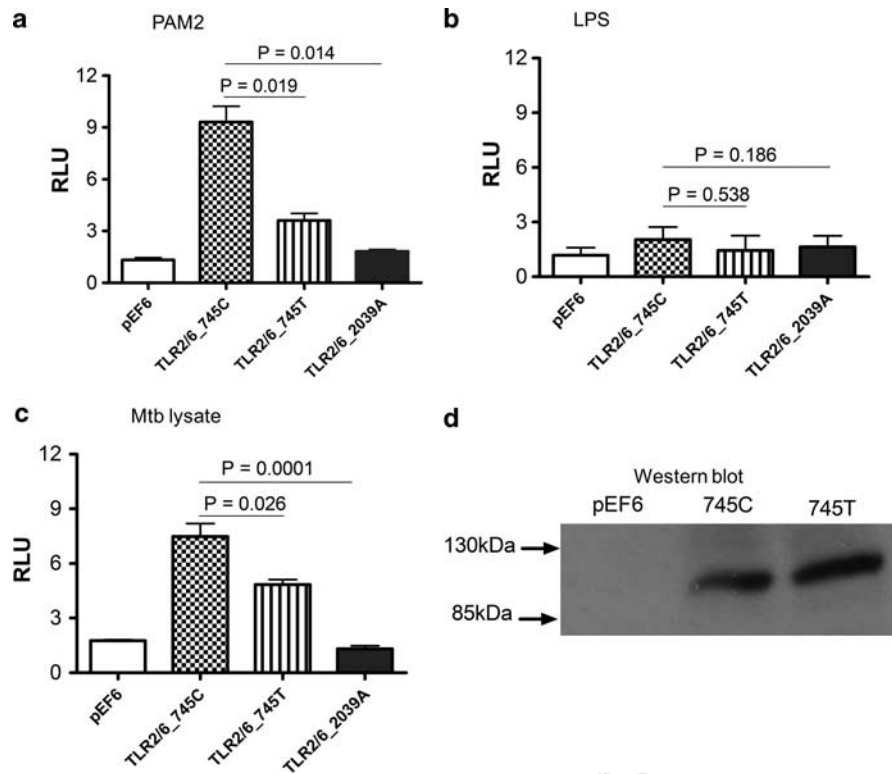


Figure 5 Regulation of NF- κ B signaling by a TLR6 polymorphism. HEK293 cells were transfected with an NF- κ B luciferase reporter, a *Renilla* luciferase construct to control for transfection efficiency (pRL-TK), and CD14. Additional transfectants varied by condition and included an empty plasmid vector (pEF6), or TLR2 with one of three TLR6 constructs, 745C, 745T or 2039A. Polymorphism C2039A (P680H) is a TLR6 variant with a dominant-negative effect on NF- κ B signaling. Luciferase activity shown represents the ratio between basal activity (medium only) and that of transfected cells stimulated with (a) PAM2, (b) LPS or (c) Mtb lysate. Mean values (\pm s.d.) are depicted for three independent experiments, each performed in triplicate. *P*-values calculated with Student's *t*-test. RLU, relative luciferase units. (d) Expression of TLR6 745C (wild type) and 745T (variant) by immunoblotting using transfected but unstimulated HEK293 cells.

correlation with TLR6 promoter region and TLR1 polymorphisms (Figure 2). Although the validation results were inconclusive, the data suggest that allele 745T may be associated with decreased IL-6 secretion.

To account for the potential confounding effect of the IL-6 promoter polymorphism G-174C, which has been associated with reduced IL-6 levels,³¹ we genotyped this polymorphism in our study population. We found no association between IL-6 levels and genotype at position -174 of the IL-6 promoter (data not shown).

To further investigate a possible regulatory effect of C745T on lipopeptide signaling, we cloned the 745C (wild type) and 745T variant into the EF6-V5-His-TOPO expression plasmid vector. As a control, we also generated a dominant-negative TLR6 variant with a proline to histidine mutation at aa680 (P680H, C2039A). This mutation is equivalent to the P712H substitution in TLR4, which abrogates LPS signaling in C3H/HeJ mice.³² We examined whether the TLR6 variants were able to mediate NF- κ B signaling in HEK293 cells. In response to PAM2 stimulation, allele 745T mediated significantly decreased NF- κ B signaling activity in comparison to allele 745C (Figure 5a). The dominant-negative variant, 680H, was also associated with decreased NF- κ B signaling. As a control, we stimulated the cells with LPS and found no significant signaling above baseline levels in comparison to wells stimulated with media alone (Figure 5b). We next examined whether variant 745T mediated signaling in response to a

complex microbial ligand by stimulating with Mtb lysate. We found that allele 745T mediated decreased NF- κ B signaling activity, compared with 745C (Figure 5c). As a control, the 2039A dominant-negative variant completely abolished signaling to PAM2 and to Mtb lysate (Figures 5a and c).

We next examined whether differential localization patterns or expression levels of the two variants could mediate observed signaling differences. The TLR6 variants had similar protein levels when assessed by immunoblot (Figure 5d). Furthermore, TLR6 localization was similar for the variants with expression at or near the plasma membrane and those with expression within the cell (data not shown). The intra-cellular staining pattern excluded the nucleus and appeared vesicular, suggesting a location within organelles, rather than in the cytoplasm. As a negative control, we transfected the empty backbone pEF6 vector, and did not observe any positive staining (data not shown).

Together, these results suggest that polymorphism C745T directly regulates NF- κ B signaling and IL-6 production in response to PAM2 stimulation and Mtb lysates.

Discussion

In this study, we aimed to identify TLR6 polymorphisms occurring in Cape Town, South Africa, and to examine

whether TLR6 polymorphisms regulate innate immune responses to lipopeptides and mycobacteria. We identified a novel polymorphism, and also observed that altered IL-6 secretion from stimulated whole blood was associated with the G1083C (T381T) and C745T (P249S) polymorphisms.

The mechanism of altered IL-6 secretion associated with the G1083C polymorphism is not known. G1083C is synonymous and does therefore not alter the aa composition of TLR6. Furthermore, individuals with different G1083C genotypes had similar levels of TLR6 mRNA expression, suggesting that transcriptional regulation is an unlikely mechanism. Possible alternative mechanisms include post-transcriptional regulatory mechanisms, or differential stability of the protein or mRNA within the cell. Several studies have shown that mRNA expression and protein levels are not always correlated.^{33,34}

Alternatively, SNP G1083C may be in LD with a polymorphism that acts through a post-transcriptional mechanism. Candidate LD regions include the promoter region of TLR6, or polymorphisms in TLR1 and TLR10, TLRs that are adjacent to TLR6. To examine this possibility, we genotyped haplotype-tagging polymorphisms in TLR1, TLR10 and non-coding regions of TLR6. We found that overall there was a weak LD correlation between several polymorphisms and the G1083C and C745T polymorphisms. This suggests that the G1083C polymorphism may mediate differential IL-6 secretion through association with any of these polymorphisms. The effects of two previously characterized polymorphisms, TLR2_G2258A (R753Q)^{6,27–29} and TLR1_T1805G (I602S)^{6,8,10} could not explain our results. Together, our data suggest that G1083C is at least a useful genetic marker for regulation of IL-6 responses to di-acylated lipopeptides. Owing to our strict selection criteria for the polymorphisms to be genotyped, we might have excluded some polymorphisms that may be associated with mycobacterial and other diseases. On the basis of the current data, we do not know the causative SNP underlying these observations. It is, however, possible that this polymorphism might be in LD with a yet-to-be described polymorphism either in TLR6 or in genes adjacent to TLR6.

SNP C745T is non-synonymous and was associated with altered NF- κ B signaling in a transfected cell line and possibly with differences in IL-6 levels in response to lipopeptide stimulation of primary cells. Although the latter observation was not clearly reproducible, this may have been because we were unable to test sufficient numbers of persons with the rare homozygous 745TT genotype. On the basis of the NF- κ B signaling data in a reconstitution system, this non-synonymous SNP appears to directly alter TLR6 signaling. Given the location of C745T (P249S) in the extracellular domain, we speculate that this polymorphism alters ligand recognition. Alternatively, the substitution could result in a conformational change that affects the assembly of a TLR2/6 signaling complex. A recent study reported no differences in NF- κ B signaling between the two C745T variants after PAM2 stimulation of HEK293T cells.³⁵ Compared with our experiments, these investigators used 10-fold more TLR6 DNA for transfection (100 ng), and 25-fold less PAM2 (10 ng ml⁻¹) as TLR6 ligand. These observations suggest that the effect of C745T on NF- κ B

signaling in a reconstituted system may be partial and results may vary in different *in vitro* experimental conditions.

Interestingly, the C745T polymorphism was associated with altered IL-6 production in response to stimulation with FSL-1, but not PAM2. Although both of these lipopeptides are di-acylated, they have different peptide moieties, which may affect TLR6 binding.³⁶ Furthermore, PAM3 stimulation induced significantly different IL-6 levels in individuals with the different TLR6 genotypes, an unexpected finding given that PAM3 is predominantly a TLR2/1 ligand. These results may be due to our incomplete understanding of ligand specificity of these receptors. Previous studies indicate that the specificity of lipopeptides for TLR1 and TLR6 is not solely dependent on the number of acylation side groups. Other structural features such as length of the fatty acid chain, chirality of the diacyloxypropyl carbon, position of the acyl group and aa composition of the terminal peptides have also been shown to affect receptor specificity.^{11,12,14,15,36,37} The specificity of our findings was supported by the finding that IL-6 produced in response to LPS, a TLR4 ligand, was not associated with differences when comparing TLR6 genotypes.

Two previous studies have examined the functional role of TLR6 polymorphisms. A recent study showed that rare SNPs in TLR6 were associated with altered NF- κ B signaling and an increased risk of TB disease in certain ethnic populations.²⁶ We did not find any of these polymorphisms in our sample of 100 individuals. Recently, three SNPs in TLR6 (rs5743795, rs1039560 and rs3775073) were associated with differential responses to PAM3.¹⁰ PAM2 and FSL-1 were not tested in this study, so it is difficult to directly compare our results. We also examined one of these SNPs, A1263G (rs3775073), but it was not associated with differential responses to PAM3. We did not identify the other two SNPs in our cohort. Possible explanations for apparent discrepancies in results may be due to protocol differences including PAM3 dose (10 ng ml⁻¹ vs 300 ng ml⁻¹), incubation time (6 h vs 20 h) and different population genetic backgrounds (Seattle, WA, USA vs Cape Town, South Africa). This suggests that other variables including dose of lipoproteins and genetic background may also influence TLR signaling responses.

Although the role of TLR1 in Mtb recognition is well established, the role of TLR6 has been less clear and has not been studied well.¹⁹ Our NF- κ B signaling data support a role for TLR6 in Mtb recognition. In addition, we found that TLR6_G1083C genotypes were associated with different levels of IL-6 after stimulation with Mtb lysate and BCG. We were surprised that the response to a combination of ligands may be associated with TLR6 genotypes. As TLR6 recognizes di-acylated lipopeptides, this suggests the presence of these lipopeptides in Mtb and also that these lipopeptides may have a major role in immunity to Mtb. Previous studies on lipoproteins in TB show that the inactivation of the Mtb LspA by allelic replacement has an important role in lipoprotein synthesis and the pathogenesis of Mtb.³⁸ The cell wall-associated lipoprotein LprA of Mtb was also shown to regulate innate immunity and inhibits antigen presentation in macrophages.³⁹ In addition to Mtb, TLR6 is likely to mediate immune responses to a wide variety of pathogens.^{40,41} TLR6 polymorphisms show some

evidence of association with IL-6 secretion on stimulation with lipopeptides and mycobacteria. These results require further replication to confirm the findings. This work thus suggests a role for TLR6 in regulating mycobacterial signaling and offers a strong impetus to evaluate the relationship between TLR6 polymorphisms and susceptibility to mycobacterial diseases including TB.

Materials and methods

Participant recruitment, enrollment, blood collection and processing

Healthy adults were enrolled at the South African Tuberculosis Vaccine Initiative clinical site, near Cape Town in South Africa. Exclusion criteria included HIV and other chronic infections, pregnancy and active tuberculosis. Heparinized blood was collected, for whole blood incubations (below) and for PBMC isolation by density gradient centrifugation. This study was approved by the Research Ethics Committee of the University of Cape Town, the Western Institutional Review Board (USA) and by the Institutional Review Board of the University of Washington. Written informed consent was obtained from all participants. The study population included individuals from different backgrounds, including Black African ($n=24$), Caucasian ($n=12$) and South African Mixed Ethnicity ($n=64$). The latter is a distinct group that emerged more than 300 years ago and received genetic input from Malaysia, Indonesia, European Caucasoid and Black Africans.⁴²

Ligands and antigens

Ultrapure LPS (TLR4 ligand, used in whole blood assays at 10 ng ml^{-1} , concentration in other assays mentioned below) isolated from *Salmonella minnesota* R595 was obtained from List Biological Labs Inc. (Campbell, CA, USA). The lipopeptides PAM2 (PAM2CSKKKK, S-[2,3-bis(palmitoyloxy)-propyl]-(R)-cysteinyl-(lysyl)3-lysine, TLR2/6 ligand, 100 ng ml^{-1}), FSL-1 (TLR2/6 ligand, 300 ng ml^{-1}) and PAM3 (Pam3CSKKKK, N-palmitoyl-S-[2,3-bis-(palmitoyloxy)-propyl]-(R)-cysteinyl-(lysyl)3-lysine, TLR2/1 ligand, 300 ng ml^{-1}) were synthetic lipopeptides obtained from EMC Microcollections (Tuebingen, Germany). Lysate from Mtb strain H37Rv ($25\text{ }\mu\text{g ml}^{-1}$) was obtained from J Belisle (Colorado State University, Fort Collins, CO, USA; NIAID reagent contract). Lyophilized live BCG ($20 \times 10^6\text{ CFU ml}^{-1}$) was obtained from Statens Serum Institute (Copenhagen, Denmark).

Whole blood incubation and IL-6 ELISA

Whole blood diluted 1:5 in RPMI medium 1640 (BioWhittaker, Walkersville, MD, USA) was incubated with TLR ligands, BCG or Mtb lysate for 20 h. Polymixin B (BioChemika, $10\text{ }\mu\text{g ml}^{-1}$; Steinheim, Germany) was added to the antigen wells containing the lipopeptides (except to the LPS condition), to minimize any possible effects of LPS contamination. IL-6 secreted into the supernatant was measured by ELISA (Human IL-6 OptEIA, BD Biosciences, San Diego, CA, USA), according to the manufacturer's instructions.

DNA isolation and sequencing

Genomic DNA was isolated from 5 ml blood using the Qiagen Blood Maxi Preparation kit (Valencia, CA, USA). TLR6 was PCR-amplified using Pfu polymerase (Promega, Madison, WI, USA) in two fragments (1600 and 900 base pairs, respectively) and nucleotide sequencing was performed (commercially by Macrogen, Seoul, South Korea). The following primers were used to amplify the two fragments: TLR6-2 (5'-GTGGAGGTTTGAGAGT AACCATCCG-3'), TLR6-15 (5'-GTGGGCTTCCTCTAT AACTTTCTGGG-3'), TLR6-5 (5'-GAGGTCAATAAAAG CAGGGGACAATCC-3') and TLR6-16 (5'-GGCTAACCT CACCGCCTAGCTCAGTTCCCC-3'). The sequencing reactions were performed with the following forward primers: TLR6-3 (5'-CACATGCTGTGTCTCATGCAC CAAGC-3'); TLR6-4 (5'-CACCCAAGTATTTATTTCGC TATCC-3'); TLR6-6 (5'-CCTGCCATCCTATTGTGAGTT CAGGC-3') and TLR6-7 (5'-GAGGAACTTTGTCCTG GCAAGAGC-3'). The sequences were aligned and analyzed using the programs Phred/Phrap and Consed (University of Washington, Seattle, WA, USA).^{43,44}

DNA expression vectors

For functional studies, the single exon coding region of TLR6 was amplified from genomic DNA using Pfu Turbo polymerase (Promega) and primers hTLR6-start (5'-ATGACCAAAGACAAAGAACCTATTG-3') and hTLR6-nostop (3'-CAGTGACTTTTGTACTACTTTTAGA-5'). The amplified fragment was then cloned into the pEF6/V5-His-TOPO expression cloning vector with a V5 epitope tag (Invitrogen, Carlsbad, CA, USA). The different polymorphic variants were generated with a whole plasmid PCR mutation strategy with techniques described earlier.⁴⁵ The following primers were used to generate the 745T (249S) variant, hTLR6_745T-F 5'-ACCAGAGGGTCAACCTTACTGAATTTTACC-3', and hTLR6_745T-R 5'-ATTCAGTAAGGTTGACCCTCTGGT GAGTTCTG-3'. For the dominant-negative control mutant, 2039A (680H), the primer set of hTLR6 2039A-F 5'-GAGGAACTTTGTCATGGCAAGAGCATTGTGG-3' and hTLR6 2039A-R 5'-CCACAATGCTCTTGCCATG GACAAAGTTCTCTCATG-3' was used. Subsequent digestion with the restriction enzyme *DpnI* removed the methylated DNA before transformation into JM109 competent *Escherichia coli* cells (Invitrogen). Human TLR2 was cloned into pEF6/V5-His-TOPO vector after fragment amplification with the following primers, hTLR2-F 5'-ATGCCACATACTTTGTGG-3' and hTLR2-R 5'-GGACTTTATCGCAGCTCTCAG-3'. All plasmid constructs were verified by sequencing.

HEK293 cell transfections

HEK 293 cells (ATCC #CRL-1573) were grown according to ATCC recommendations. HEK293 cells were cultured in Dulbecco's modified Eagle medium (BD Biosciences, Lenexa, KS, USA), supplemented with 10% heat inactivated fetal bovine serum, 1% L-glutamine (Life Technologies, Carlsbad, CA, USA) and maintained at 37°C in 5% CO_2 . HEK293 cells were transfected for 16–20 h using Polyfect Transfection Reagent (Qiagen) at a cellular concentration of 2×10^4 cells per well in a 96-well plate. Cells were transfected with the following plasmids: 100 ng ELAM-luciferase (NF- κB reporter with ELAM promoter and firefly luciferase), 10 ng pRL-TK luciferase (thymidine kinase promoter with *Renilla*

luciferase reporter used as a control for transfection efficiency), 40 ng CD14 as co-receptor to enhance signaling, 10 ng TLR6 or TLR6 variants and 50 ng TLR2. The final concentration of transfected DNA was normalized with empty vector, pEF6. After overnight incubation, the transfected cells were stimulated for 4 h with PAM2 (250 ng ml⁻¹), Mtb lysate (25 µg ml⁻¹) or LPS (100 ng ml⁻¹). The cells were lysed with cell lysis buffer (Promega) according to the manufacturer's instructions and luciferase activity was measured with a dual luciferase system (Promega). To control for transfection efficiency, firefly luciferase activity was normalized to *Renilla* luciferase activity.

Immunoblotting and immunofluorescence

For immunoblotting, cells were transfected for 16–20 h and then lysed with 1% Triton X-100 buffer containing a cocktail of protease inhibitors (Sigma-Aldrich, Steinheim, Germany). The lysates were blotted on a membrane and probed with a mouse anti-V5 epitope antibody (Serotec, Oxford, UK) that recognizes the V5-His tag on the TOPO cloning vector expressing the protein, followed by HRP-conjugated rabbit anti-mouse IgG (Zymed, San Francisco, CA, USA) and then developed with luminol chemiluminescent reagents (Roche, Basel, Switzerland).

For immunofluorescence, HEK293 cells were transfected for 16–20 h with TLR6 constructs and plated in a 24 well plate on coverslips. After fixing with 10% neutral-buffered formalin solution (Sigma-Aldrich), the cells were lysed with 0.25% Triton X-100 in PBS, probed with the mouse anti-V5 epitope antibody and then with an FITC-conjugated rabbit anti-mouse conjugated secondary antibody. The coverslips were then washed and mounted for microscopy with mounting medium containing 25% glycerol, 10% polyvinyl alcohol (Sigma-Aldrich), 0.1 M Tris-Cl pH 8.5 and 2.5% 1,4-diazabicyclo-[2.2.2]-octane (DABCO).

mRNA expression

In all, 5 × 10⁵ PBMCs were incubated with PAM2 (100 ng ml⁻¹) and LPS (10 ng ml⁻¹) or left unstimulated for 20 h. RNA was isolated from the stimulated PBMCs using RNeasy Mini kit (Qiagen) and complementary DNA was synthesized from the extracted RNA using Omniscript RT kit (Qiagen) with Oligo dT_{12–18} primers (Invitrogen). Recombinant RNaseOUT Ribonuclease inhibitor (Invitrogen) was used to inhibit ribonuclease activity. Quantitative real-time PCR was performed using SensiMix dT (Quantace, Watford, UK) containing 2 × SensiMix dT, 50 × SYBR Green solution and 50 mM MgCl₂ solution with the following primers for TLR6, 5'-C TGTGTCCTCATGCACCAAG-3' (forward) and 5'-TC AACCCAAGTGCAGTTTC-3' (reverse) and glyceraldehyde 3-phosphate dehydrogenase: 5'-TTCACCACTGGAGAGAGGC-3' (forward) and 5'-GGCATGGACTGTGGTCATGA-3' (reverse). A standard curve was generated for each run with cloned fragments of TLR6. Results were normalized to relative expression of glyceraldehyde 3-phosphate dehydrogenase.

Genotyping and LD

Genotyping was done using Sequenom's MassARRAY technique to identify polymorphisms in the promoter or coding region of TLR6 or in TLRs 2, 1 and 10, as described earlier.⁴⁶ This technique uses allele-specific

primer extension reactions to discriminate genotypes. We identified haplotype-tagging SNPs from the YRI and CEU populations from the International HapMap Project (<http://www.hapmap.org>) and other public databases with the Genome Variation Server (<http://www.ncbi.nlm.nih.gov/SNP/> and <http://www.innateimmunity.net>). We searched a region on chromosomes 4, 50 kb upstream and downstream of genes for TLR1, TLR6, TLR10, as well as TLR2 for tagged SNPs using an R² cut-off of 0.8 for LD and a minor allele frequency cut-off of 5%.

Stata/Intercooled v10.0 software program PWLD (Stata Corp LP, College Station, TX, USA) was used to calculate R² and D' as measurements of LD between the polymorphisms.

We also genotyped the G-174C polymorphism in the IL-6 promoter region in our population as described earlier.^{31,47} Briefly, the region flanking position -174 was PCR amplified and the PCR product digested with the restriction enzyme, *Hsp92* II (Promega), which cleaves at the C allele, but not the G allele.

Data analysis

The observed allelic frequencies for the SNPs were determined by Hardy–Weinberg equilibrium equation for Black African and mixed ethnicity groups. A Mann–Whitney *U*-test was used for statistical analysis of IL-6 secretion in whole blood whereas Student's *t*-test was used for the luciferase signaling assays. The differences were considered significant if the *P*-value was <0.05 using a two-tailed test.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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