Investigating defects in monocyte responses to *Mycobacterium tuberculosis* in HIV-TB co-infection

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DECLARATION

I, Narjis Khatoon Thawer, hereby declare that the work on which this dissertation is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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LIST OF ABBREVIATIONS

AMs	Alveolar macrophages
AIDS	Acquired Immunodeficiency Syndrome
ALS	Alternating lysing solution
APC	Antigen presenting cells
APC-Cy7	Allophycocyanin-Cy7
APOBEC3	Apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3
ART	Antiretroviral therapy
AX488	Alexafluor488
AX700	Alexafluor700
BCG	Bacille Calmette Guérin
BFA	Brefeldin-A
BV	Brilliant violet
CCR	Chemokine receptor
CD	Cluster of differentiation
CLRs	C-type lectin receptors
CVD	Cardiovascular disease
DC	Dendritic cells
DC-SIGN	Dendritic cell specific intercellular adhesion molecule-3-grabbing non-integrin
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorter
FBS	Foetal Bovine Serum
FcR	Fc Receptor
FITC	Fluorescein isothyocyanate
FMO	Fluorescence minus one
FSC	Forward Scatter
h	hour
HAART	Highly active antiretroviral treatment
HIV/HIV-1	Human Immunodeficiency Virus type 1
Hsp	Heat shock protein
ICS	Intracellular cytokine staining
IFN-α	Interferon alpha
IFN-γ	Interferon gamma
IgG	Immunoglobulin G

IL	Interleukin
IRF	Interferon regulatory factor
IRAK	IL-1 receptor-associated kinases
JAK-STAT	Janus kinase-Signal trnasducer and activator of transcription
LAM	Lipoarabinomannan
LBP	LPS-binding protein
LTBI	Latent TB infection
LP	Lipoprotein
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
MDM	Monocyte-derived macrophages
MOI	Multiplicity of infection
MR	Mannose receptor
mRNA	Messenger ribonucleic acid
MFI	Median Fluorescent Intensity
MHC	Major histocompatibility complex
min	minutes
ml	millilitre
mm	millimeter
M.tb	*Mycobacterium tuberculosis*
MyD88	Myeloid differentiation primary response gene 88
Nef	Negative regulatory factor
NF-κB	Nuclear factor kappa B
NK	Natural killer
NO	Nitric oxide
NOD	Nucleotide binding oligomerisation domain
ng	Nanogram
PAMPs	Pathogen-associated molecular patterns
Pam3Cys	Palmitic-acid-3-cystein acid
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PE	Phycoerythrin
PE-Cy5	Phycoerythrin-Cy5
PE-Cy7	Phycoerythrin-Cy7
PG	Peptidoglycan
PIM	Phosphatidylinositol mannoside
PPD	Purified protein derivative
PRRs	Pathogen recognition receptors

RLRs	RIG-like receptors
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute cell culture medium
SR	Scavenger receptor
STF	Soluble tuberculosis factor
ss	Single-stranded
SSC	Side scatter
TAK1	TFGβ-activated protein kinase 1
TB	Tuberculosis
Th	T helper
TIR	Toll/IL-1 receptor
TLR	Toll-like receptors
TNF-α	Tumor necrosis factor alpha
TRAF	TNF receptor-associated factor
Vivid	Violet-fluorescent reactive dye
WHO	World Health Organization
µg	microgram
µl	microlitre
%	percent
°C	Degrees Celsius
x g	times gravity (centrifugation speed)

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ABSTRACT

HIV-infected persons are more susceptible to TB, and the reasons for this are not fully understood. HIV infection leads to CD4+ T cell depletion, compromising adaptive immunity to M.tb, however less is known regarding the effect on innate immunity. Monocytes play a key role in innate immune defense and are the precursors of macrophages. These cells sense pathogens through toll-like receptors (TLR), which leads to the release of pro-inflammatory mediators, triggering innate and adaptive immune responses to infection, critical events for the control of M.tb. The aim of this study was to investigate whether HIV infection induced functional defects in monocytes, impairing their ability to respond to M.tb. The focus was on TLR functioning in monocytes, by examining whether infection with HIV altered cytokine production in response to TLR stimulation. The hypothesis was that co-infection with HIV may lead to defective TLR responses to mycobacterial TLR stimuli. The rationale was that sustained stimulation of HIV-responsive TLR may influence how monocytes respond to mycobacteria-encoded TLR ligands.

Whole blood was obtained from 20 HIV-infected and 18 HIV-uninfected individuals with CD4 counts >400 cells/mm^3 and antiretroviral therapy naïve. A multiparameter flow cytometry panel was developed and used to identify monocytes using phenotypic markers CD14 and HLA-DR, and to measure the capacity of monocytes to produce the cytokines IL-1β, IL-6 and TNF-α, upon stimulation with mycobacteria-derived TLR stimuli, mycobacterial lipoarabinomannan (LAM, a TLR2 ligand), M.tb purified protein derivative (PPD, which stimulates TLR1, 2, 4 and 6), live Bacillus Calmette Guérin (BCG, a stimuli for TLR1, 2, 4, 6 and 9), as well as lipopolysaccharide (LPS, a TLR4 agonist from gram-negative bacteria).

Monocytes differed in their ability to respond to the different TLR stimuli in healthy, HIV-uninfected individuals. BCG induced the most robust response, with total cytokine production detected at a median of 83.2% of monocytes (IQR, 73-85%), followed by LAM, LPS and PPD (median 64.1, 45.4 and 8.8%, respectively). Monocytes produced mainly IL-1β and IL-6, with fewer producing TNF-α in

response to all the TLR stimuli. Considerable heterogeneity was observed among the donors in response to LAM and LPS. When monocyte cytokine responses were compared between the HIV-infected and uninfected groups, there were no significant differences to LAM, BCG or PPD stimulation. Analysis of the frequency of total cytokine production, individual cytokine production, expression levels of cytokines (median fluorescent intensity) as well as polyfunctional capacity of monocytes revealed that HIV infection did not alter the cytokine response to these three mycobacteria-derived stimuli. In contrast, in response to LPS, total cytokine production was significantly enhanced in the HIV-infected group ($p=0.002$), with the frequency of IL-1β+ ($p=0.016$), IL-6+ ($p=0.003$) and TNF-α+ ($p=0.009$) monocytes significantly increased in this group compared to HIV-uninfected individuals. However, this increase in monocyte response was not related to HIV viral load or CD4 T cell count. In addition, the HIV-infected group displayed a significantly greater proportion of monocytes producing three cytokines simultaneously ($p=0.041$) in response to LPS. HIV-infected individuals showed significantly reduced frequencies of monocytes compared to uninfected individuals ($p=0.0328$), but this did not associate with HIV viral load or CD4 T cell count. However, no difference in the expression level of CD14 was observed between the two groups. Upon stimulation, the frequency of CD14+ monocytes reduced in response to LAM, BCG and LPS ($p<0.01$, $p<0.01$, $p<0.001$), likely due to CD14 shedding. In addition, a significant reduction in the expression levels of CD14 on monocytes was observed in response to all the TLR stimuli ($p<0.001$). Interestingly, HIV infection altered the degree of CD14 shedding in response to the TLR2 ligand LAM, and the TLR4 ligand, LPS. In HIV-uninfected individuals, there was an inverse correlation between the change in the level of CD14 expression on monocytes in LAM, BCG and LPS-stimulated cultures and cytokine responses to these stimuli ($p=0.016$, $r=-0.56$; $p=0.033$, $r=-0.505$ and $p=0.013$, $r=-0.573$ respectively), whilst this relationship was disrupted in HIV-infected individuals, where no such correlation was observed.

Overall, these data demonstrate that HIV co-infection does not impair the ability of monocytes to secrete key cytokines in response to mycobacteria-derived stimuli, although there was evidence of CD14 dysregulation in response to mycobacterial LAM in HIV-infected individuals. In contrast, there was enhanced responsiveness of

monocytes to LPS stimulation during HIV infection. This may reflect specific TLR4 cross-talk from ongoing activation of the innate immune system by HIV-encoded TLR ligands or by the circulating products of microbial translocation from the gut. During HIV infection, monocytes exhibit a differential responsiveness to the degree of CD14 shedding, and the association between monocyte cytokine response and the extent of CD14 shedding from monocytes is disrupted in these individuals. With LPS stimulation, this relationship may be disturbed due to the enhanced monocyte cytokine production observed in HIV-infected individuals. However, with LAM stimulation, the reduced degree of CD14 loss observed in response to LAM in HIV-infected individuals might have long-term consequences for cytokine production or other functions of monocytes. The results of this study provide further insight into how HIV affects innate immunity, which is important for a better understanding of how a protective immune response develops against M.tb.

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1.1 Introduction

Tuberculosis (TB) remains a huge public health, social and economic burden not only in developing countries but also worldwide (Dye *et al.*, 1999). Despite various strategies being implemented to address this health challenge, morbidity due to TB still remains high (Corbett *et al.*, 2003). Recent global estimates show that around 8.6 million people developed TB and 1.3 million died from the disease in 2012 (WHO, 2013a). *Mycobacterium tuberculosis* (M.tb) infects approximately a third of the world's population, but the majority of those infected will remain asymptomatic and can control the infection. However, 5-10% of individuals with latent TB infection have a danger of reactivation and will develop the active TB disease. HIV has been an important risk factor contributing to the reactivation of latent TB (Corbett *et al.*, 2003; Glynn *et al.*, 2008; Sonnenberg *et al.*, 2005). Of the 34 million people living with HIV worldwide, those individuals infected with latent TB are 21-34 times more likely to develop active TB disease than those without HIV (WHO, 2013b), and 75% of the HIV positive TB cases are present in Africa (WHO, 2013a; **Figure 1.1**).

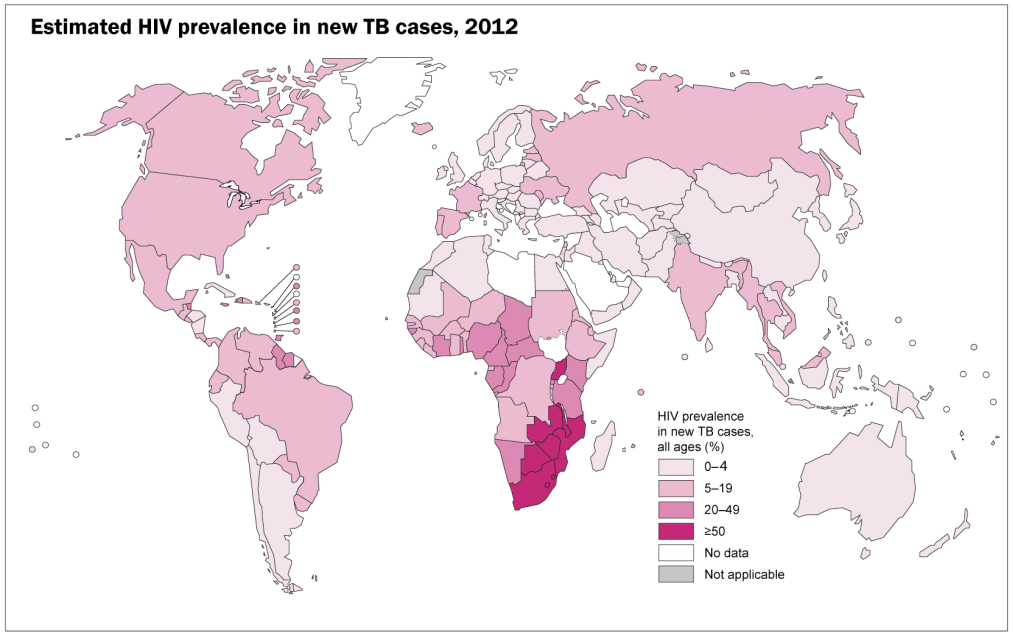

Figure 1.1: Estimated HIV prevalence in individuals newly infected with TB. This map shows the global overview of HIV positive TB cases. In parts of southern Africa, more than 50% of TB cases are co-infected with HIV. (Taken from Global Tuberculosis Report 2013- WHO, 2013a).

These alarming trends make it important to understand the mechanisms that lead from latent TB infection to active TB disease in HIV-infected individuals, which may give insights into combating the disease. HIV infection impairs the ability of the host to control M.tb infection by affecting the innate and adaptive immune system (as reviewed by Walker *et al.*, 2013; Diedrich & Flynn, 2011). Many studies have focused on how CD4 T cell depletion by HIV compromises the adaptive immune response to M.tb. Comparatively less is known regarding the effects of HIV on innate immunity to M.tb. The focus of this thesis is innate immune dysfunction during HIV infection and the consequences for TB. This chapter discusses the innate immune defects caused by HIV infection that may lead to the increased susceptibility of HIV-infected individuals to develop TB. It specifically focuses on how HIV affects the Toll-like receptor (TLR) functioning of innate cells and how this may impact the ability of these cells to respond to M.tb.

1.2 *Mycobacterium tuberculosis* (M.tb) infection

TB infection is caused by the intracellular pathogen M.tb, which is transmitted through the respiratory route (as reviewed by Philips & Ernst, 2012). The infection is initiated by the inhalation of aerosol droplets containing a few bacilli. Once these bacilli reach the lungs, the host mounts an immune response to the pathogen. Classically, TB was understood to exist as either an active disease or latent infection. However, growing evidence suggests a new paradigm of TB infection where latent TB exists as a heterogeneous spectrum of clinical states (as reviewed by Lin & Flynn, 2010). On one side of the spectrum, M.tb infection can be cleared through an effective innate immune response without the need for T cell priming, and on the other end, active disease develops due to a failure of innate and/or adaptive immunity to M.tb. In between the two ends exists the latent state, where a range of immune response can control M.tb to different degrees (as reviewed by Walker *et al.*, 2013). Individuals remain latently infected and are susceptible to disease reactivation when their immune system is compromised, as observed during HIV infection. In the following section, components of the immune response that are essential for controlling M.tb infection will be reviewed.

1.3 Immune response to M.tb

The immune response to M.tb consists of the innate and the adaptive arm, that work in concert to bring about an appropriate host response to M.tb (as reviewed by Hoebe *et al.*, 2004). However, the exact immune mechanisms mediated against this pathogen are not completely understood. Various innate immune cells and mediators are involved, and together with the cellular adaptive response, ensure that an effective response is mounted against M.tb. Since this study focuses on how the innate immune response to M.tb is affected by HIV, this section will mostly focus on this aspect of immunity to TB, followed by a brief overview of how adaptive immunity is also involved.

1.3.1 Innate immunity to M.tb

Innate immune cells form the first line of defense against M.tb infection and play an important role in immune recognition of M.tb. The innate immune response is initiated by pattern recognition of microbial structures called pathogen-associated molecular patterns (PAMPs; as reviewed by Akira e*t al.*, 2001). This is mediated by germline-encoded receptors referred to as pattern recognition receptors (PRRs) that are expressed by immune cells. Upon recognition of M.tb cell wall components, a series of signaling pathways become activated, that result into the release of various pro-inflammatory mediators that are important to mount an effective immune response to M.tb.

1.3.2 Role of innate immune cells in M.tb infection

The first cells in the airways and lungs that encounter M.tb are alveolar macrophages (AMs), which are central in innate pulmonary defense, being the major cell type responsible for killing M.tb (as reviewed by Sasindran & Torrelles, 2011). They phagocytose M.tb through receptors such as the, mannose, complement and scavenger receptors (as reviewed by Ernst, 1998; Hirsch *et al.*, 1994). These cells are also central to lymphocyte recruitment and activation and in orchestrating the granulomatous response to contain M.tb (as reviewed by Urdahl *et al.*, 2011). However, AMs are also the target cell for M.tb, and can support the replication and long-term persistence of bacteria, as M.tb can interfere with antimicrobial mechanisms of macrophages such as phagolysosome fusion and production of reactive nitrogen intermediates (as reviewed by

Flynn & Chan, 2003 and Pieters, 2008). Recent evidence showed that neutrophils are also an important target of M.tb during active pulmonary TB (Eum *et al.*, 2010). Mycobacteria that escape the destruction by host phagocytes replicate and lead to the disruption of the function of these innate cells.

Detection of M.tb by the PRRs on macrophages induces a local pro-inflammatory response that leads to the recruitment of more macrophages and other innate cells such as neutrophils, dendritic cells (DC) and monocytes to the site of infection to mount an immediate response to clear the pathogen. The monocytes differentiate into macrophages that also ingest M.tb (as reviewed by Crevel *et al.*, 2002). These cells accumulate at the site of infection to establish granulomas, which is the hallmark of M.tb infection (as reviewed by Ernst, 2012; **Figure 1.2**). DCs can phagocytose M.tb and migrate to the draining lymph nodes where they prime naïve T cells to initiate the adaptive immune response (as reviewed by Mortellaro *et al.*, 2009). The granuloma helps to contain and inhibit M.tb growth by allowing antigen-specific T cells to activate the infected macrophages (as reviewed by Saunders & Cooper, 2000 and Russell, 2007). However, granulomas can also lead to tissue destruction where they form caseous necrotic centres, and these can liquefy and erode into the bronchus, resulting in active TB disease and spreading M.tb into the environment (as reviewed by de Chastellier, 2008).

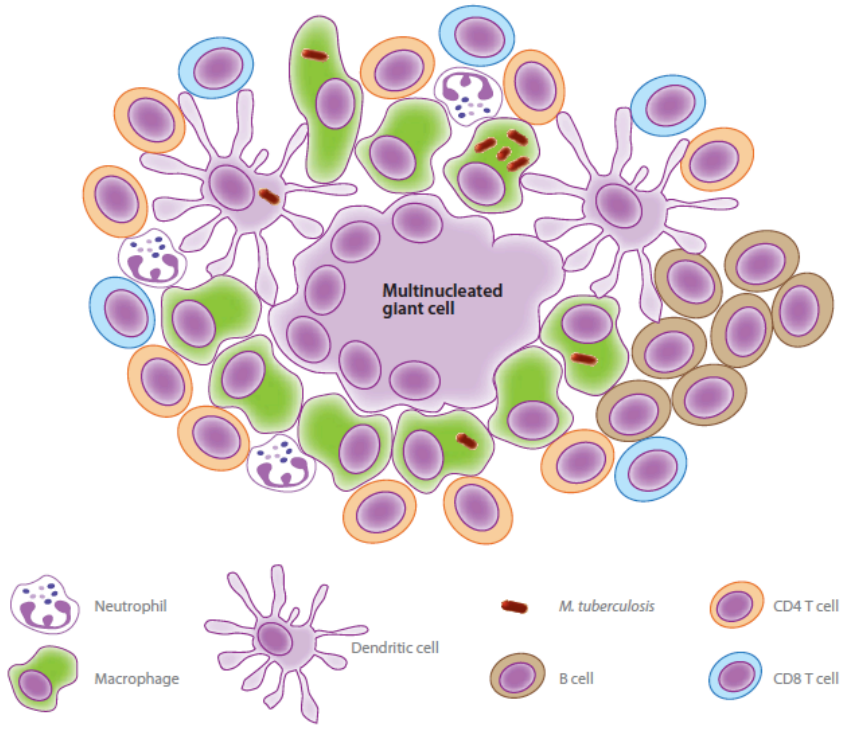

Figure 1.2: The cellular composition of a granuloma. Phagocytosis of M.tb by alveolar macrophages induces a local pro-inflammatory response that leads to the recruitment of more macrophages and other innate cells such as neutrophils and dendritic cells to the site of infection. These cells accumulate together to establish granulomas. Lymphocytes also populate the granulomas after the adaptive immune response is initiated. Granuloma macrophages can fuse together to form multinucleated giant cells as depicted in the diagram (Taken from Philips & Ernst, 2012).

1.3.3 Recognition of M.tb by pattern recognition receptors (PRRs)

The innate immune receptors play a role in the early recognition of pathogens and triggering an innate immune response. Macrophages and monocytes express a wide range of receptors that recognise diverse microbial ligands. M.tb has a complex cell wall which can lead to the simultaneous interaction and activation of a range of receptors found on the macrophage cell surface, in the phagosome and in the cytosol (as reviewed by Ernst, 1998 and Kawai & Akira, 2011; **Figure 1.3**). These include receptors such as TLRs, immunoglobulin Fc receptors (FcR), C-type lectin receptors (CLRs) and nucleotide oligomerisation domain (NOD)-like receptors (NLRs; Kang *et al.*, 2005; Divangahi *et al.*, 2008; Ferwerda *et al.*, 2005). The CLRs include among others the dendritic cell specific intercellular adhesion molecule-3-grabbing non-integrin (DC-

SIGN) and the mannose receptor (MR). Some of these receptors activate macrophages to release cytokines, chemokines and other antimicrobial factors for containing or killing M.tb and inducing adaptive immune responses (as reviewed by Kleinnijenhuis *et al.*, 2011). Other receptors impair activation and allow the entry of M.tb into the cell. This review will focus on the role TLRs play in the innate immune response to M.tb.

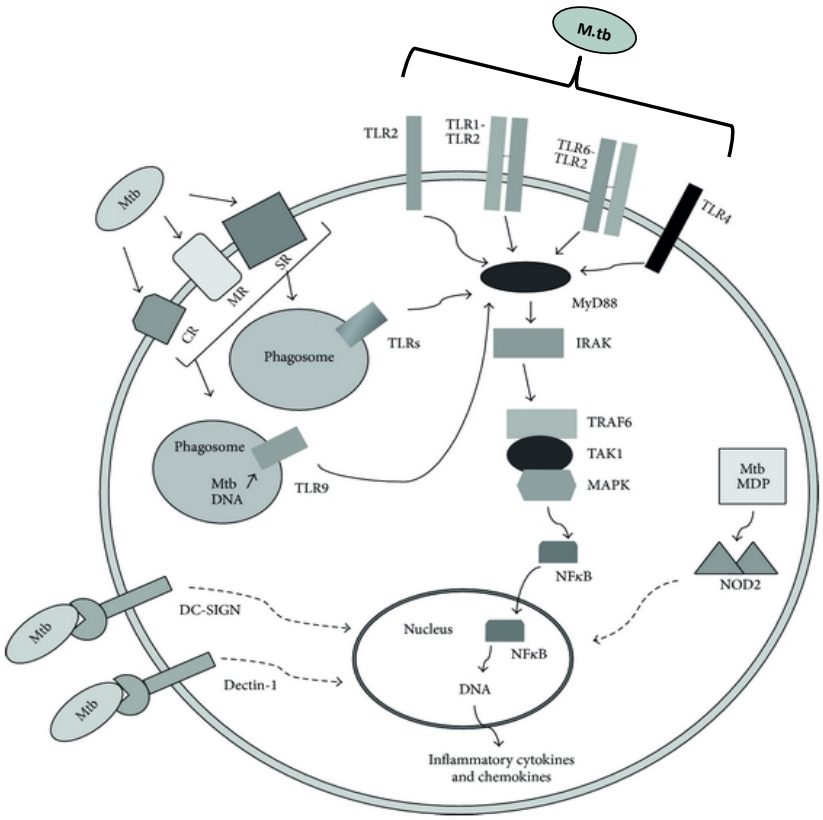

Figure 1.3: Immune recognition of M.tb by pattern recognition receptors. Mycobacteria can be recognised by various pattern recognition receptors (PRRs) found on the cell surface, in the phagosome and in the cytosol. The Toll-like receptors (TLRs) involved in recognition of mycobacterial components include TLR2, TLR1/2, TLR2/6, TLR4 and TLR9. Upon binding of an M.tb ligand to the TLRs, adaptor molecule MyD88, gets recruited and plays a role in initiating signaling pathways that lead to the production of a range of pro-inflammatory cytokines and chemokines. The complement receptors (CRs), mannose receptors (MRs) and scavenger receptors (SRs) are mainly involved in the uptake of M.tb while the cytosolic receptor NOD2 interacts with M.tb derived peptidoglycan component muramyl dipepetide (M.tb-MDP). Dashed arrow signifies various other signaling molecules involved (Figure adapted from Hossain & Norazmi, 2013).

1.3.3.1 Toll-like receptors involved in M.tb recognition

TLRs are type 1 membrane proteins that have an extracellular domain of leucine-rich repeats and a cytoplasmic Toll/IL-1 receptor (TIR) domain (as reviewed by Quesniaux *et al.*, 2004). Upon binding of an M.tb ligand to the TLRs, adaptor molecules are recruited which trigger downstream signaling cascades that lead to the production of a range of pro-inflammatory cytokines and chemokines (as reviewed by Akira *et al.*, 2001). During M.tb infection, the adaptor protein myeloid differentiation primary response protein 88 (MyD88) plays a prominent role in initiating signaling pathways by binding to the TIR domain. Subsequently, this results in the phosphorylation and activation of downstream molecules such as IL-1 receptor-associated kinases (IRAK), TNF receptor-associated factor 6 (TRAF6), TGFβ-activated protein kinase 1 (TAK1), and mitogen-activated protein (MAP) kinase (as reviewed by Akira & Takeda, 2004; **Figure 1.3**). This ultimately leads to the nuclear translocation of the transcription factor NF-kβ and transcriptional activation of cytokine genes such as TNF-α, IL-1β, IL-6 and IL-12 (as reviewed by Akira *et al.*, 2001) that are crucial to eliciting adaptive immunity to M.tb. Hence, TLR activation serves as a fundamental link between the innate and adaptive immune defense against M.tb and other microbial pathogens.

Innate cells express an array of TLRs, which allow them to sense pathogens, release pro-inflammatory mediators, and trigger innate and adaptive immune responses to infection (Muzio *et al.*, 2000). Among the TLR family, TLR1, 2, 4, 6 and 9 can sense M.tb, and have been implicated in the susceptibility or control of M.tb from genetic association studies and mouse models (as reviewed by Casanova & Abel, 2002; Doherty & Arditi, 2004; Stenger & Modlin, 2002; Krutzik & Modlin, 2004). TLR2 can recognise a range of mycobacterial structures such as M.tb hsp65, M.tb hsp70, 19kDa lipoprotein, lipoarabinomannan (LAM), components of purified protein derivative (PPD) and phosphatidylinositol mannoside (PIM; as reviewed by Doherty & Arditi, 2004). TLR2 forms heterodimers with TLR1 and TLR6 and can bind specific microbial products (as reviewed by Stenger & Modlin, 2002 and Krutzik & Modlin, 2004). TLR1/2 recognises triacylated lipoproteins (Kleinnijenhuis *et al.*, 2011; Takeuchi *et al.*, 2002) while TLR2/6 recognises the PIM component of soluble tuberculosis factor (STF; Bulut *et al.*,

2001). TLR4 recognises a heat labile ligand of mycobacteria (Means *et al.*, 1999a), while M.tb DNA containing unmethylated CpG motifs is sensed by TLR9 (as reviewed by Doherty & Arditi, 2004).

The outcome of the initial encounter between M.tb and these receptors on macrophage/monocytes may ultimately determine the outcome of infection, namely clearance, latency or disease. The development of disease may also represent a fine-tuned balance between protective and pathological immune responses (as reviewed by Dorhoi *et al.*, 2011).

1.3.3.2 CD14 and TLR signaling

TLRs also serve as transmembrane signal transducing protein for the CD14 receptor, a membrane glycoprotein found on myeloid cells which serves as an LPS-binding receptor (as reviewed by Landmann *et al.*, 2000). It exists as membrane-bound form (mCD14) as well as a soluble form (sCD14; Wright *et al.*, 1990). The soluble form plays a role in signal activation in cells that lack CD14, such as endothelial and epithelial cells (Yu *et al.*, 1998). Plasma LPS-binding protein (LBP), a soluble serum protein, binds to LPS and forms a complex with the CD14 receptor, leading to cellular activation. CD14 is a glycophosphatidylinositol-linked protein and lacks the transmembrane and intracellular domains, and therefore cannot induce signals on its own (as reviewed by Triantafilou & Triantafilou, 2002). Thus, TLRs transduce signals for the CD14 receptor, with TLR4 required for the signaling of LPS. Besides LPS, CD14 also acts as a receptor for recognition of other bacterial components, such as LAM from mycobacteria (Savedra *et al.*,1996; Pugin *et al.*, 1994). CD14-mediated signaling by LAM and LPS requires TLR2 and TLR4, respectively (Means *et al.*, 1999b). Hence, distinct CD14 ligands require different TLR proteins for intracellular signaling.

Several mediators can regulate and alter CD14 synthesis and expression on myeloid cells upon stimulation. Membrane-bound CD14 is shed from the cell surface to the soluble form following activation of monocytes (Bazil & Strominger, 1991). Upon LPS stimulation *in vitro*, CD14 expression on monocytes was found to be decreased after 3-6

hours (Landmann *et al.*, 1996). At this point, CD14 mRNA was down regulated and an increase in sCD14 was observed. Additional studies demonstrated an increase in serum sCD14 levels and decreased CD14 expression on peripheral monocytes during sepsis and other disorders (Burgmann *et al.*, 1996; Landmann *et al.*, 1995; Oesterreicher *et al.*, 1995). Higher sCD14 levels have also been reported in HIV-infected individuals, which correlated with disease progression (Lien *et al.*, 1998). This issue is discussed in more detail in section 1.5.2.3.

1.3.4 Cytokines and chemokines involved in M.tb infection

M.tb recognition by phagocytic cells leads to the activation of genes that encode various pro- and anti-inflammatory cytokines (Ragno *et al.*, 2001), which recruit cells such as T cells, neutrophils, monocytes and natural killer (NK) cells to the site of infection, leading to activation of these cells (as reviewed by Cooper *et al.*, 2011; Berrington & Hawn, 2007; Flynn & Chan, 2001; Crevel *et al.*, 2002). The cytokines involved in M.tb infection include the pro-inflammatory cytokines TNF-α (Flynn *et al.*, 1995), IL-1β (Yamada *et al.*, 2000), IL-6 (Ladel *et al.*, 1997), the anti-inflammatory cytokines IL-10 (North, 1998), the Th1 cytokine IFN-γ (Flynn *et al.*, 1993) and the Th1-inducing cytokine IL-12 (Khader *et al.*, 2006). The formation of the granuloma is shaped by the induction of these cytokines, which initiate the adaptive immune response to M.tb.

TNF-α is produced by monocytes and macrophages exposed to microbial products from M.tb (as reviewed by Berrington & Hawn, 2007). It activates macrophages to kill intracellular M.tb and plays a role in the formation of the granuloma (Kaneko *et al.*, 1999; Roach *et al.*, 2002). Mice deficient in TNF-α or TNF-α receptor had an increased susceptibility to TB, and granuloma formation was impaired (Flynn *et al.*, 1995). Patients using TNF-α inhibitors were more susceptible to TB disease, indicating that TNF-α plays an important role in host defense to M.tb (Keane *et al.*, 2001). However, excessive TNF-α production can be detrimental and can cause immunopathology (Jacobs *et al.*, 2007).

IL-1β is another pro-inflammatory cytokine that is first produced in the cell as an inactive precursor form (pro-IL-1β) (as reviewed by Sims & Smith, 2010). This gets cleaved to an active form upon stimulation, which is then secreted from the cell. Stimulation with M.tb ligands triggers the conversion of pro-IL-1β to IL-1β (as reviewed by Berrington & Hawn, 2007). This cytokine acts through the IL-1R type 1 receptor that activates the production of other pro-inflammatory cytokines (as reviewed by Sims & Smith, 2010). Mice deficient in IL-1β and IL-1α were not able to clear M.tb infection and formed larger granulomas than wild type mice (Yamada *et al.*, 2000).

M.tb infection also induces the production of IL-6, which is a potent cytokine that plays a role during the early innate response to M.tb before adaptive T cell immunity has fully developed (as reviewed by Berrington & Hawn, 2007). In the absence of IL-6, mice had an increased M.tb burden in the lungs and decreased IFN-γ production compared to wild type controls (Ladel *et al.*, 1997). As for TNF-α, IL-6 may also be harmful, with a role in suppressing T cell responses having been reported (Vanheyningen *et al.*, 1997).

IL-12 production in M.tb infection promotes the differentiation of naïve T cells into Th1 cells by inducing production of IFN-γ (as reviewed by Berrington & Hawn, 2007). Mice that were deficient in IL-12 had increased susceptibility to M.tb infection (Cooper *et al.*, 1997). IL-10 is an immunoregulatory cytokine that suppresses the pro-inflammatory response and plays a regulatory role in many infections (de Waal Malefyt *et al.*, 1991; as reviewed by Saraiva & O'Garra, 2010). Studies in mice revealed that IL-10 promoted TB disease progression and can promote reactivation of TB (Beamer *et al.*, 2008; Turner *et al.*, 2002). In humans, polymorphisms in the promoter region of IL-10 gene were associated with enhanced production of IL-10 from T cells and monocytes, and these polymorphisms have been shown to lead to increased susceptibility to TB (Awomoyi *et al.*, 2002; as reviewed by Berrington & Hawn, 2007 and Mege *et al.*, 2006; Turner *et al.*, 1997).

IFN-γ is a crucial cytokine of the Th1 cell response for effective control of M.tb (as reviewed by Berrington & Hawn, 2007). It is secreted primarily by CD4+ T cells, CD8+ T cells and NK cells, and activates macrophages to release cytokines. In the absence of IFN-γ, mice showed disseminated mycobacterial infection and defective macrophage activation (Cooper *et al.*, 1993; Flynn *et al.*, 1993). In humans, individuals with mutations in IFN-γ receptor genes are more susceptible to infection with M.tb (as reviewed by Ottenhoff *et al.*, 1998).

In addition, many other cytokines and mediators have also been implicated in the response to M.tb, such as IL-17 (Lockhart *et al.*, 2006; Yoshida *et al.*, 2010), granulysin (Dieli *et al.*, 2001; Stenger *et al.*, 1999) and vitamin D (Liu *et al.*, 2007; Nnoaham & Clarke, 2008) amongst others, and may play important roles in influencing the outcome of M.tb infection.

1.3.5 Adaptive immunity to M.tb

The best-characterised adaptive response to M.tb is the Th1 immune response, that involves CD4+ T cells, CD8+ T cells and cytokines, with CD4+ T cells considered to be central for host defense (as reviewed by O'Garra *et al.*, 2013). Memory T cells recruited to the lung are stimulated by M.tb antigens presented by macrophages and exert their protective effect through the production of cytokines such as IFN-γ, that induce effector immune responses in macrophages which help to contain the infection (Cooper *et al.*, 1993). IFN-γ and TNF-α stimulate antimicrobial functions in infected macrophages, leading to intracellular bacterial killing through phagolysosome formation and production of reactive nitrogen and oxygen intermediates (as reviewed by O'Garra *et al.*, 2013). M.tb-specific CD8+ T cells also produce cytokines and can lyse infected macrophages (as reviewed by Lazarevic & Flynn, 2002 and Woodworth & Behar, 2006; Lewinsohn *et al.*, 2003). The cytokines and chemokines released during infection and the host-pathogen interactions will determine whether infection is cleared, controlled or whether active TB disease develops (as reviewed by Cooper *et al.*, 2011).

1.4 Monocytes

The studies in this thesis specifically focus on monocytes, the precursors of macrophages, and how HIV affects TLR functioning in these cells. Hence, this section gives a brief introduction to these innate cells, that will be further elaborated on in the subsequent section in the context of HIV infection.

Monocytes are key innate cells that represent approximately 5-10% of peripheral blood leukocytes in humans (as reviewed by Tacke & Randolph, 2006). They originate in the bone marrow from myeloid precursors and are then released into the blood where they circulate with a half-life of about three days. Monocytes migrate and enter tissues and differentiate into antigen presenting cells (APCs) such as macrophages and myeloid DCs (as reviewed by Auffray *et al.*, 2009; Krutzik *et al.*, 2005). The recruitment of monocytes to sites of infection is facilitated by chemokines such as monocyte chemoattractant protein 1 (MCP-1), which is recognised by CCR2, expressed on monocytes. Non-haematopoietic bone marrow cells are able to detect TLR ligands in the bloodstream during infections and express MCP-1, thereby promoting the entrance of monocytes into the circulation (Shi *et al.*, 2011).

The TLRs expressed on monocytes include TLR1, 2, 4, 5, 6, 8 and 9 (Muzio *et al.*, 2000; O'Mahony *et al.*, 2008; Juarez *et al.*, 2010; Hornung *et al.*, 2002). Dysregulation of pathogen sensing by monocytes may have serious consequences for acquisition of opportunistic and other infections, and development of disease. Human blood monocytes form a heterogeneous group of cells, and three different subsets have been described based on the expression of cell surface markers CD14 and CD16 (the latter, a low affinity receptor for IgG; as reviewed by Ziegler-Heitbrock *et al.*, 2010). The CD14++CD16- monocyte population is referred to as 'classical' monocytes and these are the most prevalent subset in blood (as reviewed by Ziegler-Heitbrock, 2000). This subset maintains tissue macrophages and DC populations. The CD16+ monocyte population represents only 5-10% of the total circulating monocytes and comprise the CD14++CD16+ intermediate subset and the CD14+CD16++ non-classical monocyte subset. These different subsets of monocytes appear to display distinct functions. The

non-classical subset has been shown to have patrolling behavior *in vivo*; they survey the endothelium for signs of inflammation or damage (Cros *et al.*, 2010). The intermediate CD14++CD16+ subset is characterised as being 'pro-inflammatory', due to enhanced production of cytokines (Frankenberger *et al.*, 1996). This subset has higher MHC class II expression and greater TNF-α production upon stimulation with LPS or TLR2 ligand, Pam3Cys (Belge *et al.,* 2002). The CD16+ monocytes are increased in frequency in various diseases such as rheumatoid arthritis, atherosclerosis, sepsis, TB and HIV infections, amongst others (as reviewed by Wong *et al.*, 2012 and Ziegler-Heitbrock, 2007).

1.5 Effect of HIV on the immune response to M.tb

HIV-infected persons are more susceptible to TB disease, and the reasons for this are not fully understood (as reviewed by Diedrich & Flynn, 2011). Evidence from human and animal studies have identified aspects of the immune response to M.tb that are targeted by HIV. Many studies on HIV/TB co-infection have focused on how HIV affects adaptive immune responses to M.tb, and have described defects in M.tb-specific T cell responses. There were fewer M.tb specific memory CD4 T cells in the peripheral blood of HIV-infected individuals with latent TB infection, indicating that M.tb-specific CD4+ T cells are depleted early in HIV infection (Geldmacher *et al.*, 2008, 2010). A recent study also demonstrated fewer M.tb-specific CD4+ T cells in the lungs in advanced HIV infection (Jambo *et al.*, 2011). Most studies have focused on immune responses to M.tb in the blood. However, a better understanding of the effect of HIV on immunity in the lungs, the site of TB disease, is required.

In addition to targeting the adaptive immunity, HIV also leads to dysfunction of innate responses to M.tb. The following section summarises the literature surrounding the defects in innate immunity during HIV infection, specifically focusing on TLR functioning of monocytes and macrophages.

1.5.1 Alveolar macrophage function

AMs are the first cells in the lung to encounter M.tb, and play a central role in innate immunity to M.tb (as reviewed by Kleinnijenhuis *et al.*, 2011). This section briefly highlights studies that have examined defects in AM function during HIV infection, which may lead to a deficit in innate immunity and result in the increased susceptibility to TB in HIV-infected individuals.

An impairment in TNF-α production from AM has been observed during HIV infection or after infecting macrophages with HIV *in vitro*. Decreased secretion of TNF-α from AM in HIV-infected patients on highly active antiretroviral therapy (HAART) was observed in response to both TLR2 and TLR4 stimulation, compared to healthy controls (Nicol *et al.*, 2008). Decreased expression in TLR1, TLR2 (and to some extent TLR4) genes in the AM of HIV-infected patients was also observed, which may explain the decreased cytokine production (Nicol *et al.*, 2008). Furthermore, impaired TLR4-mediated MyD88-dependent signaling was present in AM from HIV-infected individuals (Tachado *et al.*, 2010). *In vitro* infection of AM with HIV, reduced M.tb-induced apoptosis of macrophages by inducing IL-10, which in turn reduced TNF-α production (Patel *et al.*, 2007). Another study showed that HIV Nef protein inhibited activation of the TNF-α promoter and affected the stability of TNF-α mRNA, which led to a reduced M.tb-induced apoptosis of macrophages (Kumawat e*t al.*, 2010). Moreover, direct HIV infection of macrophages infected with M.tb also decreased their ability to acidify vesicles (Mwandumba *et al.*, 2004). It is likely that there may be both indirect effects of HIV infection, as well as direct affects in HIV-infected macrophages, on their functioning.

HIV infection of innate cells can alter the manner in which these cells respond to M.tb. Some *in vitro* studies demonstrated that HIV-infection of monocyte-derived macrophages (MDM) promoted M.tb growth and reduced the viability of macrophages (Imperiali *et al.*, 2001; Pathak *et al.*, 2010). Also, in these HIV/M.tb co-infected cultures, viral replication was enhanced and higher levels of pro-inflammatory cytokines was observed. It was recently shown that HIV-1 Nef activates the TRAF pathway by

interacting with TRAF2, TRAF5 and TRAF6, and favors the replication of HIV in macrophages (Khan *et al.*, 2013). In addition, recent evidence demonstrates that HIV can evade recognition and sensing by innate receptors and favors its replication in macrophages through recruitment of the cofactors cleavage and polyadenylation specificity factor subunit 6 (CPSF6) and cyclophilins (Rasaiyaah *et al.*, 2013). However, it is important to note that very few macrophages get directly infected with HIV *in vivo*, and it may be more likely that HIV leads to dysfunction of these cells indirectly as a result of the inflammatory environment of the lung in HIV co-infection (as reviewed by Twigg & Knox, 2007 and Borrow, 2011). In fact, it has been estimated that as few as 3 per 100,000 AMs contain integrated HIV-1 DNA (Collini *et al.*, 2010; Lewin *et al.*, 1998). HIV is frequently detected in the lungs of infected individuals and can replicate to high levels, however it is likely that the major cellular source of HIV in the lungs are the T cells and not the AMs (Wood *et al.*, 2003; Brenchley *et al.*, 2008; Twigg *et al.*, 2008).

Studying the function of AMs in the context of HIV infection is extremely relevant to understanding not only the effect on the control of M.tb but also other bacterial pathogens. Ongoing studies in our laboratory are examining the effects of HIV co-infection on transcriptional profiles of AMs. The study presented in this thesis examined the functioning of blood monocytes from HIV-infected and uninfected individuals, thus the remainder of this review will be centered on monocytes.

1.5.2 Monocyte function

Monocytes form a heterogeneous group of cells and play an important role in the defense against M.tb (as reviewed by Fenton & Vermeulen, 1996). Their innate ability to deal with M.tb determines the outcome of the infection. A few previous studies have demonstrated that monocyte function is altered following HIV infection, and this may contribute to defects in the ability to respond to co-infections (as reviewed by Noursadeghi *et al.*, 2006), such as M.tb.

Some mechanisms by which HIV infection may alter monocyte (or macrophage) function to the advantage of M.tb include reduced oxidative burst capacity (Spear *et al.*, 1990; Muller *et al.*, 1990), diminished oxygen radical production (Dobmeyer *et al.*, 1995), downregulation of receptors for chemotactic ligands that are important for monocyte recruitment to site of infection (Wahl *et al.*, 1989), and dysregulation of cytokine production such as IL-1β, IL-6 and TNF-α (Tilton *et al.*, 2006; Lathey *et al.*, 1994).

These studies highlight the interaction between HIV and monocytes, leading to functional impairment in their role as innate cells of the immune system. As for macrophages, direct effects of HIV infection of monocytes on their function have been studied, as well as indirect effects affecting their phenotype and functioning which may compromise the innate immune response to M.tb

1.5.2.1 HIV infection of monocytes

Monocytes are susceptible targets for HIV infection, and function as a reservoir for persisting virus even during antiretroviral therapy (as reviewed by Coleman & Wu, 2009; Sonza *et al.*, 2001). However, they are likely to form only a small reservoir of actively replicating virus compared to memory CD4+ T cells, since a very small fraction of the monocyte population becomes productively infected. HIV replication appears to be restricted in most monocytes due to multiple host factors that hinder viral replication, such as high levels of apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3A and 3G (APOBEC3A, APOBEC3G), amongst others (as reviewed by Bergamaschi & Pancino, 2010). Estimates show that <0.1% of monocytes contain HIV-1 DNA *in vivo* and this persists despite HAART (Collini *et al.*, 2010; Sonza *et al.*, 2001). Since the half-life of monocytes is approximately 3 days *in vivo* (before they differentiate into macrophages or DCs in tissues), the HIV detected from blood monocytes implies an ongoing renewal of infection in these cells (Zhu, 2002). Infection of bone marrow hematopoietic progenitor cells with HIV may keep renewing the viral pool by passing the virus to the progeny monocytes (as reviewed by Alexaki & Wigdahl, 2008).

Certain subsets of monocytes may be more permissive to infection than others. Several studies have revealed that the CD16+ monocytes are more permissive to HIV infection due to higher expression of CD4 and CCR5, the cell entry receptors for HIV, and lower restrictive antiviral activity within these cells (Ellery *et al.*, 2007; Han *et al.*, 2009). *In vitro* experiments demonstrated that the CD16+ monocyte subset were more permissive to HIV infection compared to other monocyte subsets (Ellery *et al.*, 2007), with enhanced viral entry and replication in this subset. Furthermore, HIV DNA was detected more readily in the CD16+ monocytes compared with the CD14++CD16- classical monocytes isolated from HIV-infected individuals on HAART. However, compared to memory CD4+ T cells, the HIV DNA copy number in CD16+ monocytes was 2-fold lower, suggesting that monocytes make up a very tiny reservoir for HIV and their contribution to the total viral pool is small. However, monocytes may serve to transmit the virus to other susceptible cells such as CD4+ T cells when they migrate into tissues (Lambotte *et al.*, 2000; Sonza *et al.*, 2001; Zhu *et al.*, 2002).

1.5.2.2 Role of CD16+ monocytes in TB and HIV infection

Studies in HIV-infected individuals and non-human primate models have revealed significant shifts in the different monocyte subsets. The CD14++CD16+ subset expands following HIV infection (Thieblemont *et al.*, 1995), and a greater frequency of these cells is associated with higher plasma HIV viral loads (Kim *et al.*, 2010; Han *et al.*, 2009). An increase in CD16+ monocytes has also been reported in TB patients, and this was associated with severity of TB disease and higher TNF-α plasma levels (Balboa *et al.*, 2011). This subset was less able to differentiate into DCs *in vitro* and instead differentiated into DCs that lacked CD1a and DC-SIGN (Balboa *et al.*, 2013). Since monocytes are essential sources of APCs, HIV may interfere with their ability to differentiate to DCs, which could in turn compromise the development of effective and timely adaptive responses to co-infections such as M.tb.

In HIV-infection, higher levels of IL-23 production by CD16+ monocytes were observed upon *in vitro* stimulation of PBMCs with *Escherichia coli* compared to HIV-uninfected individuals (Manuzak *et al.*, 2013). In addition, the frequency of CD16+

TLR4+ monocytes and the expression levels of TLR4 expressed on CD16+ monocytes were higher in HIV infection compared to healthy controls, which may be responsible for the enhanced IL-23 production. Thus, the stimulation of monocytes by microbial products and HIV *in vivo,* may drive these cells to produce IL-23, a heterodimer comprised of IL-12p40 and p19 subunits and activates similar signaling pathways to IL-12 (as reviewed by Lankford & Frucht, 2003). This cytokine functions in the expansion and activation of Th17 cells. These cells have been shown to play an important role in M.tb infection and in maintaining the granuloma structure (as reviewed by Khader & Cooper, 2008). However, excessive Th17 responses may lead to enhanced IL-17 production that can cause tissue damage and have detrimental effects in TB disease (Cruz *et al.*, 2010; as reviewed by Torrado & Cooper, 2010). Thus, the increased IL-23 production in HIV infection may impair the Th17 balance in TB co-infection.

In HIV infection, CCL2/MCP-1 (a pro-inflammatory chemokine produced by monocytes) may be one of the factors contributing to chronic inflammation and immunopathology in HIV (as reviewed by Ansari *et al.*, 2011). Recently, it was shown that there was a preferential expansion of the intermediate CD16+ monocyte subset with an increased production of pro-inflammatory chemokine CCL2 in HIV-infected individuals compared to healthy controls (Ansari *et al.*, 2013). In M.tb infection, CCL2/MCP1 leads to the recruitment of lymphocytes and monocytes to the lung that may cause increased inflammation (Lin *et al.*, 1998). Indeed, MCP-1 mRNA and protein were expressed at higher levels in CD14+ monocytes from patients with active TB compared to healthy individuals (Lin *et al.*, 1998). Furthermore, functional polymorphisms in the CCL2 promoter were associated with increased susceptibility to TB and these patients had elevated amounts of plasma CCL2 and lower IL-12 levels (Flores-Villanueva *et al.*, 2005). Hence, the increased monocyte-derived CCL2 during HIV infection may favour the development of TB.

Taken together, these data highlight the dramatic effect that HIV infection can have on monocyte phenotypic heterogeneity, which may alter the ability of these cells to respond to co-infections.

1.5.2.3 HIV and innate immune activation

A range of defects associated with pathogen sensing in monocytes have been described in HIV infection. These include altered TLR expression (Heggelund *et al.*, 2004; Hernandez *et al*., 2012), aberrant downstream signaling in response to TLR ligands, and changes in TLR ligand responsiveness (Lee *et al.*, 2008; Pathak *et al.*, 2009).

Immune hyperactivation is a characteristic immune pathology of HIV infection and is linked to disease progression and pathogenesis (as reviewed by Paiardini & Müller-Trutwin, 2013). The TLR pathway is thought to contribute to the persistent immune activation that is seen in individuals chronically infected with HIV. HIV encodes TLR7/8 ligands that can lead to direct activation of a range of immune cells (Chang *et al.*, 2012). Single-stranded (ss) RNA of HIV contains multiple uridine-rich sequences that directly stimulated TLR7 and TLR8 on monocytes and DCs to produce pro-inflammatory cytokines such as IL-6 and TNF-α in a MyD88-dependant manner (Meier *et al.*, 2007). This could result in the chronic production of pro-inflammatory cytokines and be a major contributor to the generalised immune activation observed in HIV infection.

Interestingly, pre-stimulation of monocytes with HIV TLR ligands *in vitro,* can enhance pro-inflammatory cytokine release from monocytes in response to other microbial TLRs, including TLR2 and TLR4 (Mureith *et al.,* 2010). The likely explanation for this is the significant cross talk that exists between TLRs, where stimulation of one TLR may enhance or dampen responses through another TLR (as reviewed by Trinchieri & Sher, 2007). Studies have shown that the phenomenon of cross-talk can occur between other TLRs too, such as TLR2, TLR3, TLR4, TLR7, TLR8 and TLR9 among others (Bagchi *et al.*, 2007; Mäkelä *et al.*, 2009; Napolitani *et al.*, 2005; Sato *et al.*, 2000). Thus, HIV infection may directly or indirectly modulate expression and function of a range of TLRs, and this could affect the innate sensing of pathogens such as M.tb **(Figure 1.4)**.

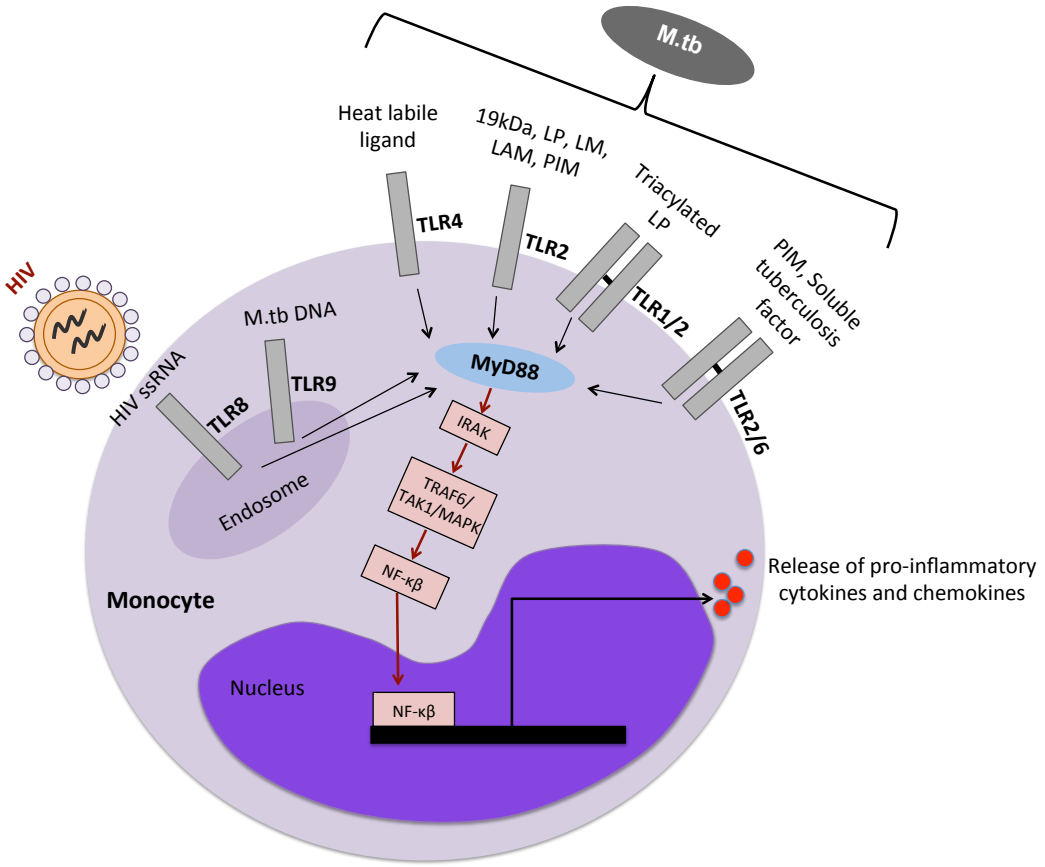

Figure 1.4: Recognition of M.tb components and HIV ligands by TLRs on monocytes. Single-stranded (ss) RNA of HIV contains multiple uridine-rich sequences that stimulate TLR8 on monocytes. HIV may have indirect effects on monocyte functioning, whereby sustained stimulation of HIV-responsive TLRs may influence how monocytes respond to other TLRs, in a process termed "TLR cross talk". Thus, HIV infection may indirectly modulate expression and function of a range of TLRs involved in the innate sensing of M.tb.

Monocytes from HIV-infected individuals showed increased expression of TLR2 (Heggelund *et al.*, 2004). In addition, *in vitro* stimulation of monocytes with HIV envelope protein gp120 increased the expression of TLR2 on these cells from HIV-infected patients. These findings were confirmed by another study that examined expression patterns of TLR2 and TLR4 on monocytes from HIV-infected individuals (Hernandez *et al.*, 2012). There was increased expression of TLR2 in monocytes from HIV-infected individuals compared to healthy controls, but no difference was observed in the expression pattern of TLR4 (Hernandez *et al.*, 2012). The increase in TLR2 expression was associated with higher plasma viral load in HIV-infected individuals. Moreover, *in vitro* HIV infection of MDMs from healthy donors resulted in an increased

expression of both TLR2 and TLR4 upon stimulation with TLR2 and TLR4 agonists, and this led to higher levels of IL-6 and IL-8 production compared to uninfected MDMs. Thus, the increase in TLR expression on monocytes during HIV infection can increase the pro-inflammatory cytokine production which can play a role in immune activation. In line with these data, circulating monocytes in HIV infection had a sustained TLR2 gene signature (increased expression of genes for pro-inflammatory cytokines and chemokines among others) compared to controls, suggesting that monocytes are constitutively activated in HIV infection (Gekonge *et al.*, 2012). This constitutively activated profile may affect the functioning of monocytes when they are recruited to tissues, or their differentiation into macrophages or DCs.

HIV has also been shown to affect TLR9 responses from monocytes. TLR9 is involved in the recognition of unmethylated CpG motifs in bacterial DNA (Hemmi *et al.*, 2000). Monocytes from HIV-infected individuals demonstrated impaired maturation (reduced induction of CD40 and CD83) and interferon α/β responses upon stimulation with CpG ODN (a TLR9 agonist) compared to monocytes from uninfected individuals, and the defects were related to plasma viral load (Jiang *et al.*, 2005). Hence, HIV can lead to TLR9 dysfunction and affect the innate immune function of monocytes to bacterial infections (as reviewed by Noursadeghi *et al.*, 2006), including M.tb infection.

A recent genome wide transcriptome analyses of monocytes showed that TLR signaling pathways are down-regulated in viremic HIV-infected individuals on HAART, as demonstrated by decreased TLR expression, reduced expression of cytokine genes and decreased expression of genes encoding MAPK, NF-kβ, JAK-STAT and IRF signaling cascades (Wu *et al.*, 2012). However, these pathways were up regulated in non-progressive antiretroviral therapy (ART) naïve patients, in line with the findings that also showed increased TLR expression on monocytes from HIV-infected individuals (also ART naïve) (Heggelund *et al.*, 2004; Hernandez *et al.*, 2012). In addition, dysregulation of TLR signaling involving IRAK-4 has been described, where it was found suppressed in HIV-infected THP-1 cells (Pathak *et al.*, 2009). Another study reported dysregulation of the JAK-STAT and MAPK signaling in HIV-infected pediatric

patients (Lee *et al.*, 2008). All together, these studies highlight the defects in TLR signaling pathways caused by HIV infection which may impair the ability of these innate cells to respond to pathogens such as M.tb.

Apart from HIV-encoded TLR ligands, it is hypothesised that microbial translocation through the gastrointestinal tract allows components of gut bacteria to enter the blood stream, and this may lead to activation of monocytes (Brenchley *et al.*, 2006a, 2006b). HIV leads to the depletion of CD4 T cells in the gut early in HIV infection (Brenchley *et al.*, 2004). This results in a compromised mucosal barrier that leads to an increase in translocation of microbial products into the systemic circulation, such as LPS and bacterial DNA that stimulates monocytes to produce cytokines via TLR4 and TLR9 (Brenchley *et al.*, 2006a; Jiang *et al.*, 2009). Higher levels of sCD14 were associated with the increased levels of plasma LPS (Brenchley *et al.*, 2006a).

Interestingly, monocyte activation has been linked to various non-AIDS co-morbidities in chronically HIV-infected individuals, such as dementia and cardiovascular disease (CVD) (as reviewed by Marchetti *et al.*, 2013). Recently, a study reported that increased IL-1β production from monocytes leads to high levels of IL-6 responses (a biomarker of CVD) in HIV-infected individuals on ART (Jalbert *et al.*, 2013). This may lead to systemic inflammation and increase the risk for all-cause mortality in these individuals. Importantly, plasma levels of sCD14 were associated with an increased risk of mortality in HIV infection (Sandler *et al.*, 2011).

The scavenger receptor CD163 is expressed by monocytes and macrophages and the receptor gets shed as sCD163 upon TLR2, TLR4 or TLR5 activation (Weaver *et al.*, 2006, 2007). Elevated levels of sCD163 were found in the plasma of HIV-infected individuals, which was positively correlated with plasma viral loads and negatively correlated with CD4+ T cell counts (Burdo *et al.*, 2011). In addition, the levels of sCD163 were positively correlated with the percentage of the intermediate CD14++CD16+ monocyte subset. Elevated levels of sCD163 have also been described in TB infection, and this was associated with increased mortality in active TB patients

(Knudsen *et al.*, 2005). Therefore, the high levels of sCD163 in HIV infection can have detrimental effects in M.tb co-infected individuals.

All together, the presence of HIV ligands, particularly ssRNA, a potent activator of TLR7/8, in the circulation during chronic infection, together with PAMPs from opportunistic pathogens that enter the host due to compromised immune barriers and translocation of microbial products from the gastrointestinal tract, may influence responses to other TLRs. This may alter monocyte function during co-infection with pathogens such as M.tb.

1.6 Aims and objectives of the thesis

This study sought to understand whether infection with HIV induced functional defects in blood monocytes, impairing their ability to respond to mycobacteria. The focus was on TLR functioning in monocytes, as these receptors are central in orchestrating a protective response to pathogens like M.tb. The specific objective was to examine whether infection with HIV altered TLR responsiveness and inflammatory cytokine secretion in response to stimulation with mycobacteria-derived TLR ligands.

The hypothesis was that co-infection with HIV may lead to defective TLR responses to mycobacterial TLR stimuli. The rationale behind this was that sustained stimulation of HIV-responsive TLR may influence how monocytes respond to mycobacteria-encoded TLR ligands.

Investigating how HIV infection affects innate immunity to M.tb may be important for understanding why HIV-infected individuals are more susceptible to TB. Much remains to be learnt about what constitutes a protective immune response to M.tb, and how this response fails during HIV infection. Understanding the functional defects associated with HIV infection may provide insights into how a protective immune response develops against M.tb.

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2.1 Study design

This was a cross-sectional study in which 20 HIV-infected individuals and 18 HIV-uninfected individuals were recruited, all with suspected latent TB infection (LTBI). Volunteers were recruited from the Ubuntu HIV-TB clinic in Khayelitsha, Cape Town for peripheral blood draw after a written informed consent was received from all participants. Exclusion criteria for the study included antiretroviral therapy use, pregnancy, age <18 years, recent TB disease (within five years prior to enrollment in this study), treatment for TB, and any other acute or chronic disease. Age and TB history of the participants were collected through a questionnaire. LTBI was diagnosed based on the following criteria: no signs or symptoms of active TB disease by a screening questionnaire, a positive IFN-γ release assay by a QuantiFERON® Gold In-Tube assay that measures M.tb-specific immunity, and no evidence of active TB by chest X-ray. Ethical approval for this study was obtained from the Research Ethics Committee of the Faculty of Health Sciences, University of Cape Town (Reference number 158/2010).

2.2 Determining clinical characteristics of study participants

2.2.1 QuantiFERON® Gold In-Tube assay

The QuantiFERON®-TB Gold In-Tube assay (Cellestis Laboratories, USA) is a whole blood test for cell-mediated immune responses to M.tb. Blood drawn from the participants was collected directly into QuantiFERON® tubes coated with M.tb antigens (ESAT-6, CFP-10 and TB 7.7), a mitogen positive control and negative control tubes. The tubes were incubated for 24 h at 37°C after which they were centrifuged for 7 min at 800 x g. The plasma was then collected and used to perform QuantiFERON® enzyme-linked immunosorbent assay (ELISA). A positive test was considered as ≥ 0.35IU/ml and ≥ 25% of the Nil value (negative control), whilst a negative result was defined as <0.35IU/ml and <25% of the Nil value. If the Nil value exceeded 8.0 IU/ml or if the Mitogen minus the Nil was less than 0.5IU/ml, the result was considered indeterminate and these individuals were not recruited to the study.

2.2.2 CD4 count determination

The absolute CD4 cell count from EDTA treated whole blood was determined by using the Flow-CARE^{TM} PLG CD4 test (Beckman Coulter, Ireland). Viral load and CD4 counts were performed by the National Health Laboratory Service (NHLS) (Groote Schuur Hospital). Only individuals with CD4 counts >400 cells/mm^3 were recruited into the study, since the aim was to study defects in response to mycobacteria before profound CD4 depletion.

2.2.3 Viral load determination

HIV viral load was quantified in the plasma of the HIV-infected participants by evaluating HIV RNA copies using an Abbott® m2000 RealTime HIV-1 assay (Abbott Laboratories, USA) with a detection limit of 40 copies/ml.

Plasma from whole blood was collected by Ficoll-Hypaque (Sigma-Aldrich, USA) density gradient centrifugation. This technique was used to isolate peripheral blood mononuclear cells (PBMC) from whole blood collected in acid dextrose (ACD) tubes from the participants of this study as part of another ongoing project in the lab. The method involves layering blood onto Ficoll, a hydrophilic polymer that produces a density gradient for the separation of cells and plasma. Upon centrifugation, erythrocytes and granulocytes aggregate and sediment, while mononuclear cells do not aggregate and form a layer between the plasma and Ficoll.

Briefly, 15 ml of Ficoll was added to a 50 ml Leucosep tube (Greiner Bio-one, Germany) containing a filter disc and centrifuged at 800 x g for 1 min to force the Ficoll to settle below the filter disc. About 30 ml of whole blood was then poured into the tube on top of the filter disc and centrifuged at 800 x g for 15 min. This separated the blood components, such that PBMC formed a white layer between the plasma and the Ficoll solution. The plasma formed the top most layer and was collected and stored as 2 ml aliquots in -80°C for determination of viral loads in HIV-infected individuals. Using a sterile pasteur pipette, the PBMC were carefully removed and washed twice with 1X PBS containing 1% FBS and counted to determine the cell number and viability. They were then cryopreserved and stored in liquid nitrogen for later use.

2.3 Blood collection and processing

Peripheral blood from participants was collected in sterile sodium heparin and ACD anti-coagulant vacutainer tubes (BD Biosciences, Plymouth, UK) and processed within 4 h of collection.

2.3.1 Whole blood assays

Blood collected in sodium heparin tubes were used immediately for whole blood assays optimised previously (Shey *et al.*, 2012). Whole blood (500 µl) from HIV-infected and uninfected individuals were stimulated with the TLR stimuli described below and incubated in a water bath at 37°C for 6 h, and after 3 h, the protein secretion inhibitor, Brefeldin A (BFA, Sigma-Aldrich, USA) was added at a final concentration of 10 µg/ml to inhibit cytokine release from the cells. Unstimulated cells were included as negative controls. Cells were then washed, stained with a viability dye ('Vivid', Molecular Probes, USA), fixed and cryopreserved in liquid nitrogen for batch staining at a later time point as described below.

2.3.2 Antigens

The antigens that were used for monocyte stimulations included mycobacteria-derived TLR stimuli, namely mycobacterial lipoarabinomannan (LAM, a TLR2 ligand), M.tb purified protein derivative (PPD, which stimulates TLR1, 2, 4 and 6), live Bacille Calmette Guérin (BCG, a stimuli for TLR1, 2, 4, 6 and 9) as well as the positive control, lipopolysaccharide (LPS, a TLR4 agonist from gram-negative bacteria). **Table 2.1** summarises the concentration of each antigen used.

Table 2.1: Summary of antigens used for monocyte stimulation

Antigen	Working Concentration	Supplier
LAM-MS	25µg/ml	Invivogen, USA
M.tb PPD	20µg/ml	Statens Serum Institute, Denmark
BCG	MOI* of 2	Statens Serum Institute, Denmark
LPS-SM	25ng/ml	Invivogen, USA

*MOI: Multiplicity of infection (Ratio of bacteria to target cells)

LAM is a lipoglycan found in the cell wall of mycobacteria (Briken *et al.*, 2004). The LAM used in this study was derived from the non-pathogenic *Mycobacterium smegmatis* (LAM-MS), which has phosphoinositol-capped LAM (PILAM) that activates TLR2 (Wieland *et al.*, 2004; Underhill *et al.*, 1999).

The **PPD** used in this study was a tuberculin preparation made from culture filtrates of M.tb (Batch RT50). The composition of PPD is not fully understood but it is a mixture of various M.tb antigens (Cho *et al.*, 2012). Recent proteomic and bioinformatics analysis identified approximately 354 proteins that were present in PPD derived from M.tb, having various functions such as metabolism, virulence, lipid metabolism and cell processes, amongst others (Prasad *et al.*, 2013).

BCG used in this study was an attenuated Danish strain 1331, of *Mycobacterium bovis,* and is used universally as a vaccine against TB. Since it is a viable, live pathogen, the intact cell wall and bacterial DNA can activate a range of TLRs involved in mycobacterial recognition, such as TLR1, 2, 4, 6 and 9, as well as other PRRs (Kleinnijenhuis *et al.*, 2011; Quesniaux *et al.*, 2004).

LPS is a lipoglycan and the main component of the gram-negative bacterial outer membrane, and activates the innate immune system via the TLR4, leading to the production of pro-inflammatory cytokines (Triantafilou & Triantafilou, 2002). The LPS used in this study was an ultrapure preparation derived from *Salmonella Minnesota* (LPS-SM).

2.3.3 Cryopreservation of whole blood

All reagents used for the experiments are outlined in **Table 2.2**. To preserve stimulated cells, whole blood was incubated with alternating lysing solution (ALS) to lyse red blood cells. Whole blood was transferred into 15 ml tubes containing 5 ml (10X the volume of blood) ALS and incubated at room temperature for 10 min. Cells were then centrifuged at 400 x g for 10 min at room temperature and the supernatant was discarded and the cells were washed twice with 1X PBS. The cells were then stained with a viability marker, Vivid, for 20 min at room temperature in the dark. Vivid is a fluorescent amine reactive viability dye used to discriminate dead cells

from live cells. The cells were then washed and centrifuged twice with 1X PBS and 2 ml of FACS lysing solution was added to the cells to fix them and lyse any remaining red blood cells. The cells were incubated for 10 min at room temperature, after which they were centrifuged and washed twice with 1X PBS. Cells were then resuspended in cryopreservation solution consisting of 20% DMSO in FBS. The cells were then transferred into pre-cooled labeled cryovials (Greiner Bio-one, Germany). The cryovials were then placed in pre-cooled Mr. Frosty containers (Nalgene, USA). These containers are filled with isopropanol (Sigma-Aldrich, USA) that allows a gradual freezing of cells at a rate of -1°C/min and limits cell death. DMSO helps to maintain cell membrane integrity during the freezing process. Mr. Frosty containers were stored at -80°C for 24 h, after which the cells were transferred for storage in liquid nitrogen until later use.

Table 2.2: List of reagents used in the study

Reagent	Constituent	Manufacturer
Foetal Bovine Serum (FBS)	Heat inactivated foetal bovine serum	Invitrogen
Phosphate Buffered Saline (1X PBS)	NaCl, KCl, Na_2HPO_4, KH_2PO_4	Sigma-Aldrich
Alternating Lysing Solution	NH_4Cl, $KHCO_3$, Na_4EDTA	*
RPMI-1640 Medium	200mM L-Glutamine and 25mM HEPES	Sigma Aldrich
Pencillin (Pen)	5000 units/ml penicillin	Invitrogen
Streptomycin (Strep)	5000 units/ml streptomycin	Invitrogen
R1	1% FBS in RPMI	*
R10	10% FBS in RPMI	*
Dimethyl sulfoxide (DMSO)	$(CH3)_2SO$	Sigma-Aldrich
Ficoll-Hypaque	Ficoll & sodium diatrizoate	Sigma-Aldrich
Freezing media	20% DMSO in FBS	*
FACS lysing solution (1X)	<15% formaldehyde, <50% diethylene glycol	BD

* Prepared in the laboratory

2.4. Optimization of polychromatic flow cytometry panel for detecting monocyte cytokine responses

2.4.1 Introduction

This study sought to understand how infection with HIV might affect blood monocytes, in particular their ability to respond to mycobacteria. The specific objective was to examine whether infection with HIV altered monocyte cytokine responses to mycobacterial-derived TLR stimulation. Polychromatic flow cytometry was used to identify monocytes using phenotypic markers and measuring intracellular cytokine production. To address the aims of this study, an eight-color antibody staining panel was optimised to measure cytokine responses from monocytes. This section briefly describes the steps undertaken to develop and optimise the monocyte panel.

2.4.2 Selection of markers and fluorochromes for the flow panel

Several steps were followed to develop the panel. Markers of interest and their fluorochromes were chosen according to the instrument used, that is, a four laser BD Fortessa, in this study.

2.4.2.1 Selection of markers for identification of monocytes

Human blood monocytes are divided into three different subsets based on the expression of cell surface markers CD14 and CD16, as shown in **Figure 2.1** (Ziegler-Heitbrock *et al.*, 2010). These include the classical monocytes (CD14++CD16-), intermediate monocytes (CD14++CD16+) and the non-classical monocytes (CD14+CD16++). The intermediate subset have been shown to expand during HIV infection as well as during other disease conditions (as reviewed by Wong *et al.*, 2012). This subset produces the highest levels of pro-inflammatory cytokines and expresses high levels of HLA-DR (Belge *et al.*, 2002). Hence, this study sought to investigate whether HIV infection induces functional defects in all these three monocyte subsets by measuring production of the cytokines IL-1β, IL-6 and TNF-α from these cells. The CD14 and CD16 markers were therefore included in the panel. The activation marker HLA-DR is necessary to reliably identify monocytes, for gating on the monocyte subsets and for excluding HLA-DR negative

granulocytes and NK cells that also express CD16 marker, as described previously (Abeles *et al.*, 2012; Autissier *et al.*, 2010).

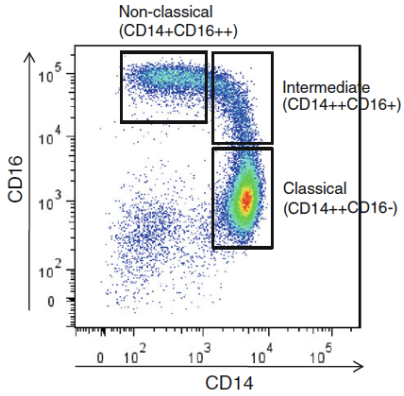

Figure 2.1: Monocyte subsets based on expression of CD14 and CD16. Flow cytometry dot plot showing the distribution of the classical, intermediate and non-classical monocyte subsets. The classical monocytes express high levels of CD14 but no CD16, the intermediate monocytes express high levels of CD14 and low CD16, while the non-classical monocytes express low CD14 but high CD16 (Taken from Wong *et al.*, 2012).

2.4.2.2 Selection of fluorochromes for the panel

Antibodies for the panel were selected based on several factors. These included the configuration of the Fortessa flow cytometer instrument. The type and number of lasers and detectors will determine whether a given fluorochrome will be excited and properly detected by the optical system according to the combination of fluorochromes used. It is therefore important to have knowledge of the instrument and how many lasers and channels are available to know how many and which fluorochromes can be included in the panel. The BD Fortessa in our core facility has four lasers available and up to eighteen colours can be detected, as shown in **Table 2.3.**

In addition, the expression levels of each cell marker, commercial availability of the markers, the level of spectral overlap from each fluorochrome are other factors to consider for selection of fluorochromes (Mahnke & Roederer, 2007). Those markers that were highly expressed were assigned to less bright fluorochromes, while those that were expressed at low levels were matched with brighter fluorochromes. The brightness of the fluorochromes can be determined based on their staining index. In this panel, fluorochromes such as PE, APC and PECy7 which have high staining index, were assigned to cytokines IL-1β, IL-6 and TNF-α that are (usually) expressed

at lower levels inside the cells compared to other surface markers in the panel. In contrast, less bright fluorochromes such as Pacific Blue and Alexafluor700 were assigned to highly expressed markers such as CD3 and HLA-DR.

Table 2.3: Configuration of the BD Fortessa showing the different lasers and detectors

Laser	Detector name	Fluorchrome detected	Dichroic Long Pass Filter	Band Pass Filter
Blue 488nm	SSC	Side scatter		488/10
	B710	Cy5.5PerCP	670LP	685/35
	B515	FITC, Alexa488, GFP, CFSE, Oregon Green	505LP	515/20
Green 532nm	G780	Cy7PE	750LP	780/60
	G710	Cy5.5PE	685LP	710/50
	G660	Cy5PE	635LP	670/30
	G610	Texas Red PE, Texas Red, Alexa594	600LP	610/20
	G560	PE	Empty	585/15
	Empty	na		
	Empty	na		
	Empty	na		
Red 640nm	R780	H7APC, Cy7APC	740LP	780/60
	R710	Alexa680, 700	685LP	730/45
	R660	APC, Alexa647	Empty	670/30
Violet 407nm	V800	QD800	750LP	780/60
	V705	QD705	685LP	705/70
	V655	QD655	630LP	670/30
	V605	QD605, 585, BV605	595LP	605/40
	V585	QD565	556LP	585/42
	V545	QD545	535LP	560/40
	V525	AmCyan, QD525, Horizon V500	505LP	525/50
	V450	PacBlue, Vivid, Horizon V450	Empty	450/50

2.4.2.3 Other considerations

Other factors that were also considered were the stability of the markers with the staining conditions and experimental procedures used in sample preparation. All markers in this study were stained intracellularly, as described before (Shey *et al*., 2012). Thus, any downregulation of markers such as HLA-DR and CD14 in response to stimulation did not compromise the ability to detect monocytes. Also, all stimulations were performed on whole blood assays rather than PBMC, to mimic the whole blood conditions *in vivo*. Since the whole blood samples in this study were

only stained and analysed in batches at a later time point after blood collection, it was necessary to fix the cells to preserve their stability and expression of markers (Davis *et al.*, 2011). The panels were therefore tested in the same conditions as the final assay as some aspects of experimental procedure may have an impact on sensitivity. This section briefly discusses certain markers that were included in the panel to increase the accuracy of the results obtained.

Inclusion of a brilliant violet fluorochrome for the CD14 marker:

Initially, the FITC fluorochrome was assigned to the CD14 marker. However, a clear separation from the CD14 negative population was not observed **(Figure 2.2)** and therefore it was necessary to include a brighter fluorochrome for this marker. Since monocytes are identified based on their expression of CD14, it was necessary to select a fluorochrome that could reliably identify this population to meet the aims of this study. Hence, a brilliant violet (BV) dye was assigned to the CD14 marker, since BVs have been shown to be extremely bright with excellent signal to noise ratio (Chattopadhyay *et al.*, 2012). BVs are a new class of dyes and are excited by the violet laser at 405 nm. The BV570 was selected as it emits at 570 nm and can be detected by the Fortessa using the 585/42 bandpass filter. With the anti-CD14-BV570 antibody, improved separation of the CD14 population was observed **(Figure 2.2).**

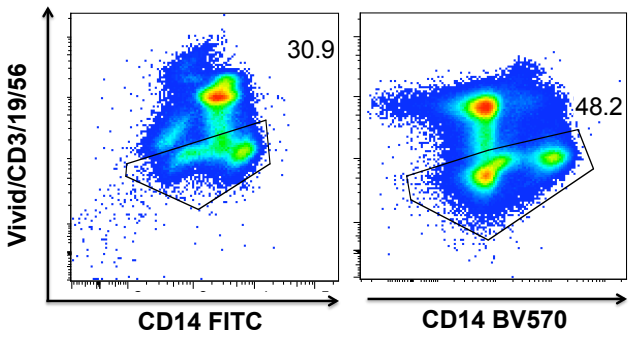

Figure 2.2: Staining of whole blood with anti-CD14-FITC or anti-CD14-BV570 antibody. Gates showing the separation of live CD14+ population from CD14- population after staining of whole blood samples with CD14-FITC and CD14-BV570 conjugated antibodies.

Inclusion of a viability marker and a dump channel:

The viability marker, Vivid, was used to discriminate between dead and live cells. Since cryopreserved whole blood samples were used, it was important to exclude dead cells to improve the quality of data obtained in terms of true cytokine responses. The viability marker helps to increase the sensitivity for detecting rare events such as cytokines, and prevents false positives, as antibody conjugates can non-specifically bind to dead cells (Perfetto *et al.*, 2006).

Furthermore, the markers CD19, CD3 and CD56 were all included as part of a dump channel to exclude B cells, T cells and natural killer cells. This helped to improve the accuracy of the panel in identifying monocytes by excluding background. These antibodies were selected for use in the same channel as Vivid (Pacific Blue) so that a single channel could be used to exclude all unwanted events.

Inclusion of a granulocyte marker:

A previous study showed that stimulation of cells with BCG or LPS led to a decrease in side scatter fluorescence for granulocytes and an increase for monocytes which prevented separation of monocytes from granulocytes using forward and side scatter parameters (Shey *et al.*, 2012). Since the CD16 marker is also expressed by neutrophils, they may contaminate the CD16+ monocyte subsets. In addition, some granulocytes express high levels of HLA-DR, and therefore the granulocyte marker CD66a/c/e was also included in the panel to ensure exclusion of all granulocytes (Shey *et al.*, 2012; Ziegler-Heitbrock *et al.*, 2010**; Figure 2.3**).

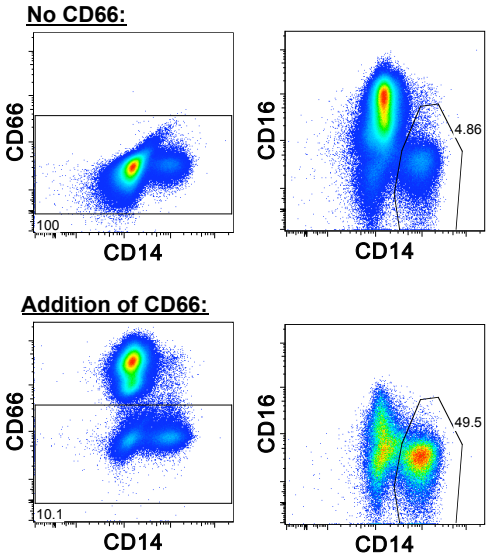

Figure 2.3: Flow plots showing the staining of whole blood samples with anti-CD66 antibody. The anti-CD66 antibody led to the exclusion of a large population of granulocytes, which would otherwise have contaminated the CD16+ monocytes in the CD16 vs. CD14 flow plot.

Table 2.4 summarises the markers and their fluorochromes chosen for the monocyte panel. Markers were selected that were bright and had minimal spectral overlap as spillover into other detectors can reduce sensitivity of measuring a particular fluorescence in those detectors.

Table 2.4: List of antibodies and fluorochromes selected for the monocyte panel

Antibody	Fluorochrome	Clone	Cat. #	Titer (µl)	Manufacturer
CD14	BV 570	M5E2	301831	0.25	Biolegend
CD16	Cy5-PE	3G8	302010	0.25	Biolegend
HLA-DR	Alexa700	L243	307626	0.50	Biolegend
CD66	Alexa488	ASL-32	342306	0.20	Biolegend
CD3	Pacific Blue	UCHT1	300431	0.30	Biolegend
CD19	Pacific Blue	SJ25-C1	MHCD1928	0.625	Invitrogen
CD56	Pacific Blue	MEM 188	304629	0.30	Biolegend
IL-6	APC	MQ2-13A5	561441	2.00	BD
TNF-α	Cy7-PE	Mab11	25-7349-41	0.0625	ebioscience
IL-1β	PE	AS10	340516	0.25	BD
Vivid	Pacific Blue		1428		Life Technologies

2.4.3 Panel development

All antibodies and the viability dye were titrated to determine their optimal titer that provides the best separation and lowest background signal. Antibody panels were evaluated with cells incubated with the full panel as well as ones in which each antibody was added sequentially. This allowed identification of antibody conjugates that reduced sensitivity. The panel was also compared between different donors to ensure sensitivity and reliability of the panel. FMO (Fluorescence minus one) controls were performed for all antibodies to confirm antibody compatibility and that there was no significant fluorescent spillover into other channels.

2.4.3.1 Challenges encountered with the CD16 marker

The objective of this study was to distinguish between the various monocyte subsets in order to investigate the effect of HIV infection on monocyte cytokine responses to mycobacterial-derived TLR stimuli. However, when the full monocyte panel was stained on whole blood samples, the distribution of monocyte subsets was not observed as expected. The CD14+ classical monocytes could be clearly gated, as shown in **Figure 2.4**. However, the two CD16+ subsets could not be discriminated but rather a CD14 intermediate population was observed.

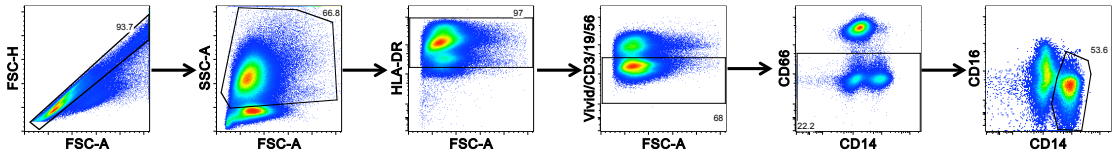

Figure 2.4: Staining of fixed whole blood samples with the monocyte panel. The flow plots show the monocyte population after they were gated on singlets, then gated based on their size on the forward scatter and side scatter parameters and then live HLA-DR+CD3-CD19-CD56-CD66-. The CD14 vs. CD16 flow plot shows a clear gate around the CD14+ classical monocytes but a gate could not be drawn for the CD16+ monocyte subsets.

This lack of being able to distinguish the monocyte subsets was not due to lack of CD16 staining, as running an FMO control for CD16 confirmed that a CD16 population was present **(Figure 2.5)**. Hence, the CD16 marker could be detected, however, the distribution of the monocyte subsets was lost and no clear separation between the subsets was observed.

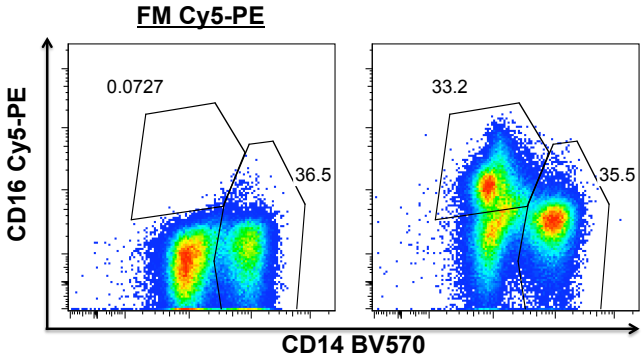

Figure 2.5: FMO control for the anti-CD16-Cy5-PE antibody. Cryopreserved whole blood was stained with the full monocyte panel as well as one in which the CD16 antibody was excluded. All dot plots were gated on live HLA-DR+CD3-CD19-CD56-CD66- cells.

The protocol and reagents used for intracellular staining of samples may affect the performance of some antibodies and change the epitope of certain proteins (Berhanu *et al.*, 2003). All samples in this study were fixed with an erythrocyte lysing buffer (FACS lysing solution) prior to cryopreservation as fixing cells helps to preserve the cellular membrane. However, the fixation buffer contains paraformaldehyde that can alter the conformation of proteins and modify the epitope structure which can affect antibody recognition of its target (Pollice *et al.*, 1992). Fixation has also been shown to alter the fluorescence intensity of certain markers (Davis *et al.*, 2011; McCarthy *et al.*, 1994; Stewart *et al.*, 2007). Therefore, the effect of fixation on CD16 marker was investigated.

To determine the effects of paraformaldehyde fixation on CD16 marker staining, fresh PBMC isolated from a donor were stained with antibodies and compared to PBMC stained post-fixation with FACS lysing solution and also compared with fixed cryopreserved whole blood from the same donor **(Figure 2.6)**. PBMC stained without fixation showed a clear distribution of the three monocyte subsets **(Figure 2.6A)**. However, when the PBMC were stained after fixation with FACS lyse solution, the monocyte subset distribution became unclear. This shows that fixation had an effect on the resolution of the CD16 marker **(Figure 2.6B)**. With fixed cryopreserved whole blood, this distribution was completely lost, indicating that some aspects of the whole blood processing and cryopreservation procedure had an effect on the CD16 marker **(Figure 2.6C)**. Fixation reduced the staining intensity of the CD16 marker such that it led to a diminished resolution between the positive and

negative populations, that made discrimination between different subsets challenging in fixed whole blood samples. It therefore appears that the monocyte populations are best analysed using fresh PBMC or whole blood with minimum processing and not using any fixatives in order to ensure that marker changes do not occur.

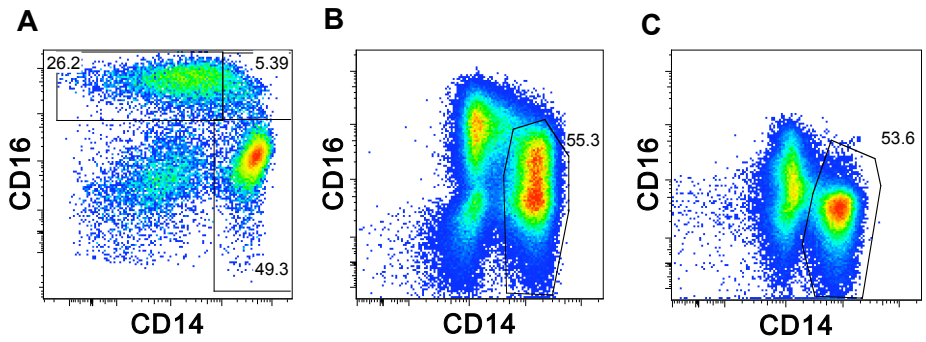

Figure 2.6: **Effect of fixation on monocyte subsets distribution. A** shows a clear distribution of the three monocyte subsets after staining of fresh PBMC without any fixatives. **B** shows that when fresh PBMC are stained with antibodies after fixation with FACS lysing solution, the distribution of the monocyte subsets changes considerably and is no longer clear. **C** shows the CD14 vs. CD16 flow plot for fixed cryopreserved whole blood, where the monocyte subset distribution is completely lost. All dot plots were gated on live HLA-DR+CD3-CD19-CD56- cells.

Due to time constraints, the panel could not be further optimised to test other clones of CD16 antibodies that may not be affected by fixation. Thus, this population was omitted from analysis due to unreliable staining and difficulty in gating on this population. This served as a limitation in this project in studying the three monocyte subsets in HIV infection.

2.4.3.2 Fluorescence minus one (FMO) controls

FMOs are used to determine whether the fluorochromes chosen for a panel can be used in combination with one another. In FMO experiments, one antibody is removed at a time to assess potential false positives due to fluorochrome interactions, spectral overlap, or tandem-dye degradations that could compromise detection in large panels (Perfetto *et al.*, 2004).

FMO controls were performed for all antibodies under the same experimental conditions as for samples, that is, whole blood that was stimulated for 6 h with the

addition of BFA after 3 h, and the samples were then stained with the viability marker (Vivid), fixed and cryopreserved. FMO controls for the monocyte panel are shown in **Figure 2.7**. Very low levels of secondary fluorescence were observed in all the channels. The highest level of background signal was seen in BV570 channel (0.346%). However, this fluorochrome was assigned to the highly expressed marker CD14, and thus, the low background fluorescence was considered negligible compared to the positively stained population.

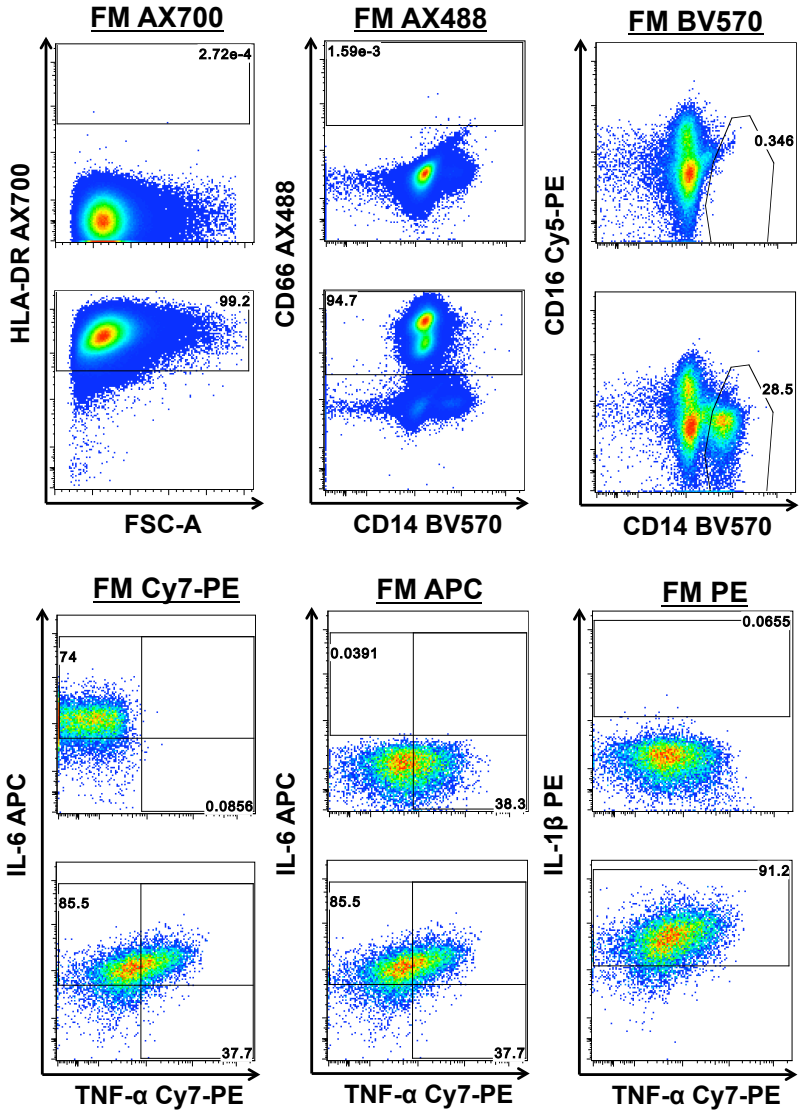

Figure 2.7: FMO controls for the monocyte panel. Cryopreserved whole blood was stimulated with LPS for 6 h with the addition of BFA after 3 h. Cells were then stained with all antibody-fluorochrome conjugates and then each antibody was sequentially removed one at a time. All dot plots were gated on live CD3-CD19-CD56- cells. The antibody excluded from the full panel is shown for each channel with its corresponding plot of cells stained with the full panel. FM: Fluorescence minus.

2.4.3.3 Gating strategy

The gating strategy used for identifying monocytes and detecting cytokine responses from monocytes is shown in **Figure 2.8**. Cells were first gated on time (for all lasers), to exclude any artifacts due to inconsistent acquisition, and then gated on singlets to exclude large cellular aggregates. Monocytes were then defined based on their size and granularity on forward scatter versus side scatter. Since some of the monocytes, such as the intermediate subset CD14+CD16++, are somewhat smaller in size than the classical monocytes, they tend to localise in the lymphocyte area and therefore the gate was extended to include part of the lymphocyte population (Heimbeck *et al.*, 2010). This was followed by gating on all HLA-DR+ cells and then the live CD3-CD19-CD56-CD66- were gated to exclude T cells, B cells, NK cells and granulocytes, respectively. The CD14+ cells were then gated and the cytokines IL-1β, IL-6 and TNF-α were quantified from this subset of cells. Gates were set based on the unstimulated control for each participant. The CD16+ subsets could not be distinguished clearly as discussed above and were therefore excluded from analysis.

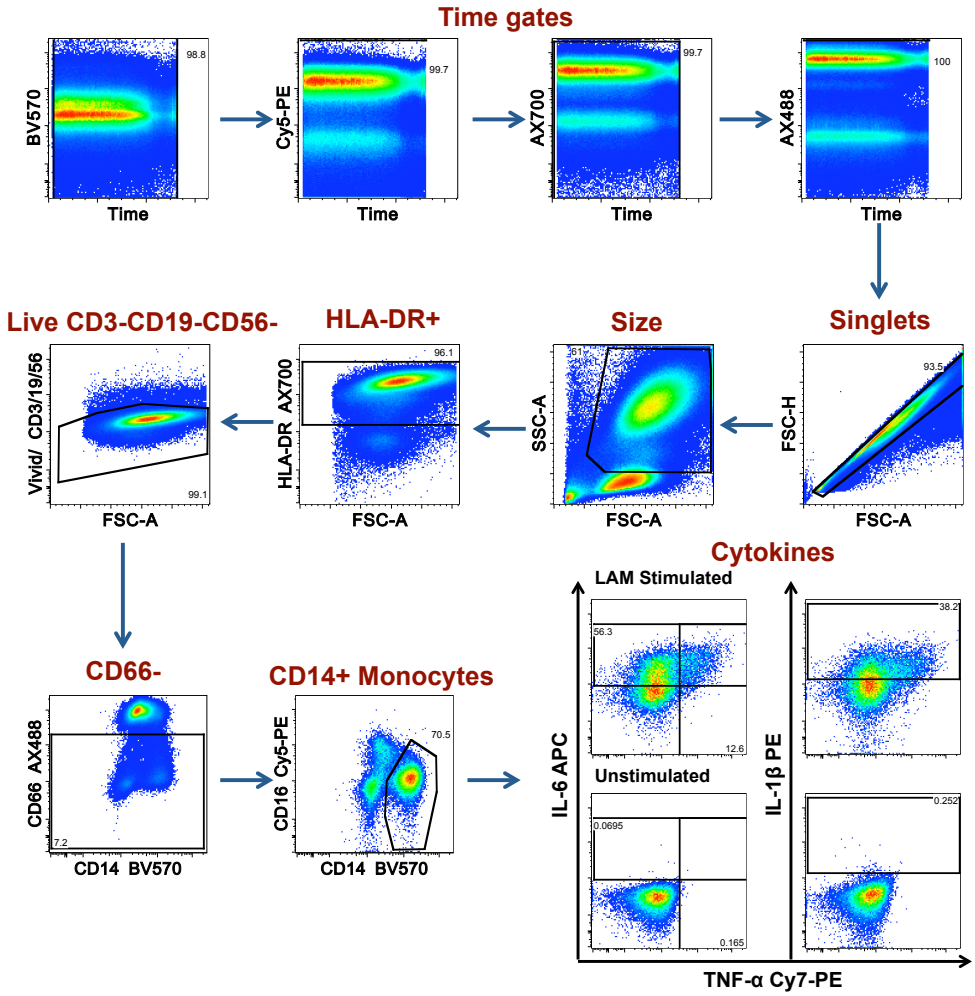

Figure 2.8: Gating strategy for the monocyte panel. Representative flow cytometry plots showing cytokine producing CD14+ monocytes upon stimulation with LAM. Cells were gated on time (for all lasers), and then gated on singlets. Monocytes were then defined based on their size and granularity on forward scatter versus side scatter. This was followed by gating on all HLA-DR+ cells and then the live CD3-CD19-CD56-CD66- were gated to exclude T cells, B cells, NK cells and granulocytes, respectively. The CD14+ cells were then gated and the cytokines IL-1β, IL-6 and TNF-α were quantified from this subset of cells. Gates were set based on the unstimulated control for each participant.

2.5. Determining blood monocyte responses to mycobacterial TLR stimuli

2.5.1 Thawing of cryopreserved samples

Cryopreserved, stimulated cells, stored in liquid nitrogen were rapidly thawed in batches by transferring the vials into a water bath at 37°C. The thawed cells were then transferred in a 50 ml centrifuge tube (Sterilin, UK) and 10 ml of pre-warmed R1 solution was then added in a drop wise fashion to the thawed cells. The cryovials

were rinsed thoroughly to recover all cells and the centrifuge tube was filled up with R1 medium to dilute the DMSO, which is toxic to the cells if the temperature increases. This was then centrifuged at 400 x g for 10 min. The supernatant was discarded and the cell pellet was washed and centrifuged again. The cells were then resuspended in 200 µ1 of BD PermWash buffer and transferred onto a 96 well-plate for antibody staining as described below.

2.5.2 Antibody staining procedure

Whole blood cells were stained using a one-step staining method. All reagents used for the staining procedure are outlined in **Table 2.5**. Cells were aliquoted in a V-bottomed 96-well plate, and centrifuged at 800 x g for 3 min at 4°C. Cells were permeabilised by adding 100 µl of PermWash buffer for 20 min in the dark at room temperature. The permeabilised cells were then pelleted as before and washed thrice prior to staining. Cells were then stained with the antibody master mix prepared in a volume of 50 µl for 20 min at room temperature in the dark. The antibody master mix consisted of all the markers shown in **Table 2.4** above. Stained cells were washed thrice with 1X PermWash and centrifuged as before. The cells were then fixed with 200 µl of 1X CellFIX (BD Biosciences). The total stained sample volume was then acquired on a BD Fortessa flow cytometer.

Table 2.5: List of reagents used in intracellular staining

Reagent	Constituent	Manufacturer
FACS wash buffer	1% FBS, 0.001% Sodium azide (Sigma-Aldrich)	*
Perm Wash (1X)	FBS, Sodium azide and saponin	BD
CellFIX (1X)	1% w/v formaldehyde and sodium azide	BD

*Prepared in the laboratory

2.5.3 Data analysis

Cells were acquired on a BD Fortessa using FACSDiva software. Compensation and data analyses was performed using FlowJo Version 9.4.10 (Tree star Inc, Ashland, OR). Additional data analysis was performed using Pestle (provided by Mario Roederer, NIH) and Spice software (freely available from http://exon.niaid.nih.gov/spice/; Roederer *et al.*, 2011).

2.6 Statistical analyses

All statistical analyses of data was performed using GraphPad Prism 5 Software (San Diego, California, USA). Non-parametric, two-tailed tests were used for all comparisons. The Mann-Whitney U test was used to determine any statistical differences between the medians of two groups for unmatched data. The Wilcoxin matched pairs test was used for matched data. Correlations were performed using Spearman Rank correlation test. Assessment of variation between groups was carried out through one-way Analysis of Variance (ANOVA) with Dunn's post-test applied to correct for multiple comparisons. A statistical significance was considered for a p-value less than 0.05.

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3.1 Introduction

HIV-infected persons are more susceptible to TB, and the reasons for this are not fully understood. Most studies have focused on how HIV infection compromises adaptive immunity to M.tb, however less is known regarding the effects on innate immunity. Monocytes play a key role in innate immune defense and are the precursors of macrophages, which are the main target cells of M.tb. If the ability of these cells to respond to M.tb is impaired, it could account for the greater susceptibility of HIV-infected individuals to develop TB. One of the ways in which these cells respond to M.tb is through TLR stimulation. TLRs are central in orchestrating a protective response to M.tb; their activation leads to the release of pro-inflammatory mediators, triggering innate and adaptive immune responses to infection, critical events for the control of M.tb. This study sought to investigate whether HIV infection induced functional defects in monocytes, impairing their ability to respond to TLR ligands that are expressed by mycobacteria. The focus was on TLR functioning in monocytes, by examining whether infection with HIV altered cytokine production in response to TLR stimulation.

In order to examine how HIV affects TLR functioning, a multiparameter flow cytometry panel was developed and optimised to characterise cytokine production from blood monocytes. Whole blood obtained from 20 HIV-infected and 18 HIV-uninfected individuals, was stimulated in a short term assay, and intracellular cytokine staining of monocytes was performed. The stimuli used included mycobacterial lipoarabinomannan (LAM, a TLR2 ligand), M.tb purified protein derivative (PPD, which stimulates TLR1, 2, 4 and 6), viable whole Bacille Calmette Guérin (BCG, a stimuli for TLR1, 2, 4, 6 and 9) as well as lipopolysaccharide (LPS, a TLR4 agonist from gram negative bacteria), the latter used as a positive control for monocyte cytokine responses.

3.2 Clinical characteristics of study participants

To assess the impact of HIV infection on monocyte cytokine responses, blood samples were collected from 20 HIV-infected and 18 HIV-uninfected individuals, all with suspected latent TB infection in a cross-sectional study approved by the University of Cape Town Research Ethics Committee. Only individuals with CD4 counts >400 cells/mm^3 were recruited into the study, since the aim was to study defects in the response to mycobacteria before profound CD4 depletion. Exclusion criteria for the study included antiretroviral therapy use, pregnancy, age <18 years, recent TB disease (within five years prior to enrollment in this study), treatment for TB, and any other acute or chronic disease. All participants gave informed consent for participation in the study.

Table 3.1 shows the clinical characteristics of the recruited participants. The participants were primarily young women; in the HIV-infected group, 80% of the participants were females with a median age of 32 (IQR: 29-39), whereas in the HIV-uninfected group, 56% of the participants were females with a median age of 22 (IQR: 19-25). HIV-infected participants were significantly older than HIV-uninfected participants ($p<0.0004$). The HIV-infected participants were relatively immune competent with a median CD4 count of 581 cells/mm^3 (IQR: 530-759), and had a wide range of viral loads in their plasma (median, 7787.5 RNA copies/ml; IQR: 2677.5-21205.8).

Table 3. 1: Clinical characteristics of study participants

HIV-infected participants (n=20)					HIV-uninfected participants (n=18)			
PID	**Age[a]**	**Sex[b]**	**CD4 count (cells/mm^3)**	**Plasma Viral load (RNA Copies/ml)**	**PID**	**Age[a]**	**Sex[b]**	**CD4 count (cells/mm^3)**
1076	25	F	543	908	1001	19	M	631
1077	47	M	526	28432	1009	21	M	820
1079	34	F	591	32485	1015	23	M	659
1080	54	F	774	14100	1024	24	M	939
1081	37	F	383	3256	1028	44	F	814
1084	40	F	619	9192	1030	25	F	858
1086	28	F	1449	4250	1031	19	F	1169
1119	30	M	545	580150	1032	21	F	NA[c]
1126	30	F	433	32994	1035	19	F	1459
1129	31	F	510	4559	1038	18	F	743
1131	32	M	785	331	1047	26	M	680
1134	37	F	599	6383	1049	40	F	655
1137	27	F	560	18797	1052	25	F	1412
1141	32	F	552	9826	1055	19	M	932
1142	53	M	441	544849	1057	20	M	871
1143	26	F	656	618	1058	35	F	866
1150	26	F	894	311	1060	23	F	902
1152	30	F	571	9697	1061	21	M	674
1153	46	F	714	2954				
1154	34	F	988	1848				
Median	32		581	7787.5		22		858
IQR	29-39		530-759	2677.5-21205.8		19-25		677-936

[a]p<0.0004; [b] % female (HIV+: 80%, HIV-:56%); [c]NA-data not available

3.3 Effect of TLR stimulation on monocyte cytokine responses

Monocytes detect microbes through the expression of various pattern recognition receptors, such as TLRs (as reviewed by Kleinnijenhuis *et al*., 2011). To determine how monocytes differed in their ability to respond to different TLR stimuli derived from mycobacteria, the profiles of cytokine production from monocytes from 18 healthy, HIV-uninfected individuals were assessed in response to TLR stimulation. The TLR2 agonist LAM forms part of the cell wall of mycobacteria (Wieland *et al.*, 2004). BCG is a viable, live pathogen, and its intact cell wall and bacterial DNA can activate a range of TLRs involved in mycobacterial recognition, such as TLR1, 2, 4, 6 and 9, as well as other PRRs (as reviewed by Hossain & Norazmi, 2013; Kleinnijenhuis *et al*., 2011; Quesniaux *et al*., 2004). PPD, prepared from culture filtrates of M.tb, is a mixture of various mycobacterial peptides and lipoproteins that

may activate monocytes through TLR1, 2, 4 and 6 (Prasad *et al.*, 2013). LPS is a lipoglycan and the main component of the gram-negative bacterial outer membrane, and activates innate immune cells via TLR4 (as reviewed by Triantafilou & Triantafilou, 2002).

Whole blood was stained *ex vivo* with phenotypic markers to identify monocytes and functional markers to measure cytokine production. The gating strategy is described in Chapter 2. Monocytes were defined based on their size and granularity on forward scatter versus side scatter, and then as live, CD3-CD19-CD56-CD66-HLA-DR+CD14+ cells. The cytokines IL-1β, IL-6 and TNF-α were then measured intracellularly from this subset of cells.

3.3.1 Frequency of cytokine responses

Upon stimulation of monocytes, different stimuli induced markedly different monocyte cytokine responses. The representative flow plots in **Figure 3.1A** demonstrate robust production of the cytokines IL-1β, IL-6 and TNF-α above background responses from monocytes in response to LAM, BCG, PPD and LPS, compared to the unstimulated control. Total cytokine responses (representing monocytes secreting any of the three cytokines) are shown in **Figure 3.1B**. Monocytes were most responsive to stimulation with BCG, where cytokine production was detected in a median of 83.2% (IQR: 72.9-85.4) of monocytes. This was followed by LAM, which induced a median of 64.1% cytokine-positive monocytes (IQR: 27.7-73.9), whilst the TLR ligand LPS stimulated nearly 2-fold fewer cytokine-producing monocytes (median 45.4%, IQR: 29.3-60.6) compared to BCG. PPD was only able stimulate a median of 8.8% (IQR: 2.3-14.4) of monocytes (10-fold less) to produce cytokines **(Figure 3.1B)**. When assessing individual cytokine responses (IL-1β, IL-6 and TNF-α; **Figure 3.1C-E**) from monocytes in response to these stimuli, the same trend was observed, where BCG was able to induce the most robust response for all three cytokines. In general, majority of the monocytes produced IL-1β and IL-6, whilst a lower frequency of monocytes produced TNF-α. Substantial variability among individuals was observed in their production of the three cytokines in response to LAM and LPS. However, this spread

among the donors was less striking for BCG or PPD, with the exception of TNF-α production, where BCG stimulation did induce a wide range of responses.

3.3.2 Expression level (MFI) of cytokine responses

The median fluorescence intensity (MFI) of cytokine production signifies the median amount of cytokine produced per cell in a population of cells positive for that cytokine. Monocytes produced the highest levels of IL-1β, IL-6 and TNF-α in response to BCG stimulation, followed by LAM and LPS **(Figure 3.2 A-C).** Cytokine production in response to PPD exhibited the lowest MFI for all three cytokines.

3.3.3 Polyfunctional capacity of monocytes

Next, the polyfunctional capacity of monocytes was compared for the different TLR stimuli. **Figure 3.3A** shows the proportions of monocytes producing one, two or three cytokines simultaneously, in response to the different stimuli, LAM, BCG, PPD or LPS. A significant difference was observed in the quality of the monocyte response between the different stimulations ($p=0.0001$ between all stimulations, except between LAM and LPS where $p<0.049$; **Figure 3.3A**). Monocytes displayed the greatest polyfunctionality (a higher proportion of three-function monocytes) after stimulation with BCG. LPS and LAM elicited nearly equal proportions of the three, two and one-cytokine-producing monocytes, however the cytokine profiles were significantly different from each other ($p<0.049$). In contrast, PPD elicited mainly monofunctional responses (a higher proportion of one-function monocytes). **Figure 3.3B** shows the frequency of the different combinations of cytokine production from monocytes. Stimulation with BCG, LAM and LPS stimulated monocytes to either produce all three cytokines simultaneously, or dual production of IL-1β and IL-6, or the single cytokines IL-1β or IL-6 only. In contrast, PPD induced mainly two cytokines, IL-1β and IL-6, and single cytokines, IL-1β or IL-6.

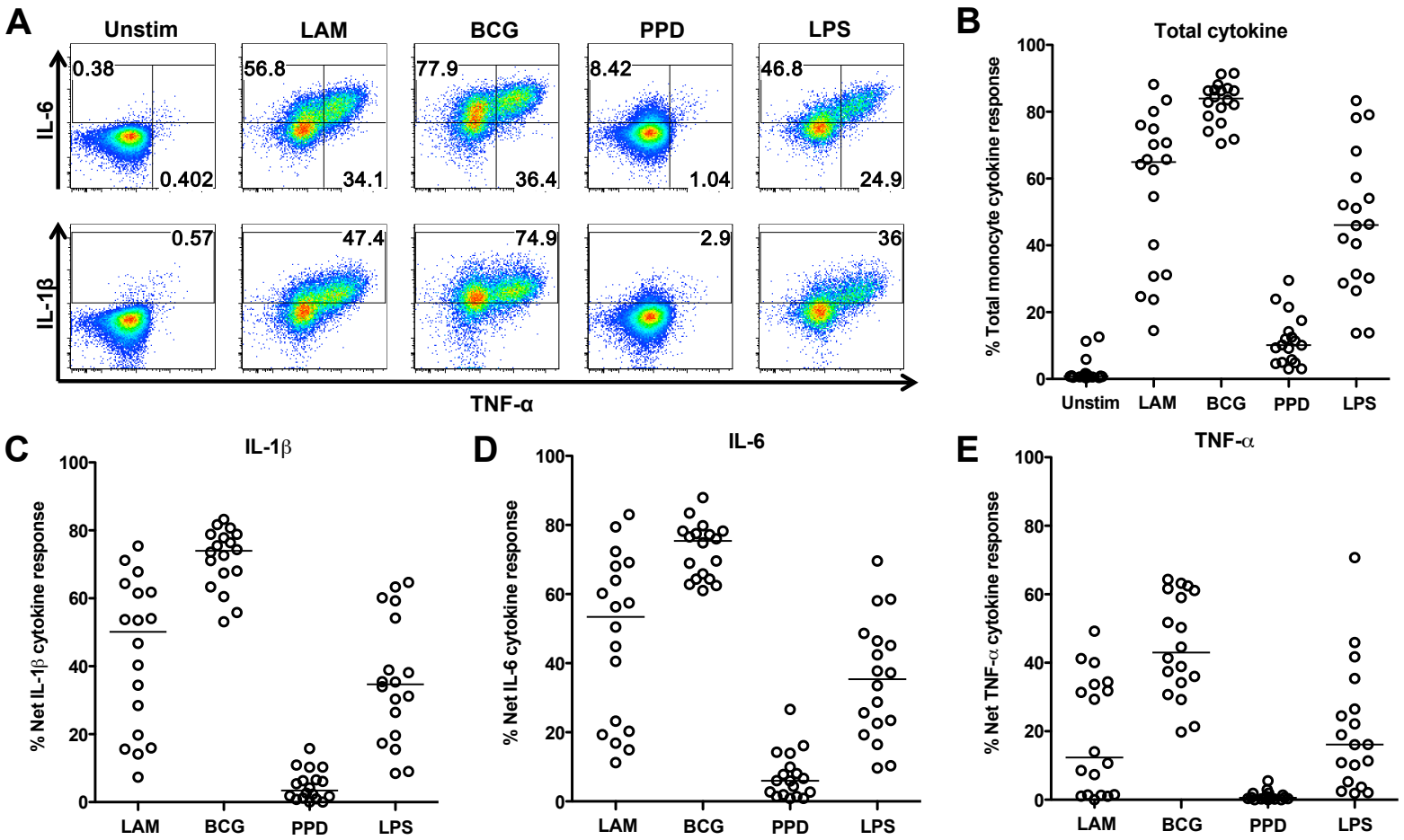

Figure 3.1: Cytokine production from stimulated monocytes. A shows representative flow cytometry plots of monocyte cytokine responses from whole blood of an HIV-uninfected individual stimulated for 6 h (with the addition of BFA after 3 hours) with LAM, BCG, PPD and LPS, or left unstimulated. The numbers on the plots show the frequency of cytokine-producing monocytes. **B** shows the frequency of the total monocyte cytokine response (background-subtracted). **C, D** and **E** show the individual cytokines IL-1 β, IL-6 and TNF-α, respectively, in response to the different stimuli (n=18; background-subtracted).

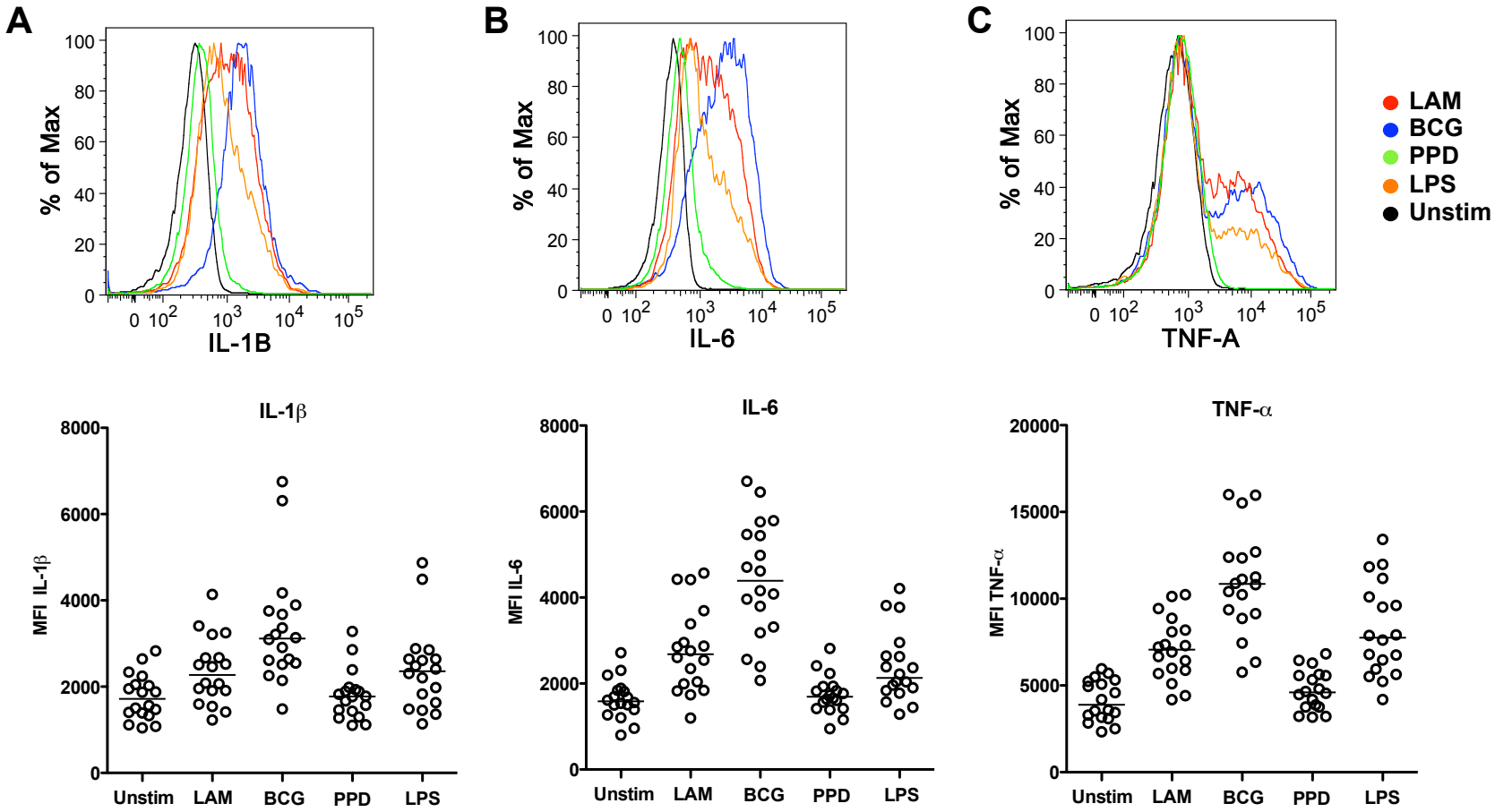

Figure 3. 2: Median fluorescence intensity of cytokine production from monocytes in response to stimulation. The MFI of IL-1β **(A)**, IL-6 **(B)** and TNF-α **(C)** is shown (lower panel) together with representative histograms (upper panel) from monocytes that were stimulated for 6 h with LAM, BCG, PPD and LPS, or left unstimulated (n=18).

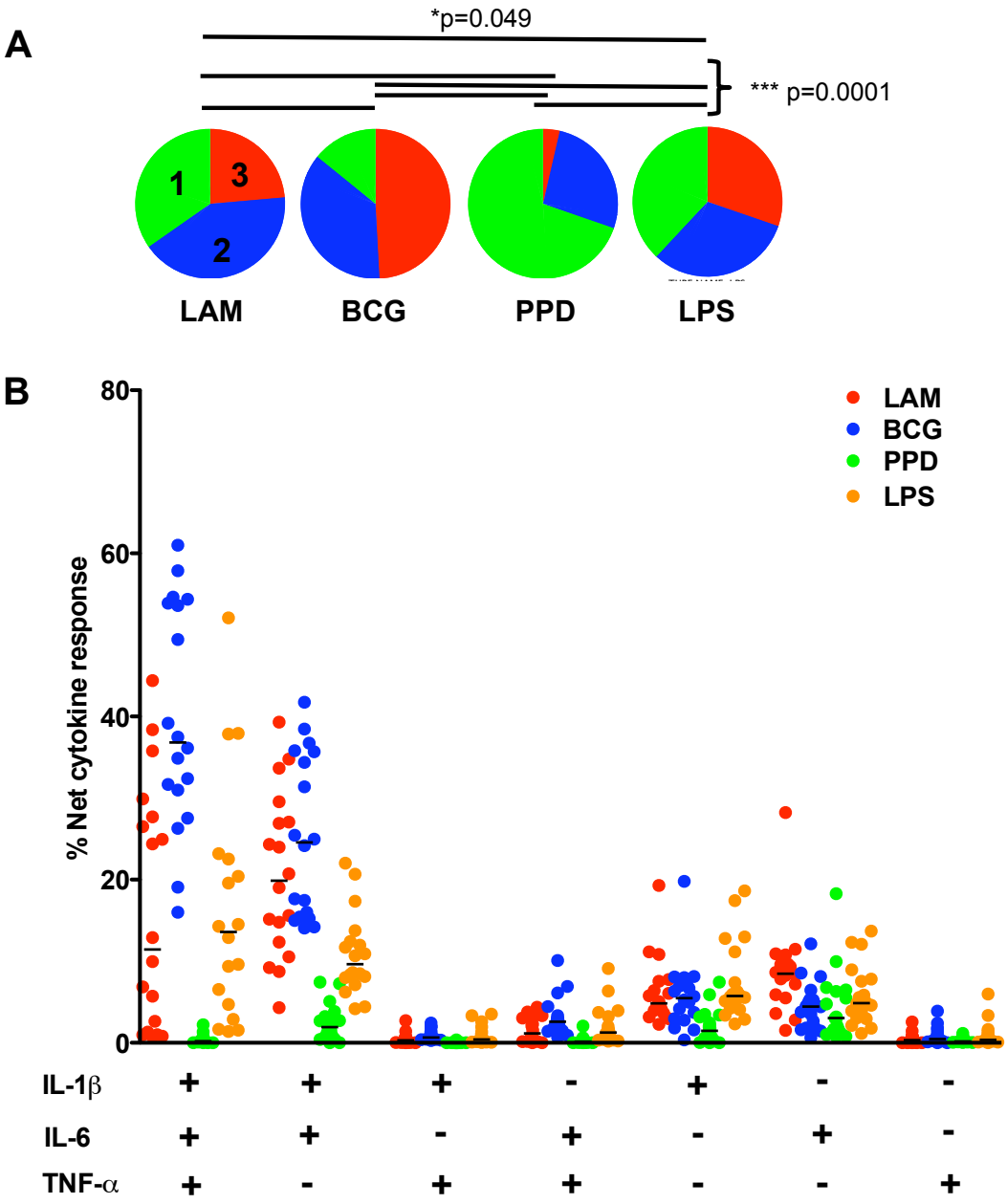

Figure 3.3: Polyfunctional capacity of monocytes in response to stimulation. A shows pie charts representing the proportion of cells producing any one cytokine (green), any two cytokines (blue) and any three cytokines (red) simultaneously. **B** shows the combinations of cytokines produced by monocytes in response to the different stimuli, LAM (red), BCG (blue), PPD (green) or LPS (orange) (n=18). Statistical comparisons were determined by a Mann-Whitney nonparametric t-test.

3.4 Effect of HIV infection on cytokine production from blood monocytes in response to various TLR stimuli

HIV may induce defects in monocyte function that could impair their ability to respond to M.tb. In an effort to determine whether monocytes from HIV-infected individuals respond differently to TLR stimulation compared to uninfected individuals, stimulation of whole blood was performed to investigate whether there were any functional defects in M.tb-responsive TLRs in monocytes, by measuring the production of cytokines from these cells.

3.4.1 LAM

To investigate the effect of HIV infection on the ability of monocytes to respond to LAM, cytokine responses were compared between HIV-infected (n=20) and HIV-uninfected individuals (n=18). **Figure 3.4A** shows representative flow plots of cytokine production from monocytes when stimulated with LAM or left unstimulated. No difference in the cytokine response to LAM (p=0.342) was observed between HIV-infected and HIV-uninfected individuals (median 56%, IQR: 26-66; median 64%, IQR: 28-74 respectively; **Figure 3.4B**) Similarly, no differences were detected in the two groups when individual cytokines IL-1β, IL-6 and TNF-α were examined in response to LAM (p=0.357, p=0.373, p=0.174 respectively; **Figure 3.4C**)**.** Considerable variability was observed among the participants in their production of the three cytokines in response to LAM. The majority of monocytes tended to produce IL-1β and IL-6, and almost 2-fold less TNF-α.

Next, the analysis was extended to assess differences in the functional profiles of monocytes between the HIV-infected and uninfected participants. No significant difference was detected in the amount of IL-1β, IL-6 or TNF-α produced per monocyte between the two groups, however, a trend of higher levels of IL-6 and TNF-α from monocytes of HIV-uninfected individuals was observed (p=0.965, p=0.066, p=0.066 respectively; **Figure 3.5A**). Also, comparing the polyfunctional capacity of monocytes between HIV-infected and uninfected individuals revealed a trend of greater proportion of monocytes in the HIV-infected individuals having the capacity to produce only one cytokine in response to LAM (p=0.0928**,** pie charts in **Figure 3.5B**)**.** Thus, these results indicate that monocyte cytokine responses to the

mycobacteria-derived TLR2 ligand LAM are not impaired in HIV infection. LAM stimulated monocytes to produce mainly a combination of IL-1β and IL-6, with a lower percentage of monocytes producing all three cytokines or single cytokines **(Figure 3.5B)**.

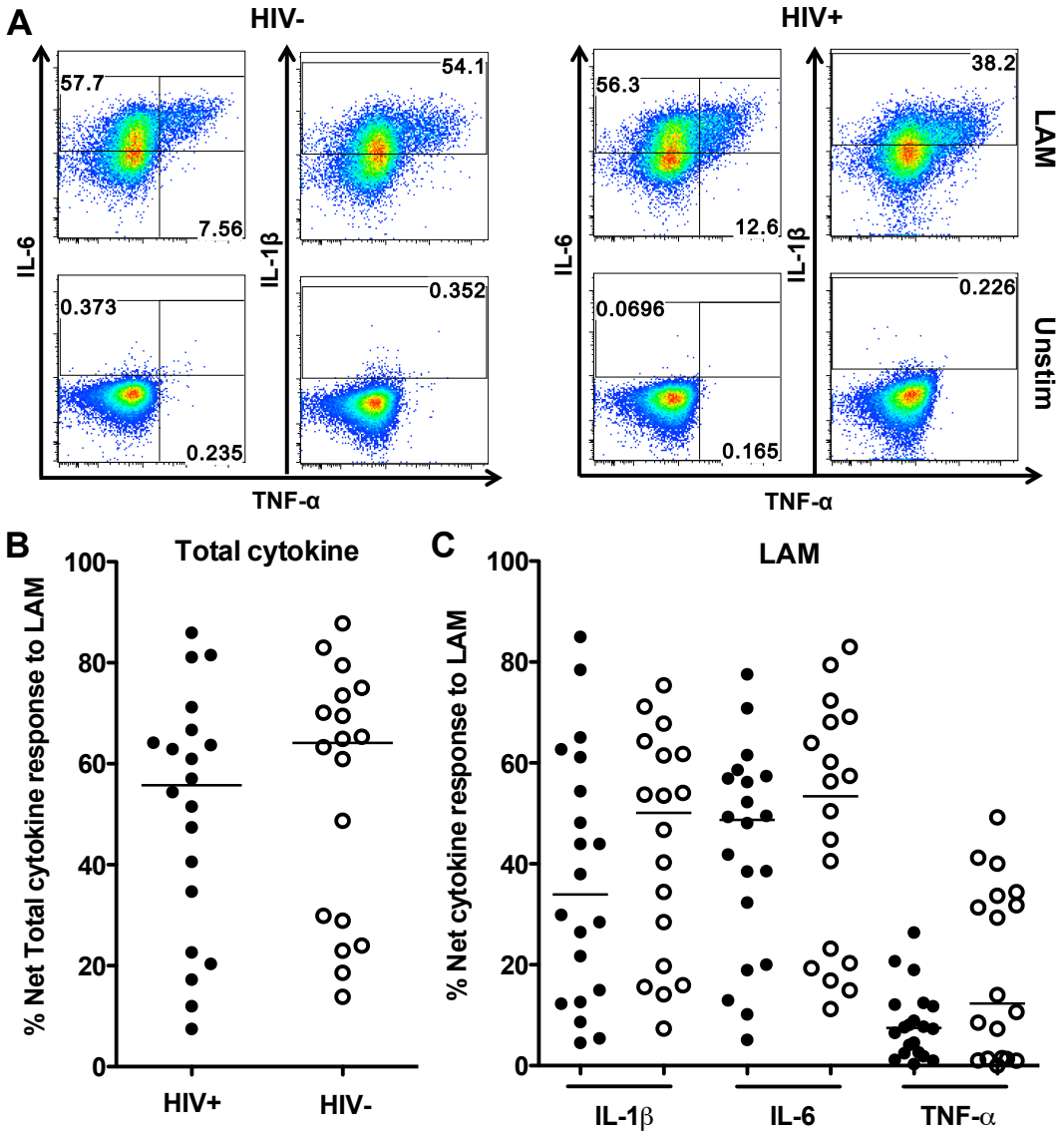

Figure 3.4: Effect of HIV on cytokine production by monocytes in response to LAM. A shows representative flow cytometry plots of LAM-stimulated and unstimulated monocytes from an HIV-uninfected and HIV-infected individual. The numbers denote frequency of monocytes producing cytokines. **B** shows the frequency of total monocyte cytokine responses for n=20 HIV-infected and n=18 HIV-uninfected individuals (background subtracted). Filled circles represent HIV-infected individuals, while open circles represent HIV-uninfected individuals. **C** shows the frequencies of cells producing IL-1β, IL-6 or TNF-α (background subtracted). Statistical comparisons were determined by a Mann-Whitney nonparametric t-test.

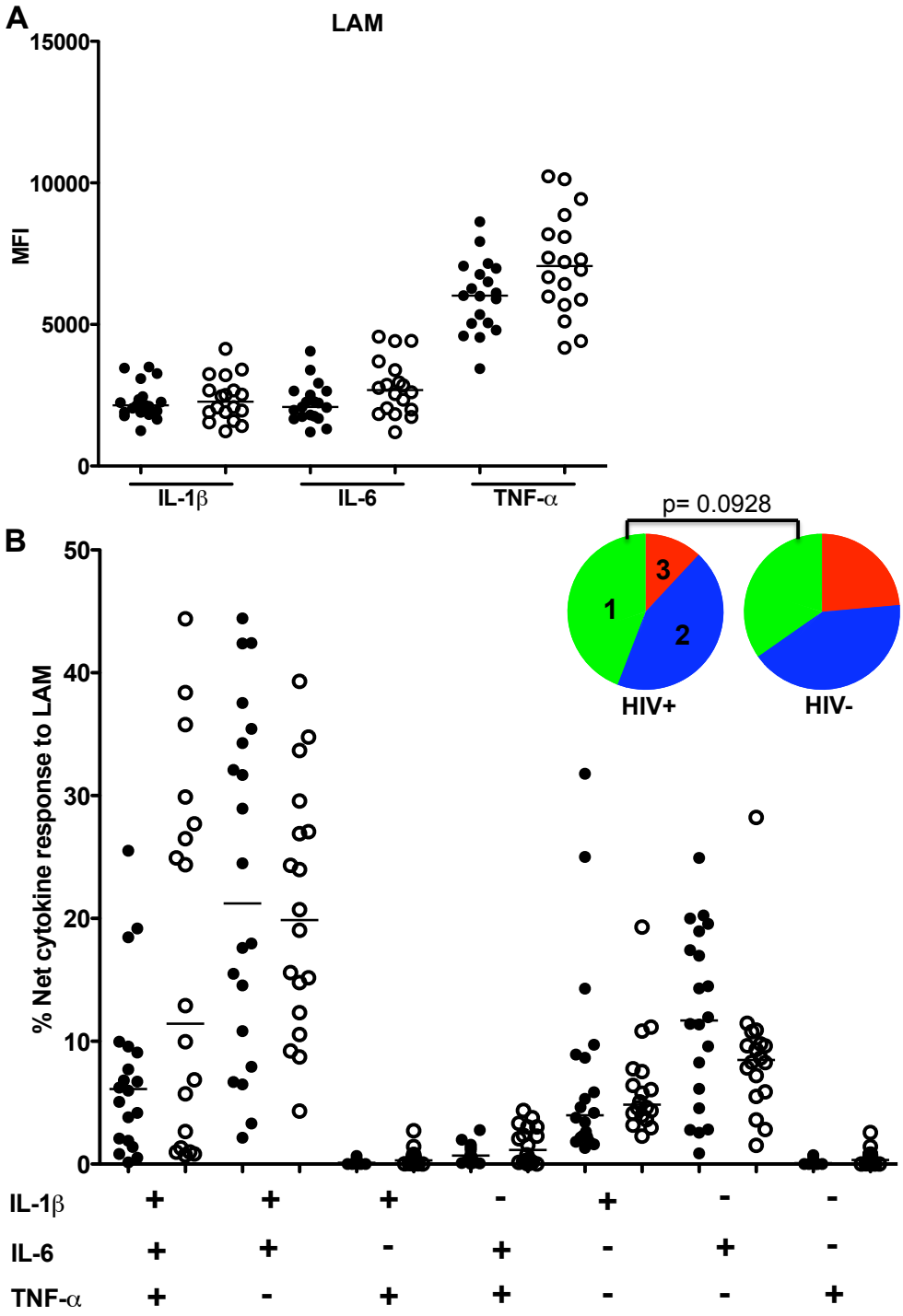

Figure 3.5: Effect of HIV on the functional profiles of monocytes in response to LAM. **A** shows MFI (median fluorescent intensity) of IL-1β, IL-6 and TNF-α in monocytes after stimulation with LAM, for n=20 HIV-infected and n=18 HIV-uninfected individuals. Filled circles represent HIV-infected individuals, while open circles represent HIV-uninfected individuals. **B** shows the polyfunctional capacity of monocytes when stimulated with LAM, where the pie charts represent the proportion of cells (of the total response) producing any one cytokine (green), any two cytokines (blue) or any three cytokines (red), and the graph shows the frequency of responses for combinations of different cytokines. Statistical comparisons were determined by a Mann-Whitney nonparametric t-test.

3.4.2 BCG

Monocyte cytokine responses to a viable mycobacteria, BCG, was next compared between HIV-infected (n=19) and HIV-uninfected individuals (n=18). One individual from the HIV-infected group was excluded due to very few events acquired in the sample. **Figure 3.6A** shows representative flow plots of cytokine production from monocytes when stimulated with BCG or left unstimulated. No difference in the cytokine response to BCG was detected (p=0.64) between HIV-infected and uninfected individuals (median 81.5%, IQR: 77-89; median 83.2%, IQR: 73-85**,** respectively; **Figure 3.6B).** Furthermore, when individual cytokines were examined, again no difference was observed in IL-1β, IL-6 or TNF-α production in response to BCG (p=0.66, p=0.82, p=0.29 respectively; **Figure 3.6C**). Variability among the donors in their production of TNF-α was observed upon BCG stimulation. Also, as with LAM, the majority of monocytes tended to produce IL-1β and IL-6, and almost 2-fold less TNF-α in response to BCG.

The functional profile of monocytes was next compared between the HIV-infected and uninfected participants. No difference was observed in the amount of IL-1β, IL-6 or TNF-α produced per monocyte between the two groups (p=0.55, p=0.48, p=0.49 respectively; **Figure 3.7A**). Furthermore, no difference was detected in the polyfunctional capacity of monocytes between HIV-infected and HIV-uninfected individuals **(Figure 3.7B).** These data suggest that HIV infection does not alter monocyte cytokine response to BCG. The majority of the monocytes produced all three cytokines simultaneously or a combination of IL-1β and IL-6, whilst a lower frequency of monocytes produced either IL-1β or IL-6 only, in response to BCG stimulation **(Figure 3.7B).**

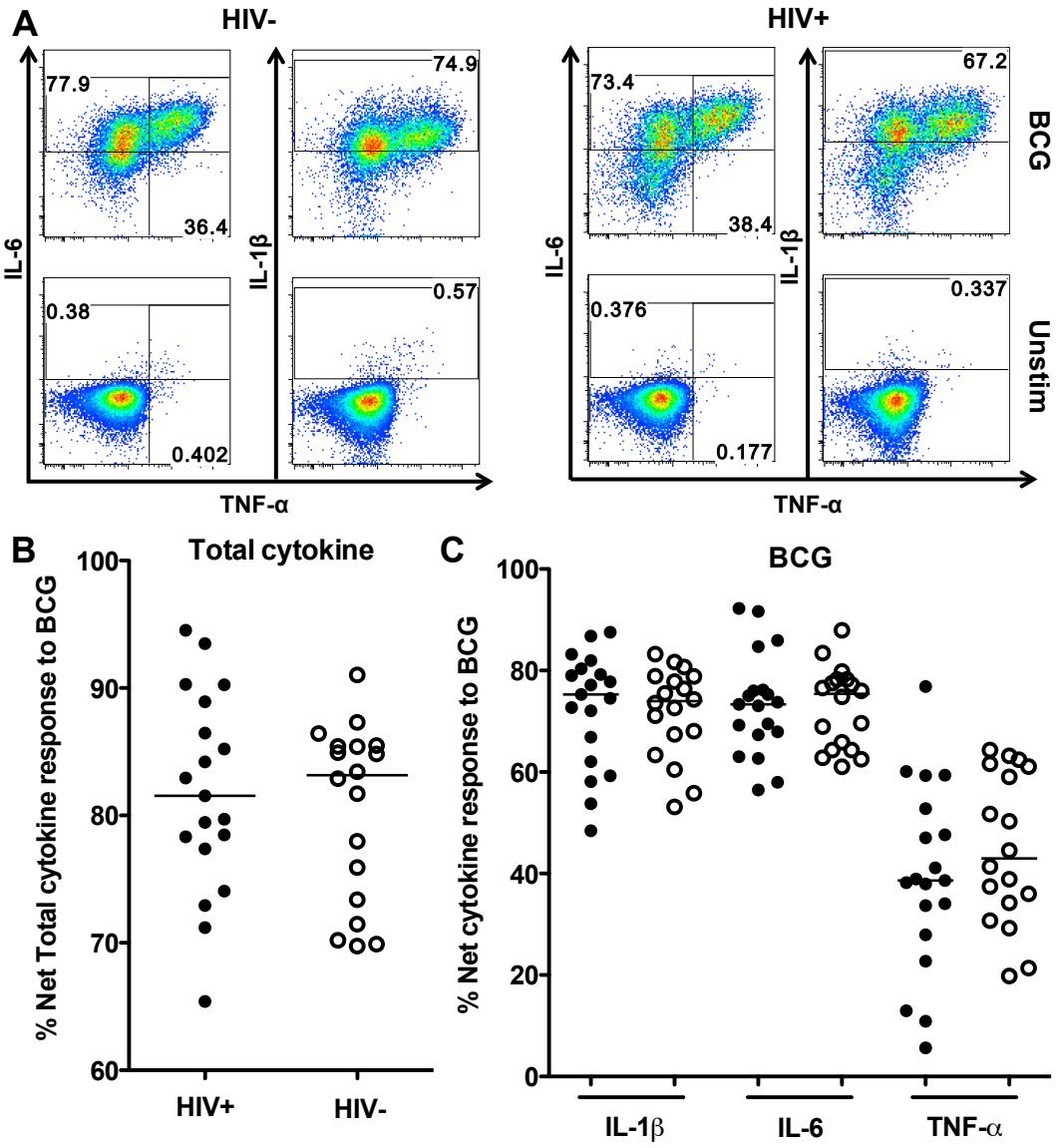

Figure 3.6: Effect of HIV on cytokine production by monocytes in response to BCG. A shows representative flow cytometry plots of BCG-stimulated and unstimulated monocytes from an HIV-uninfected and HIV-infected individual. The numbers denote frequency of monocytes producing cytokines. **B** shows the frequency of the total monocyte cytokine response for n=19 HIV-infected and n=18 HIV-uninfected individuals (background subtracted). Filled circles represent HIV-infected individuals, while open circles represent HIV-uninfected individuals. **C** shows the frequencies of cells producing IL-1β, IL-6 or TNF-α (background subtracted). Statistical comparisons were determined by a Mann-Whitney nonparametric t-test.

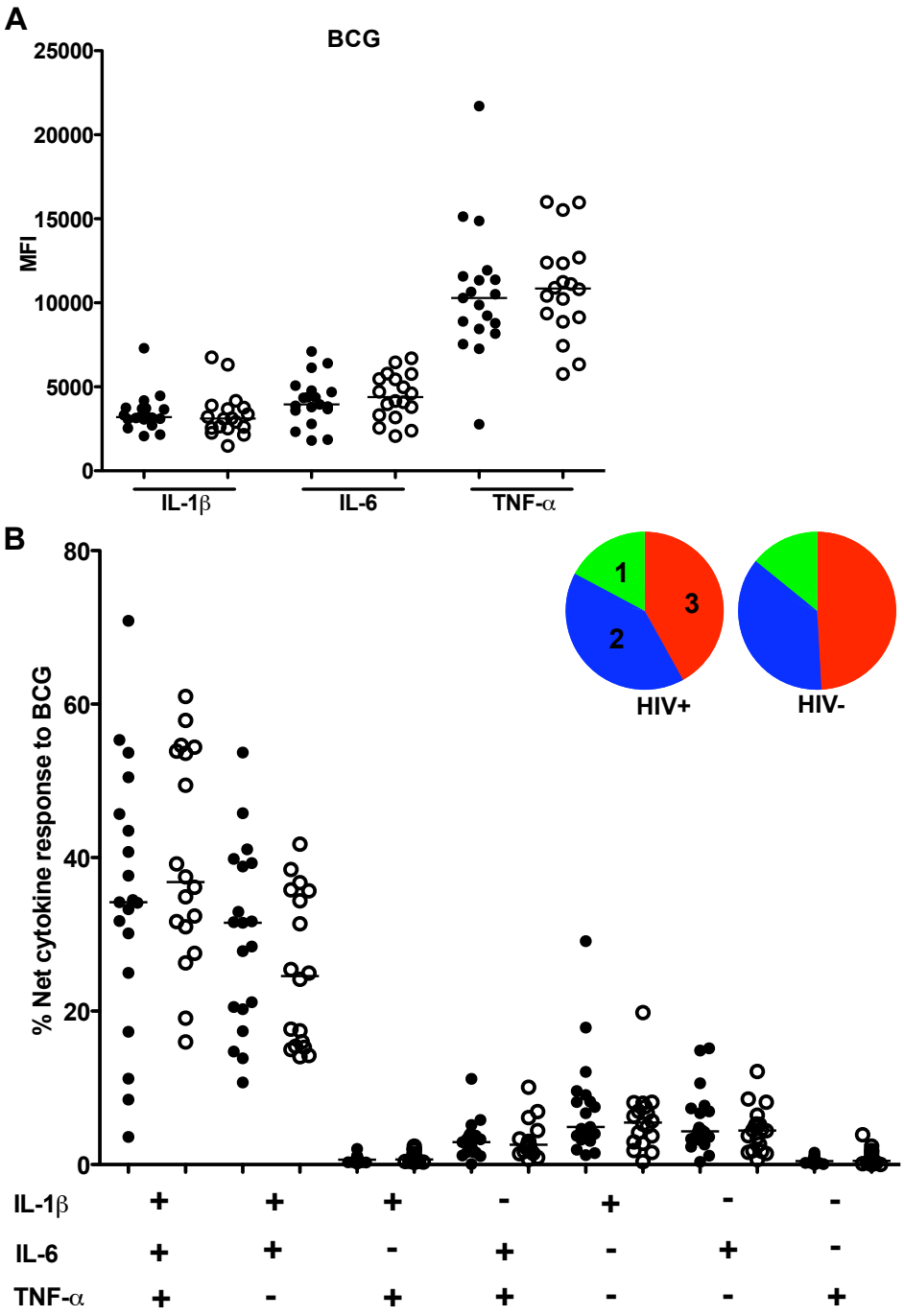

Figure 3.7: Effect of HIV on the functional profiles of monocytes in response to BCG. A shows MFI (median fluorescent intensity) of IL-1β, IL-6 and TNF-α in monocytes after stimulation with BCG, for n=19 HIV-infected and n=18 HIV-uninfected individuals. Filled circles represent HIV-infected individuals, while open circles represent HIV-uninfected individuals. **B** shows the polyfunctional capacity of monocytes when stimulated with BCG, where the pie charts represent the proportion of cells (of the total response) producing any one cytokine (green), any two cytokines (blue) or any three cytokines (red), and the graph shows the frequency of responses for combinations of different cytokines. Statistical comparisons were determined by a Mann-Whitney nonparametric t-test.

3.4.3 PPD

Monocyte cytokine responses to M.tb PPD were compared between HIV-infected (n=19) and HIV-uninfected individuals (n=18). One individual from the HIV-infected group was excluded due to very few events acquired in that sample. **Figure 3.8A** shows representative flow cytometry plots of cytokine production from monocytes when stimulated with PPD or left unstimulated. No difference in the cytokine response to PPD was observed (p=0.29) between HIV-infected and uninfected participants (median 11%, IQR: 4.7-17.4; median 9%, IQR: 2.3-14.4, respectively; **Figure 3.8B**). Furthermore, when individual cytokines were assessed, no significant difference was observed in IL-1β, IL-6 or TNF-α production in response to PPD between the two groups (p=0.35, p=0.52, p=0.64 respectively; **Figure 3.8C**). Once again, considerable variability was observed amongst the donors in their production of IL-1β and IL-6 in response to PPD. Also, as with LAM and BCG stimulation, the majority of monocytes tended to produce IL-1β and IL-6, with very few monocytes producing TNF-α.

The functional profile of monocytes was compared between HIV-infected and uninfected participants, and there was no significant difference in the amount of IL-1β, IL-6 or TNF-α produced per monocyte between the two groups, however, a trend of higher levels of IL-6 from monocytes of HIV-uninfected individuals was observed (p=0.87, p=0.07, p=0.26 respectively; **Figure 3.9A**). In addition, no difference in the polyfunctional capacity of monocytes was observed between HIV-infected and HIV-uninfected individuals in response to PPD **(Figure 3.9B).** These results demonstrate that HIV co-infection did not alter the response of monocytes to M.tb PPD. The majority of monocytes were monofunctional in response to PPD, and either produced IL-6 or IL-1β only, while a lower proportion of monocytes produced a combination of IL-1β and IL-6 **(Figure 3.9B)**.

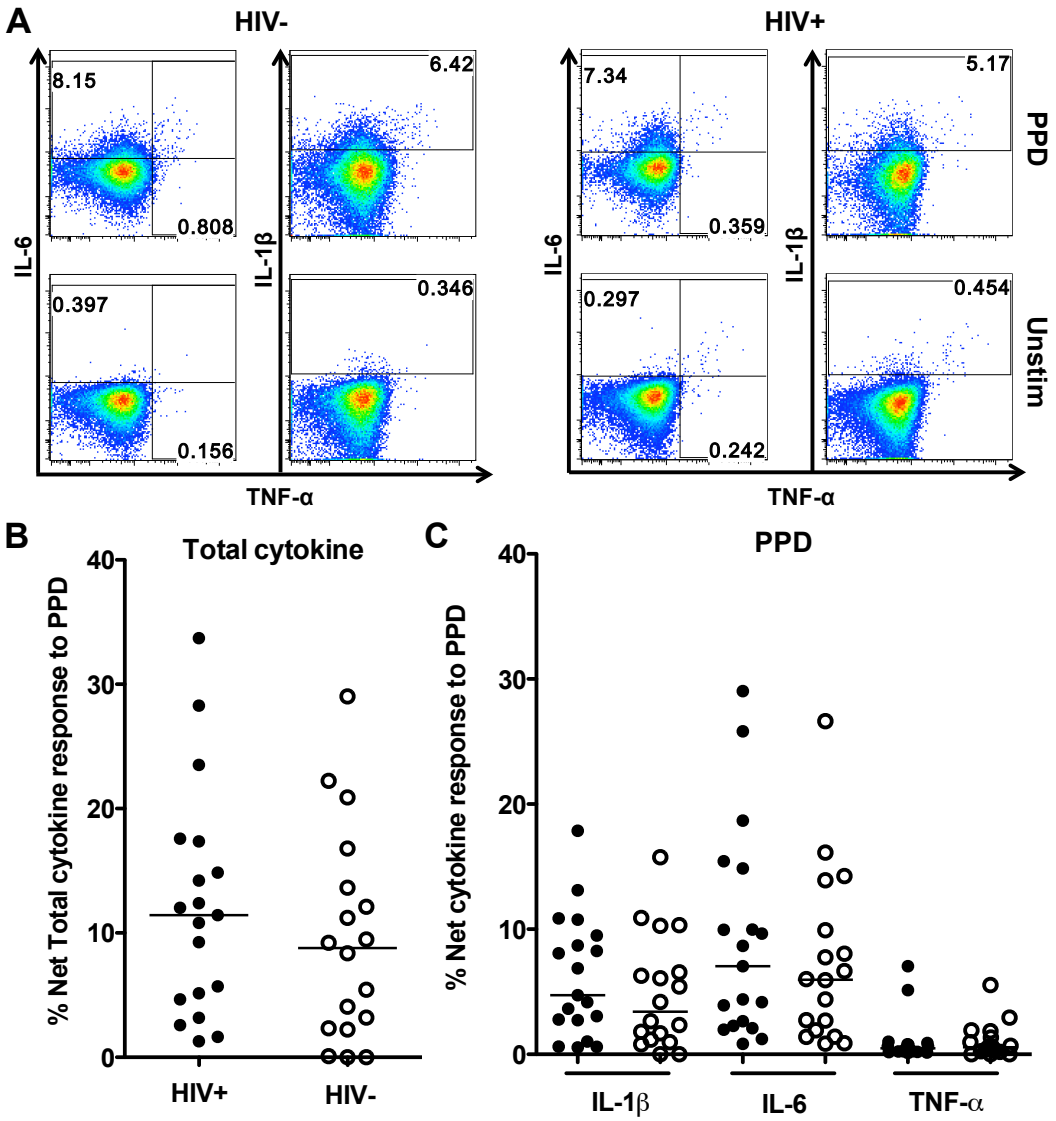

Figure 3.8: Effect of HIV on cytokine production by monocytes in response to PPD. A shows representative flow cytometry plots of PPD-stimulated and unstimulated monocytes from an HIV-uninfected and HIV-infected individual. The numbers denote frequency of monocytes producing cytokines. **B** shows the frequency of total monocyte cytokine response for n=19 HIV-infected and n=18 HIV-uninfected individuals (background subtracted). Filled circles represent HIV-infected individuals, while open circles represent HIV-uninfected individuals. **C** shows the frequencies of cells producing IL-1β, IL-6 or TNF-α (background subtracted). Statistical comparisons were determined by a Mann-Whitney nonparametric t-test.

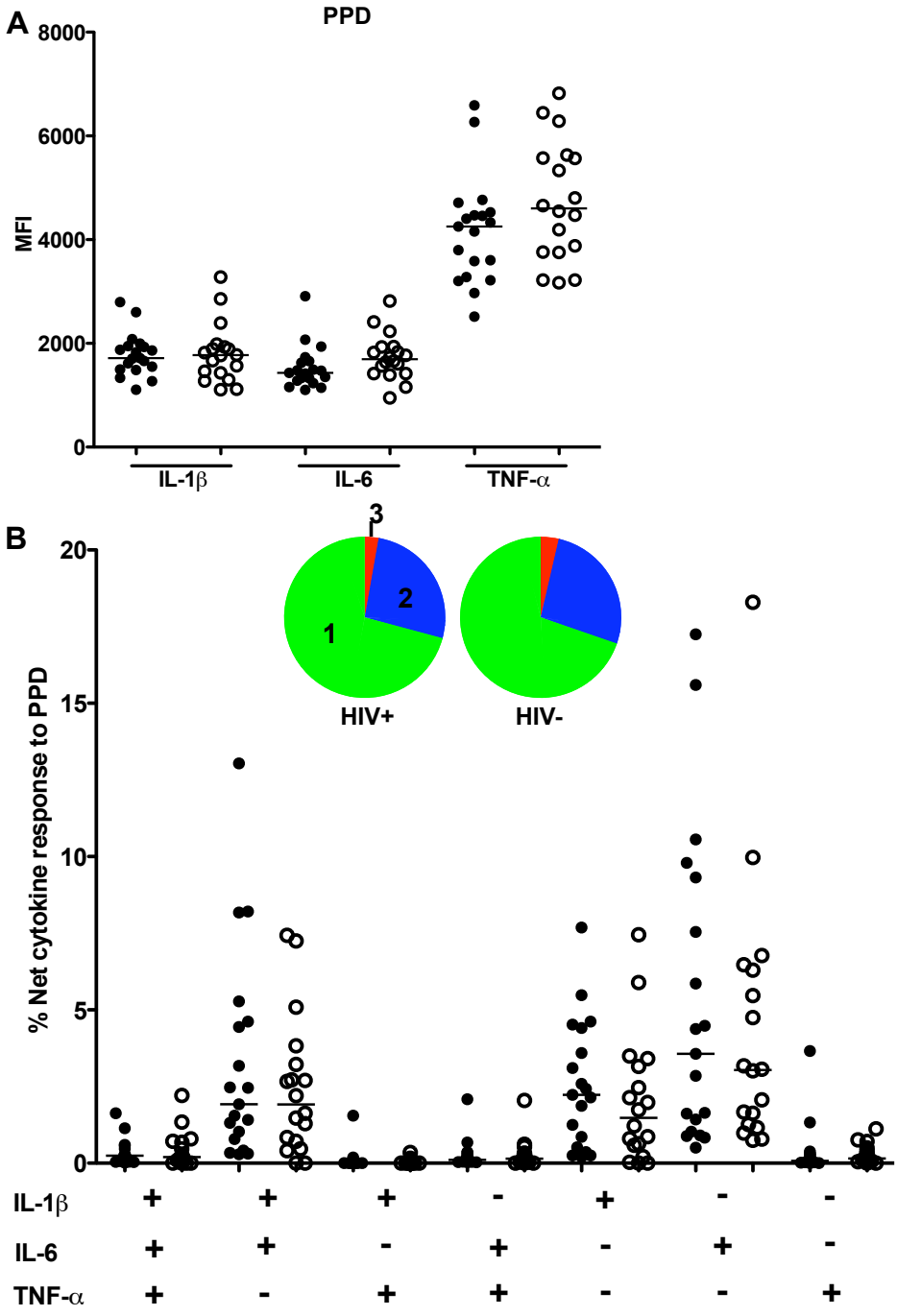

Figure 3.9: Effect of HIV on the functional profiles of monocytes in response to PPD. A shows MFI (median fluorescent intensity) of IL-1 β, IL-6 and TNF- α in monocytes after stimulation with PPD, for n=19 HIV-infected and n=18 HIV-uninfected individuals. Filled circles represent HIV-infected individuals, while open circles represent HIV-uninfected individuals. **B** shows the polyfunctional capacity of monocytes when stimulated with PPD, where the pie charts represent the proportion of cells (of the total response) producing any one cytokine (green), any two cytokines (blue) or any three cytokines (red), and the graph shows the frequency of responses for combinations of different cytokines. Statistical comparisons were determined by a Mann-Whitney nonparametric t-test.

3.4.4 LPS

Monocytes can detect the gram-negative bacterial cell wall component LPS, through its receptors CD14 and TLR4 (as reviewed by Triantafilou & Triantafilou, 2002). LPS was used as a positive control for monocyte cytokine production, as it is not a mycobacterial-derived ligand. To examine the impact of HIV infection on the ability of monocytes to respond to LPS, cytokine responses were compared between HIV-infected (n=18) and HIV-uninfected individuals (n=18). Two individuals from the HIV-infected group were excluded due to insufficient events acquired in those samples. **Figure 3.10A** shows representative flow plots of cytokine production from monocytes when stimulated with LPS or left unstimulated. In contrast to the results with mycobacterial stimulation of monocytes, HIV-infected individuals mounted a significantly higher cytokine response to LPS (p=0.002), with a median of 65% (IQR: 55.7-72.3) of monocytes producing cytokines, compared to HIV-uninfected individuals (median 45%, IQR: 29.3-60.6; **Figure 3.10B**). Furthermore, when individual cytokines were examined, the percentage of IL-1β+, IL-6+ and TNF-α+ monocytes were all significantly higher in HIV infection in response to LPS (p=0.016, p=0.003, p=0.009, respectively; **Figure 3.10C**). Considerable variability was observed among the donors in their production of the three cytokines in response to LPS. As observed previously with mycobacterial stimuli, the majority of monocytes tended to produce IL-1β and IL-6, with fewer monocytes producing TNF-α. In summary, monocyte responses to LPS stimulation were significantly elevated in individuals infected with HIV, compared to HIV-uninfected individuals.

Next, cytokine expression levels and the polyfunctional capacity of monocytes was compared between the two groups, to investigate whether any functional differences in the monocyte response to LPS was observed in HIV infection. Analysis of cytokine MFI showed no difference in the amount of IL-1β, IL-6 or TNF-α produced per monocyte between HIV-infected and uninfected individuals (p=0.223, p=0.261, p=0.359 respectively; **Figure 3.11A**). However, comparison of the polyfunctional capacity of monocytes revealed differences in the functional profiles of monocyte responses in HIV-infected and uninfected individuals. HIV-infected individuals displayed a significantly greater proportion of monocytes with the capacity to produce all three cytokines simultaneously in response to LPS (p=0.041), and a trend

towards a decreased proportion of monocytes producing one cytokine (p=0.085; pie charts in **Figure 3.11B**). The frequency of monocytes producing all three cytokines, or a combination of IL-1β and IL-6, or IL-6 together with TNF-α, was significantly greater in HIV-infected individuals (p=0.013, p=0.038 and p=0.011, respectively). These data demonstrate that the response of monocytes to the TLR4 ligand LPS was significantly enhanced during HIV infection. In contrast, the cytokine response of monocytes to LAM, PPD and BCG was similar between HIV-infected and uninfected individuals.

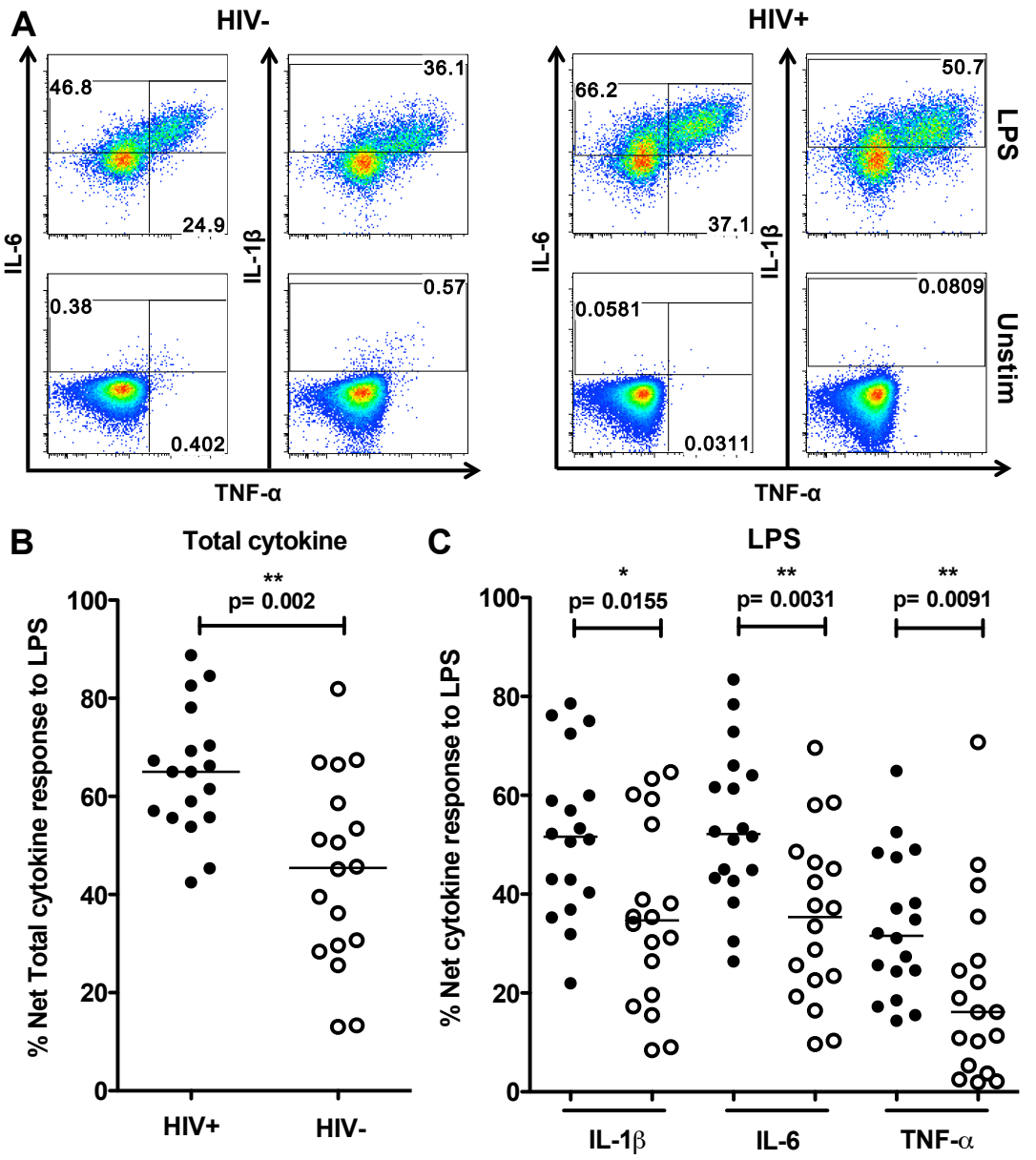

Figure 3.10: Effect of HIV on cytokine production by monocytes in response to LPS. A shows representative flow cytometry plots of LPS-stimulated and unstimulated monocytes from an HIV-uninfected and HIV-infected individual. The numbers denote frequency of monocytes producing cytokines. **B** shows the frequency of total monocyte cytokine responses for n=18 HIV-infected and n=18 HIV-uninfected individuals (background subtracted). Filled circles represent HIV-infected individuals, while open circles represent HIV-uninfected individuals. **C** shows the frequencies of cells producing IL-1β, IL-6 or TNF-α (background subtracted). Statistical comparisons were determined by a Mann-Whitney nonparametric t-test.

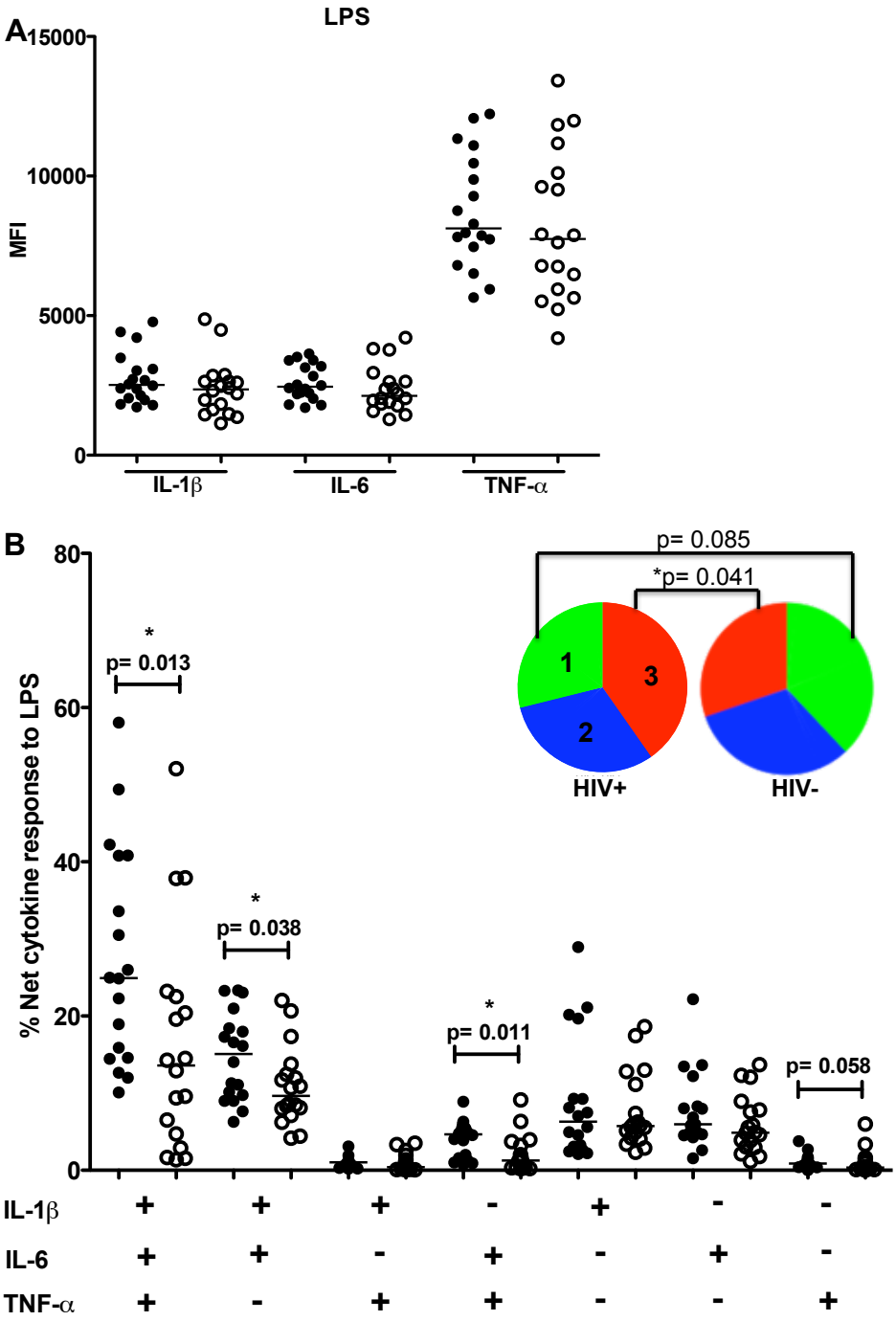

Figure 3.11: Effect of HIV on the functional profiles of monocytes in response to LPS. **A** shows MFI (median fluorescent intensity) of IL-1β, IL-6 and TNF-α in monocytes after stimulation with LPS, for n=18 HIV-infected and n=18 HIV-uninfected individuals. Filled circles represent HIV-infected individuals, while open circles represent HIV-uninfected individuals. **B** shows the polyfunctional capacity of monocytes when stimulated with LPS, where the pie charts represent the proportion of cells (of the total response) producing any one cytokine (green), any two cytokines (blue) or any three cytokines (red), and the graph shows the frequency of responses for combinations of different cytokines. Statistical comparisons were determined by a Mann-Whitney nonparametric t-test.

3.5. Association of monocyte cytokine responses with clinical characteristics

HIV-infected participants exhibited a wide range of cytokine responses, sometimes 2-fold different between individuals upon stimulation of monocytes with LAM, BCG, PPD or LPS. Since the HIV-infected participants displayed a wide range of plasma viral loads (median 3.9, IQR: 3.3-4.4 log RNA copies/ml) and CD4+ T cell counts (median 581, IQR: 530-759 cell/mm^3; **Table 3.1**), the relationship between monocyte cytokine responses and these clinical characteristics was assessed.

3.5.1 Plasma HIV viral loads

The relationship between monocyte cytokine responses in HIV-infected individuals and their plasma viral loads revealed no correlation following stimulation of monocytes with either LAM, BCG, PPD or LPS ($p=0.57$, $r=-0.134$; $p=0.95$, $r=-0.016$; $p=0.98$, $r=0.0053$; $p=0.58$, $r=-0.141$, respectively; **Figure 3.12 A-D**). When assessing the relationship of individual cytokines IL-1β, IL-6 or TNF-α with plasma viral loads, again no correlation was found (data not shown). In addition, the polyfunctional capacity of monocytes (ability to produce either one, two or three cytokines simultaneously) was examined for an association with plasma viral loads and demonstrated no correlation (data not shown). Overall, these data show that monocyte responses to TLR stimuli in the HIV-infected group were not associated with HIV viral loads.

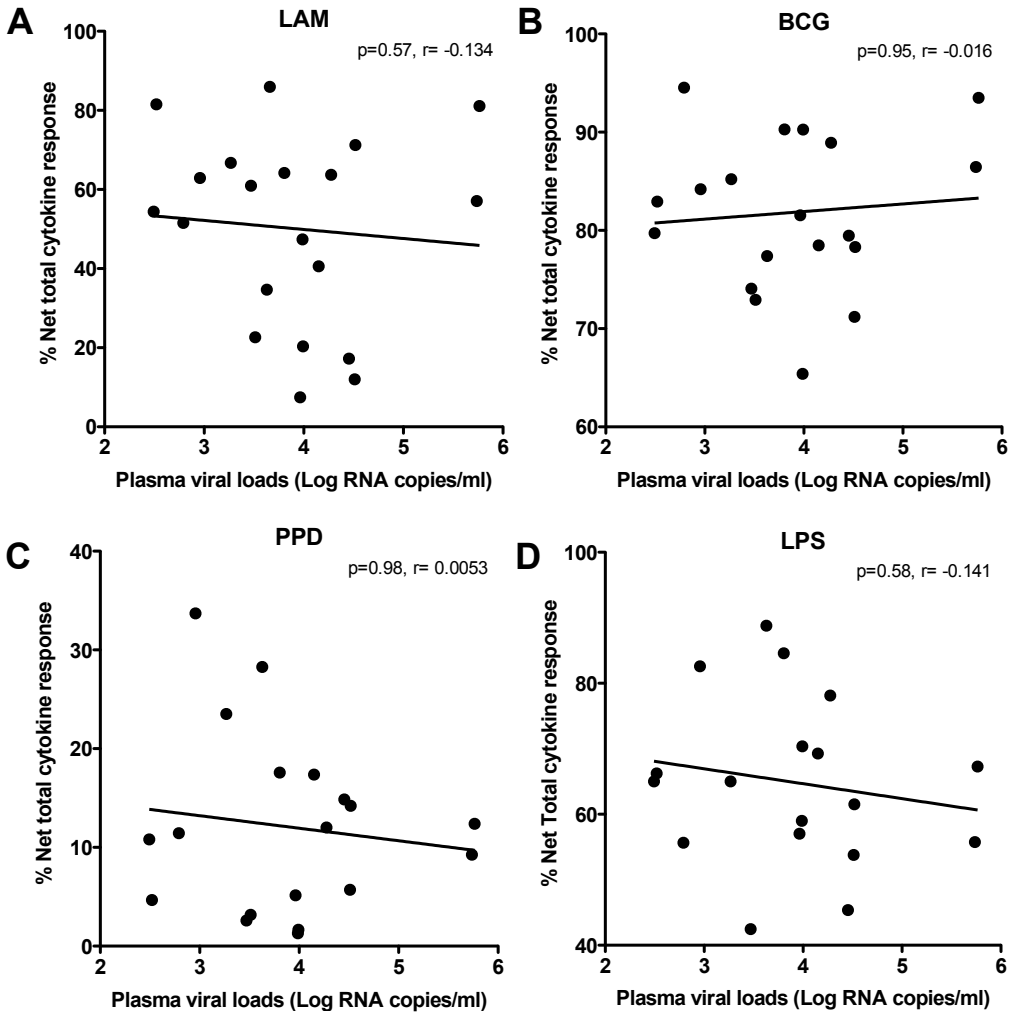

Figure 3.12: Relationship between monocyte cytokine responses and plasma viral load. Monocyte cytokine production in response to LAM **(A)**, BCG **(B)**, PPD **(C)** and LPS **(D)** were examined for an association with HIV plasma viral loads in n=18-20 HIV-infected individuals. The correlation coefficient (r) and p-values (p) are indicated on the graphs. Statistical associations were performed using a two-tailed non-parametric Spearman rank correlation.

3.5.2 CD4+ T cell count

Similarly, the relationship between monocyte cytokine responses in HIV-infected individuals and CD4 T cell count was assessed. These two parameters demonstrated no correlation following stimulation of monocytes with either LAM, BCG, PPD or LPS (p=0.82, r=-0.954; p=0.95, r=-0.016; p=0.55, r=0.147; p=0.53, r=0.16, respectively; **Figure 3.13 A-D**). When assessing the relationship of individual cytokine responses with CD4 T cell counts, again no correlation was found (data not shown).

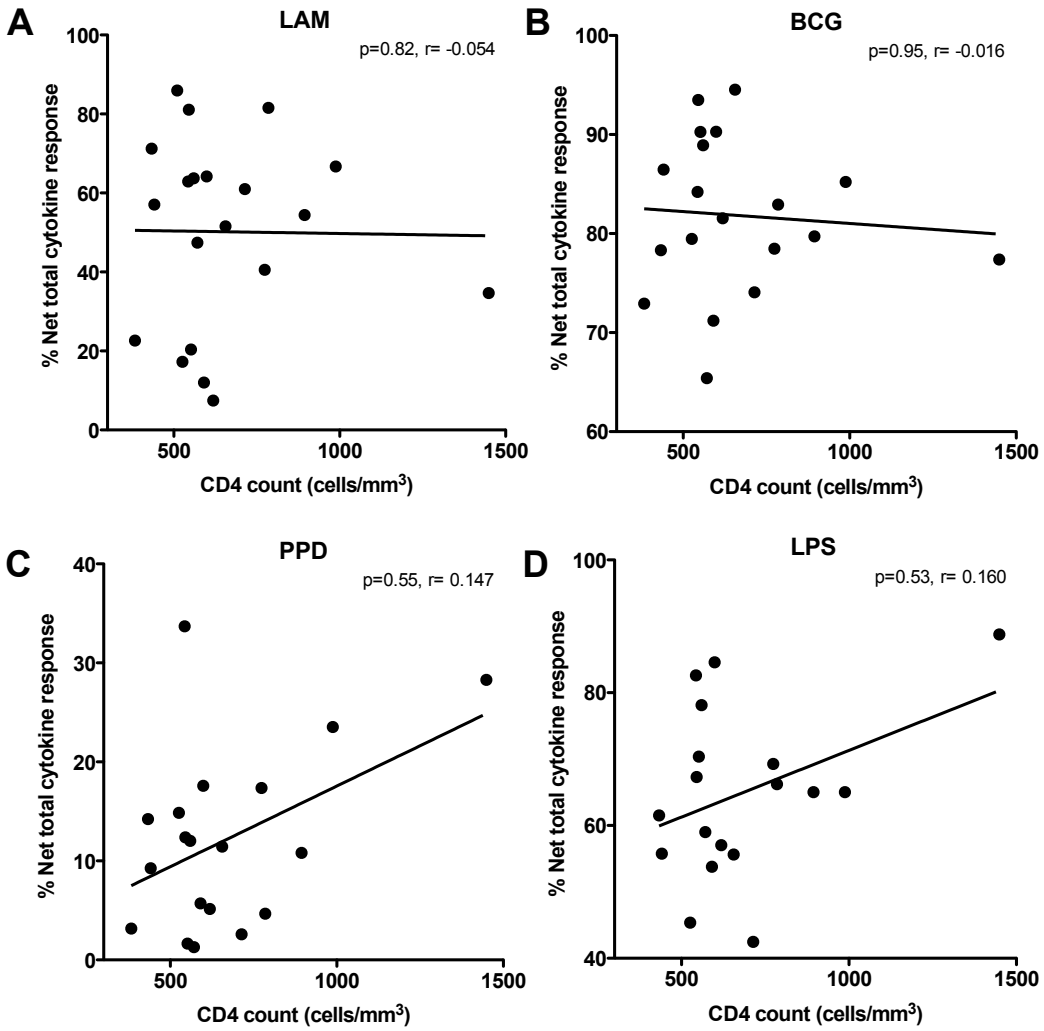

Figure 3.13: Relationship between monocyte cytokine responses and CD4+ T cell count. Monocyte cytokine production in response to LAM **(A)**, BCG **(B)**, PPD **(C)** and LPS **(D)** were examined for an association with CD4+ T cell count in n=18-20 HIV-infected individuals. The correlation coefficient (r) and p-values (p) are indicated on the graphs. Statistical associations were performed using a two-tailed non-parametric Spearman rank correlation.

3.5.3 Age

Since age was significantly different ($p<0.0004$) between HIV-infected individuals (median 32, IQR: 29-39 and HIV-uninfected individuals (median 22, IQR:19-25**; Table 3.1)** in this study, the association between monocyte cytokine responses and age was assessed. The monocyte cytokine responses did not correlate with age following stimulation with LAM, BCG, PPD or LPS ($p=0.19$, $r=-0.22$; $p=0.94$, $r=0.014$; $p=0.72$, $r=0.062$; $p=0.23$, $r=0.207$, respectively; **Figure 3.14 A-D**). When

assessing the relationship of individual cytokines IL-1β, IL-6 or TNF-α with age, again no correlation was found (data not shown).

These data demonstrate that monocyte cytokine responses to TLR stimuli were not affected by the age of the participants. Therefore, any differences in cytokine responses observed between the HIV-infected and uninfected groups were not influenced by age. Also, the cytokine responses in the HIV-infected individuals were not associated with plasma viral loads or CD4+ T cell count.

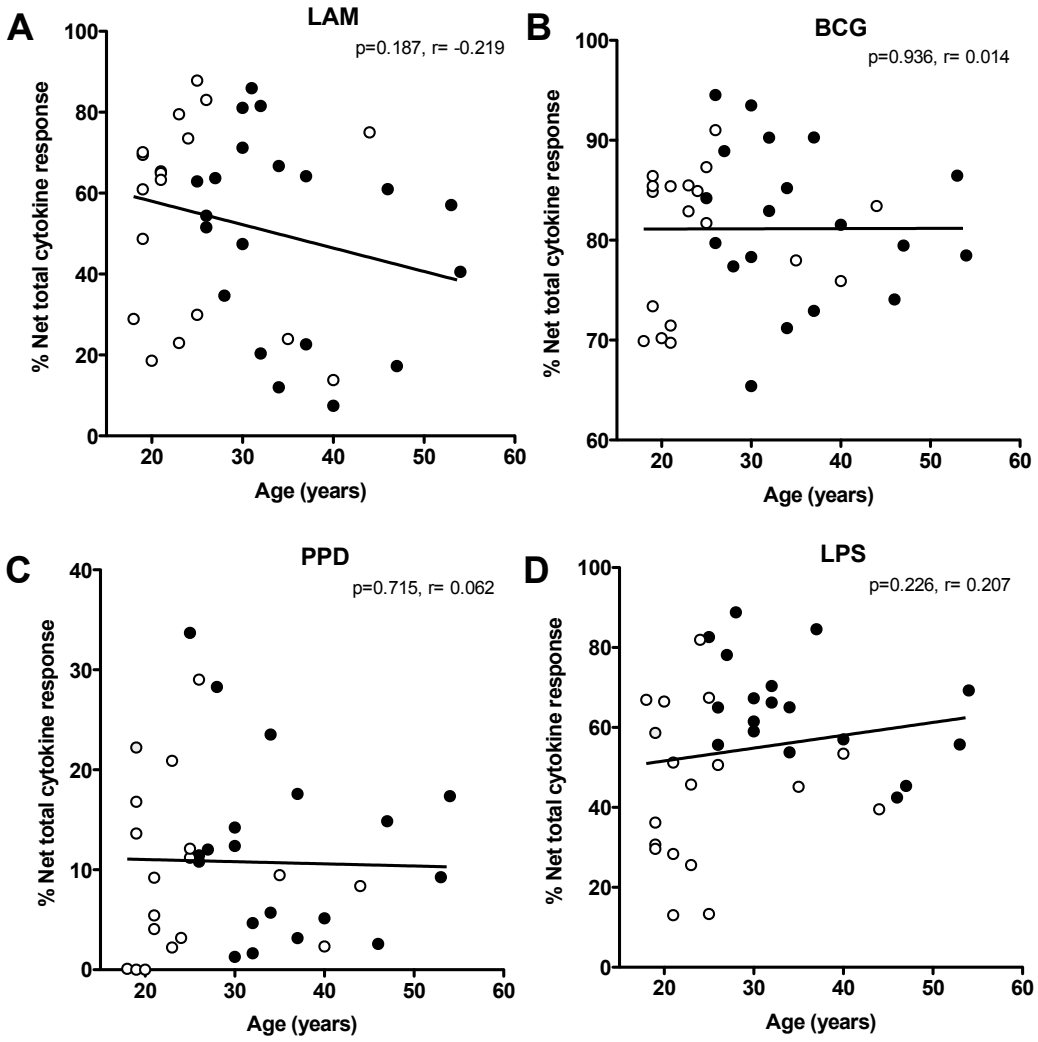

Figure 3.14: Relationship between monocyte cytokine responses and age. Monocyte cytokine production in response to LAM **(A)**, BCG **(B)**, PPD **(C)** and LPS **(D)** were examined for an association with age in n=18-20 HIV-infected and n=18 HIV-uninfected individuals. The correlation coefficient (r) and p-values (p) are indicated on the graphs. Filled circles represent HIV-infected individuals, while open circles represent HIV-uninfected individuals. Statistical associations were performed using a two-tailed non-parametric Spearman rank correlation.

3.6. Effect of HIV on CD14+ frequency and expression levels

The frequency of classical CD14+ monocytes and CD14 expression levels on monocytes were next compared between 20 HIV-infected individuals and 18 HIV-uninfected individuals.

The frequency of CD14+ monocytes was significantly lower (p=0.0328) in HIV-infected individuals (median 60%, IQR: 57-70) compared to HIV-uninfected individuals (median 70%, IQR 62-74; **Figure 3.15A**). No difference in CD14 expression levels on monocytes was observed between the two groups (p=0.77; **Figure 3.15B**). These results indicate that CD14+ monocytes are at a lower frequency during HIV infection, consistent with findings from a previous report (Han *et al.*, 2009).

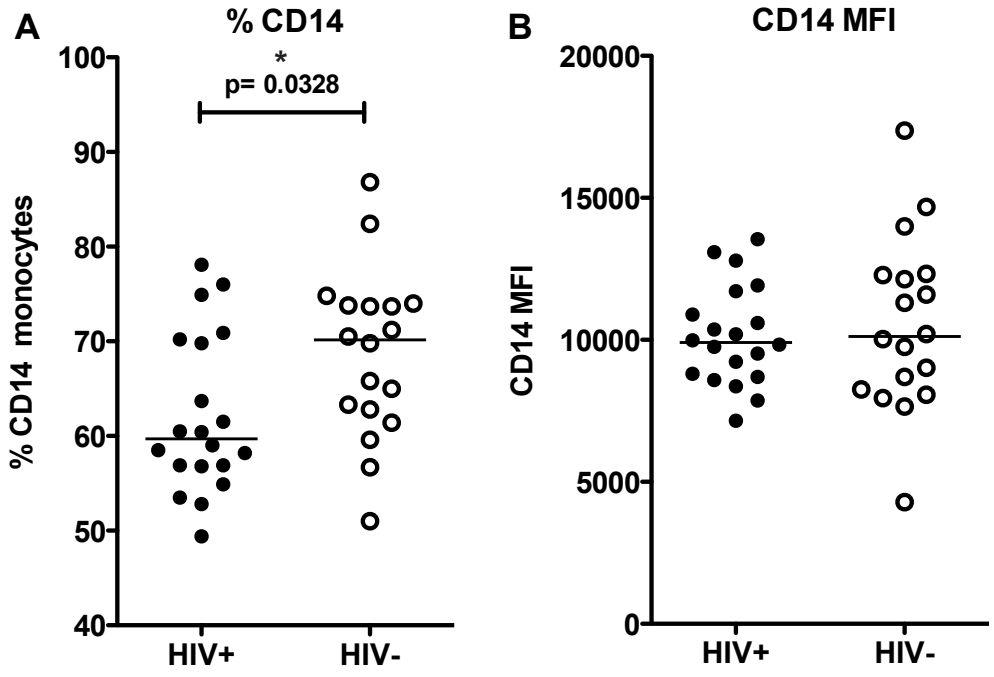

Figure 3.15: CD14+ expression on monocytes in HIV-infected and uninfected individuals. A compares the frequency of unstimulated CD14+ monocytes characterised by flow cytometry. **B** shows the expression levels (MFI) of CD14 on monocytes, from n=20 HIV-infected individuals and n=18 HIV-uninfected individuals. Filled circles represent HIV-infected individuals, while open circles represent HIV-uninfected individuals. Statistical comparisons were determined by a Mann-Whitney nonparametric t-test.

Next, the relationship between the frequency of CD14+ monocytes and plasma viral loads and CD4+ T cell counts was determined. There was no correlation between the frequency of CD14+ monocytes and HIV viral loads (p=0.37, r=0.211; **Figure 3.16A**) or with CD4 T cell counts (p=0.32, r=-0.232; **Figure 3.16B**). The association between the frequency of CD14+ monocytes with age was also determined, and no correlation was found (data not shown).

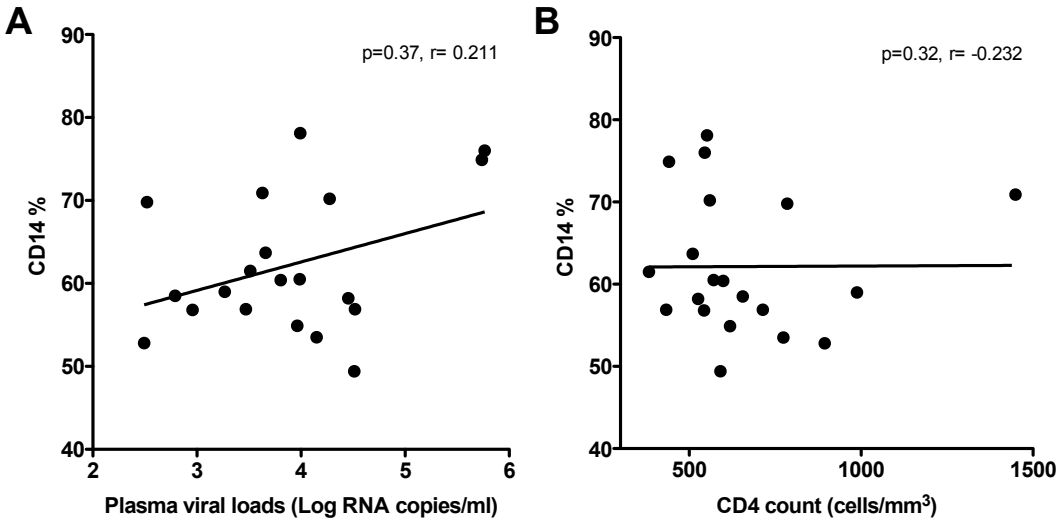

Figure 3.16: Relationship between frequency of monocytes and plasma viral loads and CD4+ T cell counts. The frequency of CD14+ monocytes were examined for an association with plasma viral loads **(A)** and CD4+ T Cell count **(B)** in n=20 HIV-infected individuals. The correlation coefficient (r) and p-values (p) are indicated on the graphs. Statistical associations were performed using a two-tailed non-parametric Spearman rank correlation.

Overall, these results indicate that there were significantly fewer CD14+ monocytes in HIV-infected individuals, and this was not associated with plasma viral loads or CD4 T cell count, consistent with findings from a previous report (Han *et al.*, 2009). These data demonstrate that HIV may have indirect effects on the frequency of classical CD14+ monocytes.

3.7. Loss of CD14 from stimulated monocytes

CD14, the receptor that is typically used for phenotypic identification of monocytes, is a myeloid membrane glycoprotein that serves as a pattern recognition molecule and participates in the host defense against various microbial pathogens (as reviewed by Antal-Szalmás, 2000; Pugin *et al.*, 1994). Specifically, it acts as a receptor for

LPS, LPS-binding protein as well as other host and bacterial components (as reviewed by Triantafilou & Triantafilou, 2002). CD14 plays a role as an accessory co-receptor not only for TLR4, but also for TLR2. Besides LPS-induced activation of TLR4/CD14, other bacterial components such as lipoteichoic acid and mycobacterial LAM activate TLR2/CD14 (Schröder *et al.*, 2003; Zhang *et al.*, 1993). In fact, it has been demonstrated that LAM acts similarly to LPS in activating monocytes through CD14 (Savedra *et al.*, 1996; Yu et *al.*, 1998). Interaction of microbial ligands with the TLR-CD14 complex leads to the activation of monocytes and results into the production of various pro-inflammatory cytokines (as reviewd by Landmann et al., 2000). Upon stimulation with these ligands, monocytes shed CD14 from their surface as the soluble form of CD14 (sCD14; Bufler *et al.*, 1995; Landmann *et al.*, 1996; Orr & Tobias, 2000). CD14 shedding is induced as a consequence of monocyte activation and serves to down-modulate CD14 on stimulated monocytes (Bazil & Strominger, 1991). This may function as a regulatory mechanism to prevent an excessive pro-inflammatory response.

3.7.1 Differential loss of CD14 in response to TLR stimulation

To investigate CD14 expression on monocytes upon activation with different TLR stimuli, the loss of CD14 from monocytes was determined from 18 healthy, HIV-uninfected individuals.

The frequency of CD14+ monocytes in response to different TLR stimuli is shown in **Figure 3.17A**. There was a significant reduction in the frequency of CD14+ monocytes after they were stimulated with LAM, BCG and LPS ($p<0.01$, $p<0.01$ and $p<0.001$, respectively), although no such reduction was observed for PPD. Since intracellular staining was performed to detect CD14, which would have labeled the marker both on the surface and intracellularly, the reduced frequency of CD14+ monocytes could not be attributed to down-regulation of the CD14 receptor, but was likely due to a complete shedding of CD14 from a proportion of monocytes, resulting in a lower frequency of CD14+ monocytes compared to the baseline, unstimulated control. As a result, the median frequency of the monocyte population was significantly reduced, from 70% when left unstimulated to 52% upon stimulation with LAM, 47% with BCG and 45% after LPS stimulation. In addition, a significant

reduction in the expression levels of CD14 on monocytes, as measured by the MFI, was observed upon stimulation with LAM, BCG and LPS (MFI of 7445, 6869 and 7558, respectively; $p<0.001$ after adjustment for multiple comparisons, compared to the unstimulated sample with an MFI of 10118; **Figure 3.17B**). This reduction in MFI of CD14 indicates partial shedding of CD14 on a proportion of the monocytes. These findings indicate that besides LPS, other bacterial cell wall constituents, namely LAM and components of BCG, also contribute to the loss of the CD14 receptor.

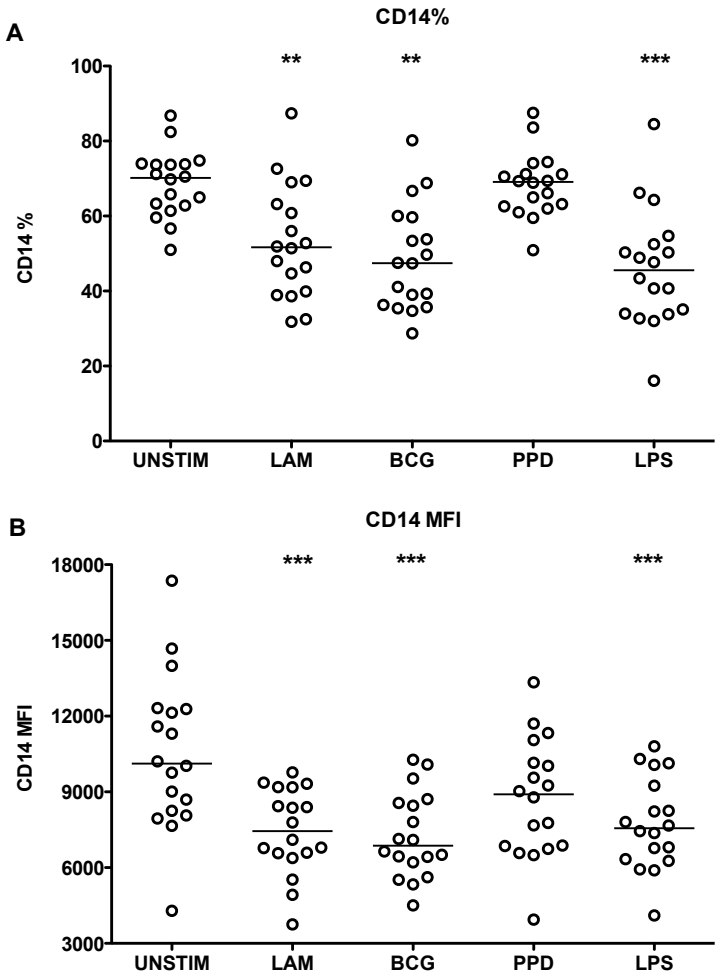

Figure 3.17: CD14 expression on monocytes in response to TLR stimulation. A shows the frequency of CD14+ monocyte population and **B** shows the expression levels (MFI) of CD14 on monocytes from n=18 healthy HIV-uninfected individuals upon stimulation with LAM, BCG, PPD and LPS, or when left unstimulated. Statistical analyses was performed using a one-way ANOVA with Dunn's correction for multiple comparisons (**$p<0.01$, ***$p<0.001$).

3.7.2 Effect of HIV infection on loss of CD14 from monocytes

To determine whether HIV infection affected the shedding of CD14 from monocytes in response to the different TLR stimulations, the frequency and expression levels of CD14 on monocytes were compared between 18 HIV-uninfected and 20 HIV-infected individuals in response to LAM, BCG, PPD and LPS **(Figure 3.18A and B)**. The frequency of the CD14+ population was reduced compared to the unstimulated baseline sample in the same manner as was observed for HIV-uninfected individuals in response to LAM, BCG and LPS stimulation (HIV-: p=0.0014, HIV+: p=0.0018; HIV-: p=0.0003, HIV+: p=0.0001; HIV-: p=0.0002, HIV+: p=0.0002, respectively). Similarly, as for HIV-uninfected individuals, no significant reduction in the frequency of the CD14 population was observed in response to PPD in HIV-infected individuals. Since PPD activated monocytes to the lowest levels compared to the other stimuli (as measured by the cytokine production from monocytes, described previously), CD14 shedding may only be occurring at very low levels.

The expression level of CD14 on monocytes from HIV-uninfected individuals **(Figure 3.19A)** and HIV-infected individuals **(Figure 3.19B)** in response to the four stimuli was investigated. Again, as with the frequency of the CD14 population, a reduction in the MFI of CD14 was observed in HIV-infected individuals, as for HIV-uninfected individuals. The CD14 expression levels were significantly reduced compared to the unstimulated sample in response to all the TLR stimuli, namely LAM, BCG, PPD and LPS (HIV-: p=0.0002, HIV+: p<0.0001; HIV-: p=0.0003, HIV+: p=0.0001; HIV-: p=0.0002, HIV+: p=0.0002; HIV-: p=0.0002, HIV+: p=0.0002, respectively). These findings indicate that CD14 is shed partially from monocytes, in both HIV-infected and uninfected individuals.

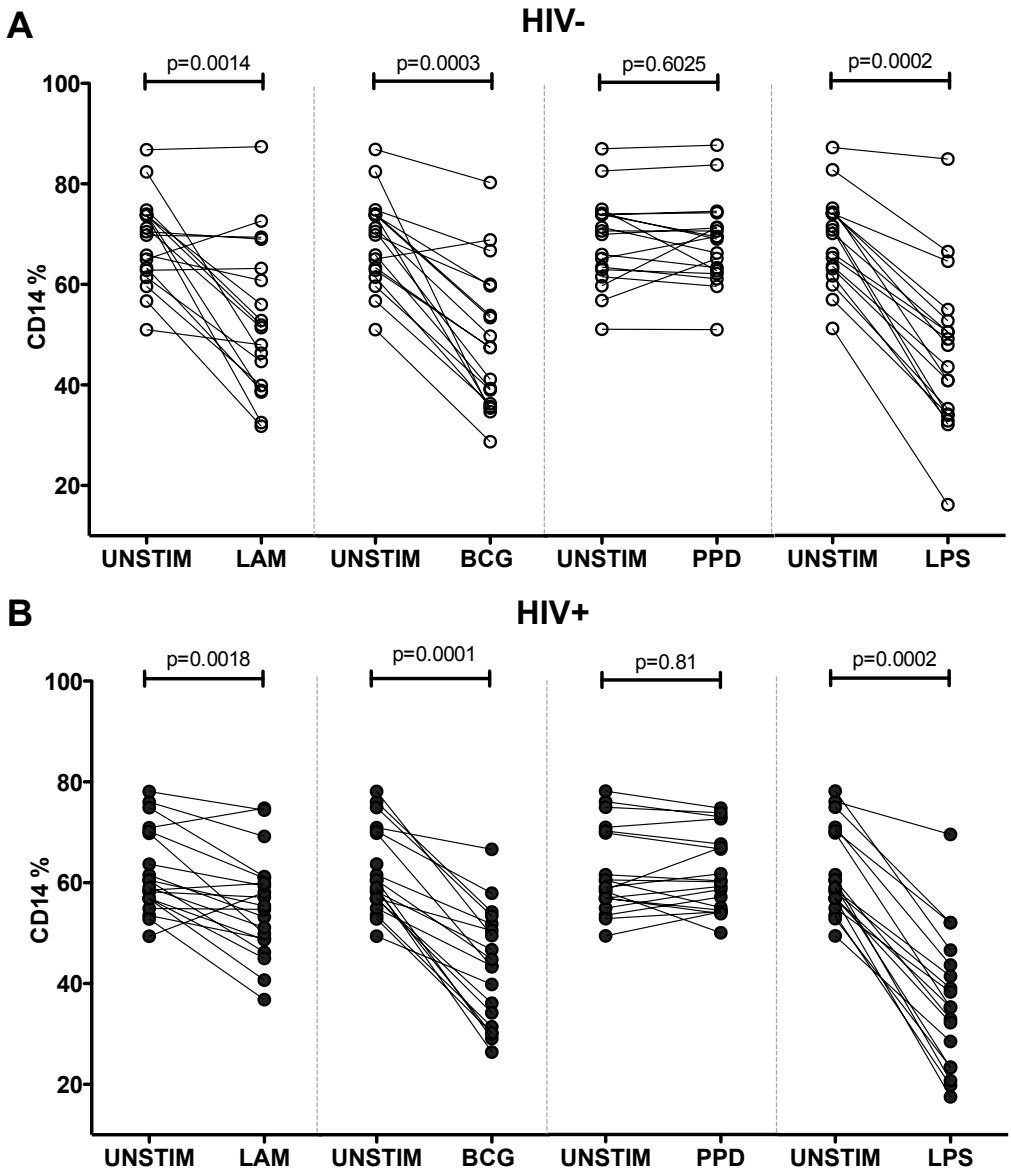

Figure 3.18: Frequency of CD14 monocytes in HIV-infected and uninfected individuals in response to TLR stimulation. The reduction in the frequency of CD14+ monocyte population was determined for n=18 HIV-uninfected **(A)** and n=18-20 HIV-infected individuals **(B)** in response to LAM, BCG, PPD and LPS. Filled circles represent HIV-infected individuals while open circles represent HIV-uninfected individuals. Statistical comparisons were determined by Wilcoxon non-parametric matched pairs test.

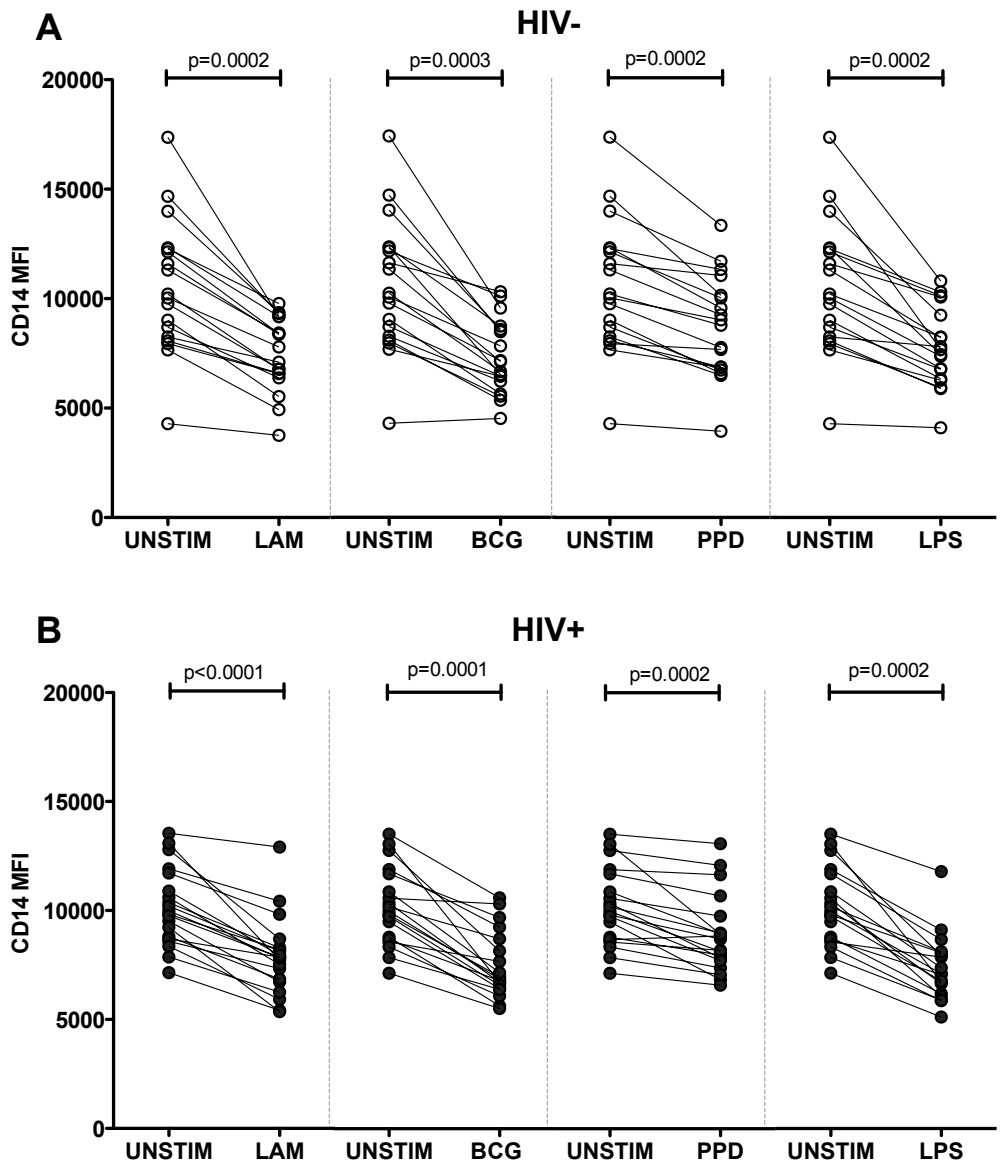

Figure 3.19: Expression levels of CD14 on monocytes in HIV-infected and uninfected individuals in response to TLR stimulation. The expression levels of CD14 (MFI) on monocytes was determined for n=18 HIV-uninfected **(A)** and n=18-20 HIV-infected individuals **(B)** in response to LAM, BCG, PPD and LPS. Filled circles represent HIV-infected individuals while open circles represent HIV-uninfected individuals. Statistical comparisons were determined by Wilcoxon non-parametric matched pairs test.

Next, the effect of HIV infection on the extent of this CD14 loss from monocytes was investigated, by comparing the fold change in the frequency of CD14 monocytes and the fold change in levels of CD14 expression between HIV-infected and uninfected individuals **(Figure 3.20).** A fold change of 1 indicated no loss in CD14 from monocytes compared to the unstimulated sample, *i.e.* maintenance of CD14 expression. An empirical fold change threshold of 20% was set, above and below

which a change would be considered significant; a change below 0.8 would indicate more than 20% loss in CD14, while a fold change greater than 1.2 would indicate more than 20% increase in CD14 on monocytes.

When the data were expressed in this way, the fold change in CD14 frequency **(Figure 3.20A)** and expression levels **(Figure 3.20B)** was <1 for all stimuli except for the frequency of CD14+ monocytes after PPD stimulation, consistent with the previous data (median **CD14%** loss: HIV+: 0%, HIV-:0%). Interestingly, differences emerged in the degree of CD14 loss to different stimuli between HIV-infected and uninfected individuals that were not apparent in the previous analyses. Although there were no significant differences between the groups in the degree of CD14 loss (either as frequency or MFI) for BCG and PPD, there was a trend towards differences in the CD14 loss for LAM and LPS **(Figure 3.20A and B)**. HIV-infected individuals had a lower fold change loss in frequency of CD14+ monocyte population in response to LAM compared to HIV-uninfected individuals (p=0.0928; **Figure 3.20A**). Two-thirds of the HIV-uninfected participants demonstrated a significant (>20%) loss in frequency of CD14+ monocytes, while only 25% of the HIV-infected individuals showed a significant loss in CD14+ frequency in response to LAM. The same trend was also observed with the fold change in CD14 expression (MFI) between the two groups in response to LAM **(Figure 3.20B)**. HIV-infected individuals showed a trend of reduced fold change in loss of CD14 expression on monocytes when compared to HIV-uninfected individuals (p=0.105). Again, almost 80% of the HIV-uninfected individuals but only 50% of the HIV-infected individuals exhibited a significant loss in CD14 expression. These findings suggest that during HIV infection, CD14 shedding from monocytes in response to the TLR2 ligand, LAM, is dysregulated.

In contrast to LAM, a trend towards greater loss in the frequency of the CD14+ monocyte population in response to LPS was observed in HIV-infected individuals compared to HIV-uninfected individuals (p=0.0967; **Figure 3.20A**), although, no difference was observed in the fold change in MFI of CD14 on monocytes between the two groups **(Figure 3.20B)**. Monocytes from majority of the participants in the two groups showed a significant loss in CD14 expression in response to LPS

(**CD14%:** HIV+: 94%, HIV-: 83%; **CD14 MFI:** HIV+: 72%, HIV-: 61%). This trend of a greater loss in frequency of CD14 during HIV infection (implying more shedding in response to LPS stimulation) may be a consequence of the heightened monocyte activation, as measured by the enhanced cytokine production from monocytes upon LPS stimulation in HIV-infected individuals, reported above. Overall, these findings indicate that HIV infection differentially alters the degree of CD14 shedding in response to the mycobacterial TLR2 ligand LAM, and the TLR4 ligand, LPS.

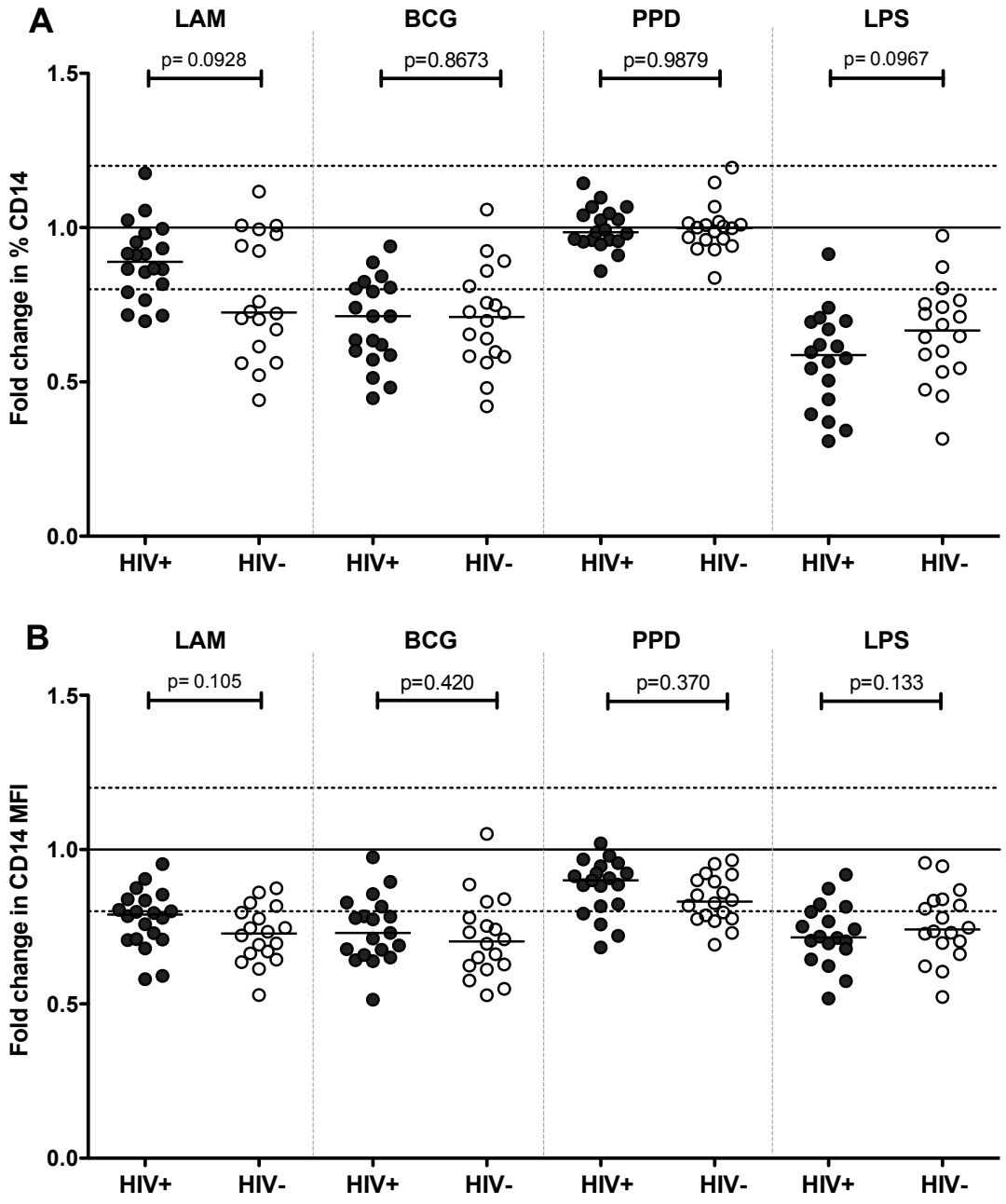

Figure 3.20: Change in CD14 expression in HIV-infected and uninfected individuals in response to TLR stimulation. A shows the fold change in frequency of CD14+ monocytes and **B** shows the fold change in CD14 expression levels (MFI) on monocytes from n=18-20 HIV-infected and n=18 uninfected individuals, in response to LAM, BCG, PPD and LPS. The solid line represents a fold change of 1 (*i.e.* no change) and the dashed lines represent a 20% change threshold. Filled circles represent HIV-infected individuals while open circles represent HIV-uninfected individuals. Statistical comparisons were determined by a Mann-Whitney nonparametric t-test.

Since a trend towards a differential degree of CD14 shedding was observed in HIV infection for some of the stimuli tested, the relationship between the change in CD14 expression on monocytes and cytokine responses from these cells was next

investigated, to determine whether cytokine production was related to the degree of shedding of CD14 upon TLR stimulation **(Figure 3.21).** Association between monocyte cytokine production and changes in the levels of CD14 expression on monocytes in response to TLR stimuli revealed that there was a significant inverse correlation in HIV-uninfected individuals between the net total cytokine production from monocytes and the change in the level of CD14 expression on monocytes in response to LAM, BCG and LPS ($p=0.016$, $r=-0.56$; $p=0.033$, $r=-0.505$; and $p=0.013$, $r=-0.573$, respectively**; Figure 3.21A, B, D**). These results indicate that more cytokine production from monocytes was associated with a greater loss of CD14 expression on monocytes in response to LAM, BCG and LPS. In contrast, this relationship was disrupted in HIV-infected individuals, where no such correlation was observed between the monocyte cytokine response and change in CD14 expression when monocytes were stimulated with LAM, BCG or LPS ($p=0.094$, $r=-0.385$; $p=0.797$, $r=-0.063$; $p=0.372$, $r=-0.224$, respectively; **Figure 3.21A, B, D**). Consistent with the previous data, for PPD, there was no association between cytokine response and change in CD14 expression for HIV-infected or uninfected individuals **(Figure 3.21C)**.

Taken together, these results suggest that during HIV infection, monocytes exhibit a differential responsiveness to the degree of CD14 shedding, and the association between monocyte cytokine response and the extent of CD14 shedding from monocytes is disrupted in these individuals. With LPS stimulation, greater CD14 shedding in HIV infection is likely linked to the enhanced cytokine production observed in these individuals compared to HIV-uninfected individuals. The altered degree of CD14 shedding observed in response to mycobacterial LAM (and to a certain extent to BCG) reveals an effect of HIV on monocytes, even though no differences in cytokine production were observed between HIV-infected and uninfected individuals in the short term assays performed in this study. This lack of CD14 loss in response to TLR2 stimulation may have implications for the functioning of monocytes and control of co-infections such as M.tb. The mechanisms associated with CD14 modulation by HIV require further investigation.

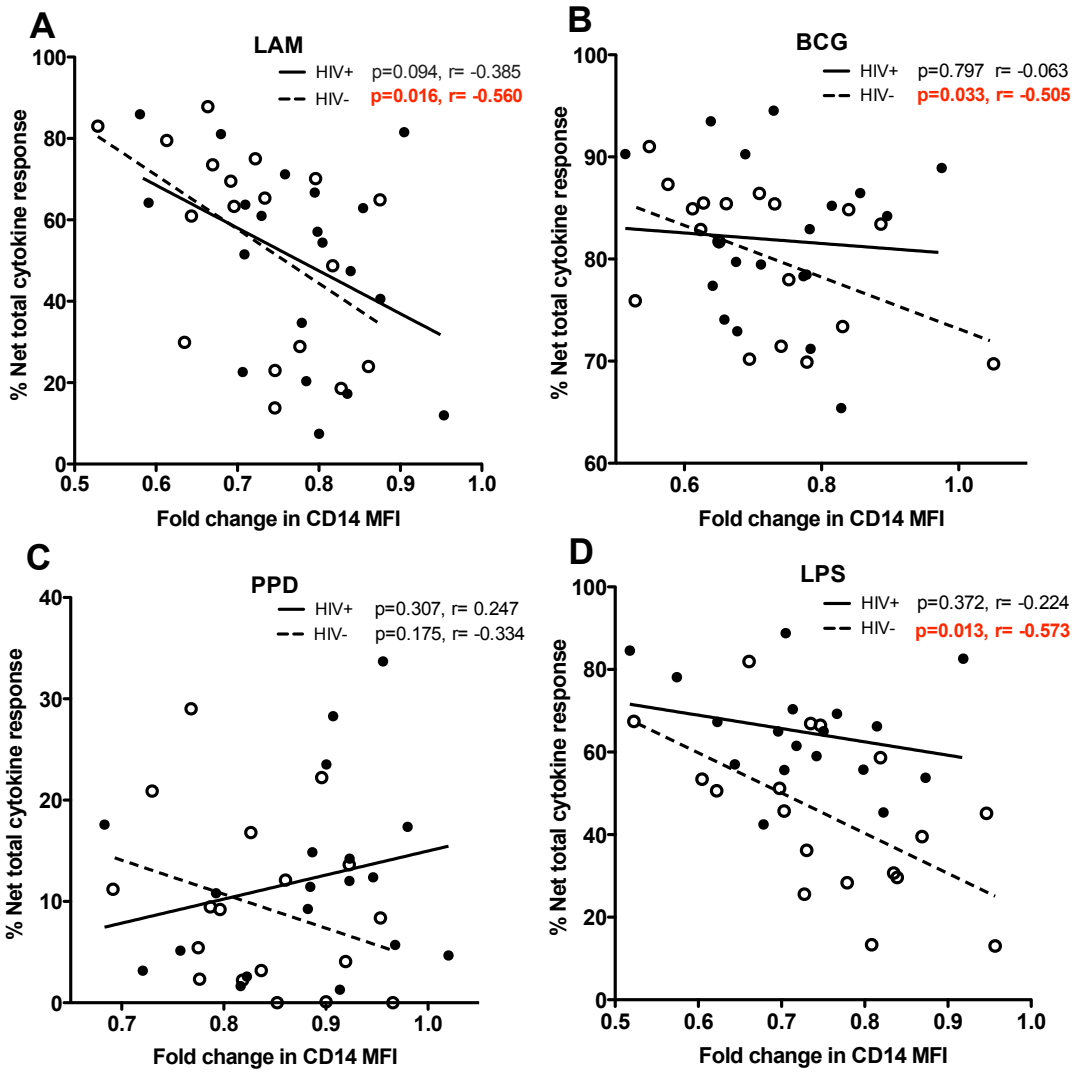

Figure 3.21: Relationship between monocyte cytokine responses and change in CD14 expression on monocytes upon TLR stimulation. The association between the net total cytokine production from monocytes and fold change in CD14 MFI in response to LAM **(A)**, BCG **(B)**, PPD **(C)** and LPS **(D)** was determined in n=18-20 HIV-infected individuals and n=18 HIV-uninfected individuals. Filled circles represent HIV-infected individuals while open circles represent HIV-uninfected individuals. The correlation coefficient (r) and p-values (p) are indicated on the graph. Significant correlations are indicated in red. Statistical associations were performed using a two-tailed non-parametric Spearman rank correlation.

3.8 Summary

The overall aim of this study was to investigate whether infection with HIV induced defects in monocytes, which impaired their ability to respond to M.tb. The focus was on TLR responses by monocytes, by determining whether cytokine production in response to mycobacteria-derived TLR ligands differed in HIV-infected individuals.

A multiparameter flow cytometry panel was developed and optimised to characterise cytokine production from CD14+ monocytes in response to TLR stimuli, LAM, BCG, PPD and LPS from 20 HIV-infected and 18 HIV-uninfected individuals.

These results showed that HIV infection did not alter the quantity and quality of cytokine responses from monocytes, after stimulation with mycobacterial antigens LAM, PPD or whole viable BCG. However, in response to the gram-negative bacterial TLR4 ligand LPS, that was included in this study as a control for TLR stimulation, cytokine responses from monocytes were significantly impaired in HIV-infected individuals. This may reflect specific TLR4 stimulation *in vivo*, from circulating products of microbial translocation from the gut, such as LPS (Brenchley *et al.*, 2006a), or sensitisation of TLR4 functioning from ongoing activation of the innate immune system by HIV-encoded TLR ligands such as ssRNA (Mureith *et al.*, 2010). Monocyte cytokine responses to TLR stimulation were not associated with either HIV viral load, CD4 T cell count or age.

Furthermore, HIV-infected individuals demonstrated significantly lower frequency of CD14+ monocytes compared to HIV-uninfected individuals, although this did not correlate with HIV viral loads or CD4 T cell counts. Upon stimulation, the frequency of CD14+ monocytes and expression levels of CD14 on monocytes was significantly reduced in both the groups, in response to LAM, BCG and LPS, likely due to CD14 shedding. Interestingly, HIV infection differentially altered the degree of CD14 shedding in response to LAM and LPS. Whilst there was an inverse correlation between the change in CD14 expression and cytokine responses to LAM, LPS and BCG in HIV-uninfected individuals, this relationship was disrupted in HIV-infected individuals, where no such correlation was observed. For LPS, this is likely explained by the enhanced cytokine production observed in HIV infection. However, no differences in cytokine production were observed to BCG or LAM stimulation, and the dysregulation of CD14 shedding may have implications for monocyte functioning not measured in the short-term cytokine assays performed in this study. The exact mechanisms of CD14 modulation by HIV require further investigation. The results of this study provides further insight into how HIV affects monocyte TLR functioning, which may have consequences for innate immune control of M.tb.

CHAPTER 4: DISCUSSION

HIV-infected persons are more susceptible to TB, and the reasons for this are not fully understood. HIV infection causes depletion of CD4+ T cells, which is thought to affect adaptive immunity to M.tb, but in addition, HIV affects the functioning of innate cells such as monocytes and macrophages. Monocytes play a key role in innate immune defense and are the precursors of macrophages, which are the main target cells of M.tb. If the ability of these cells to respond to M.tb is impaired, it could contribute to the greater susceptibility of HIV-infected individuals to develop TB. One of the ways in which these cells respond to M.tb is through TLR stimulation, that leads to the release of pro-inflammatory mediators, triggering innate and adaptive immune responses to infection, critical events for the control of M.tb. This study sought to investigate whether HIV infection induced functional defects in monocytes, impairing their ability to respond to M.tb. The focus was on TLR functioning in monocytes, examining whether HIV-infected individuals had altered monocyte cytokine responses after TLR stimulation, compared to HIV-uninfected individuals.

A multiparameter flow cytometry panel was developed and used to identify monocytes and measure their capacity to produce the cytokines IL-1β, IL-6 and TNF-α, upon stimulation with mycobacteria-derived TLR ligands. The TLR2 ligand LAM, M.tb PPD (which stimulates TLR1, 2, 4 and 6), viable whole BCG (a stimuli for TLR1, 2, 4, 6 and 9) were used, as well as LPS, a TLR4 agonist from gram negative bacteria, as a positive control. Monocytes form a heterogeneous group of cells and three different subsets have been described based on the expression of cell surface markers CD14 and CD16 (Ziegler-Heitbrock *et al.*, 2010). The intermediate CD14++CD16+ population has been previously demonstrated to expand during various infections and inflammations, including HIV and TB (Wong *et al*., 2012; Balboa *et al*., 2011; Thieblemont *et al*., 1995; Crowe & Ziegler-Heitbrock, 2010). This study, however, was limited to measuring cytokine responses from the classical CD14+ monocyte subset, due to technical limitations on the use of the CD16+ antibody in the fixed whole blood samples used in this study. Fixation reduced the staining intensity of the CD16 marker such that it led to a diminished resolution and

lack of discrimination between the different subsets, as discussed in Chapter 2. Nonetheless, classical monocytes make up the bulk of the blood monocyte population and produced robust cytokine responses when activated with various stimuli.

Monocytes varied in their ability to respond to the different TLR stimuli in HIV-uninfected individuals. BCG was the most potent inducer of pro-inflammatory cytokines from monocytes, and responses were highly polyfunctional, producing all three cytokines measured, simultaneously. This is not surprising, given that BCG is a live whole pathogen composed of many PAMPs that can activate monocytes through various pattern recognition receptors. Mycobacterial cell wall components and mycobacterial DNA can activate a range of TLRs found on the cell surface and in the cytosol of monocytes, such as TLR1, TLR2, TLR4, TLR6 and TLR9 (Kleinnijenhuis *et al*., 2011). In addition to TLR activation, mycobacteria can also activate other PRRs found on monocytes, such as the C-type lectin receptors (CLRs) and NOD-like receptors (NLRs; as reviewed by Kawai & Akira, 2011). Co-operation between TLRs and other PRRs can lead to the activation of various host signaling pathways (Ferwerda *et al.*, 2008; as reviewed by Trinchieri & Sher, 2007), which may account for the robust response observed with BCG stimulation. In contrast, LAM and LPS stimulate innate cells through the single TLRs, TLR2 and TLR4 respectively, and the lack of involvement of multiple TLR pathways may explain the lower cytokine responses compared to BCG.

Monocytes produced mainly the pro-inflammatory cytokines IL-1β and IL-6, with fewer producing TNF-α in response to all the stimuli tested. Previous studies have demonstrated that cytokines interact with one another to regulate the production of each other, which may help to fine-tune the inflammatory response (Aderka *et al.*, 1989; Dinarello *et al.*, 1986; Tosato & Jones, 1990). For example, IL-6 has been shown to inhibit TNF-α production from monocytes (Aderka *et al.*, 1989; Bailly *et al.*, 1990), which may explain the lower levels of TNF-α production from monocytes.

Considerable heterogeneity was observed among the donors in terms of cytokine production by monocytes in response to LAM and LPS. These differences may be attributed to a range of factors, such as age, genetic differences, ethnic background and history of infections. Even physiological conditions such as physical stress can modulate TLR expression and function, whereby after strenuous exercise, TLR expression was decreased on CD14+ monocytes, and this may in turn affect cytokine production from monocytes (Lancaster *et al.*, 2005). In addition, differences in monocyte subset distributions can be influenced by ethnicity and past exposure to pathogens (Appleby *et al.*, 2013). Furthermore, polymorphisms in the genes encoding TLRs themselves, CD14, TNF and IL-1 (Lin *et al.*, 2007; Louis *et al.*, 1998; Danis *et al.*, 1994) may also influence the production of cytokines, and were found to be related to the inter-individual variability observed among donors. Moreover, it has also been demonstrated that age is associated with dysregulation of cytokine production, with reduced production of pro-inflammatory cytokines in response to LPS stimulation observed in the elderly compared to young individuals (Bruunsgaard *et al.*, 1999). Although there was a significant difference in ages between the HIV-infected and uninfected groups in this study (median of 32 vs. 22 years), there was no association between cytokine response and age, likely because these are all relatively young individuals. Thus, a range of diverse factors could account for the heterogeneity among individuals in their monocyte cytokine responses observed in this study.

Comparison of monocyte cytokine responses between the HIV-infected and uninfected groups in this study revealed that there were no significant differences in the frequency of total cytokine production, individual cytokine production, expression levels of cytokines (median fluorescent intensity) as well as polyfunctional capacity of monocytes to the mycobacteria or mycobacterial-derived stimuli LAM, BCG or PPD. Thus, HIV infection did not lead to defective cytokine responses to mycobacterial TLR stimuli. In contrast, the monocyte cytokine response to LPS was significantly enhanced in the HIV-infected group compared to uninfected individuals. This hyper-responsiveness of monocytes from HIV-infected individuals in response to LPS is discussed later in this chapter.

Stimulation of monocytes with LAM, BCG and LPS resulted in a reduced frequency of CD14+ monocytes in both HIV-infected and uninfected individuals, likely due to complete shedding of CD14 from a proportion of monocytes in the population. In addition, the expression levels of CD14 on monocytes from both the groups were also reduced upon stimulation with these ligands, indicating partial shedding of CD14 on some monocytes. Shey *et al*. (2012) also found markedly lower frequencies of CD14+ monocytes upon stimulation with LPS and BCG, and suggested that this could be due to either down regulation of CD14, shedding of CD14 or cell death. Since all markers in this study were stained intracellularly, any down regulation of CD14 in response to stimulation did not compromise the ability to detect monocytes. CD14 shedding may therefore explain the reduced CD14+ frequency upon stimulation. Measuring sCD14 in the stored supernatants of these monocyte cultures would confirm this phenomenon. Shedding of CD14 is induced as a consequence of monocyte activation and serves to down modulate CD14 on stimulated monocytes (Bazil & Strominger, 1991). This functions as a regulatory mechanism to prevent an excessive pro-inflammatory response. The shedding of CD14 from monocytes in response to LAM, BCG and LPS is thus likely a mechanism to down modulate CD14 to prevent excessive cytokine responses. These findings confirm previous studies that have demonstrated that besides LPS, other bacterial cell wall constituents such as those from gram-positive bacteria and mycobacteria also regulate the CD14 receptor (as reviewed by Antal-Szalmás, 2000; Landmann *et al.*, 1996; Pugin *et al.*, 1994).

In contrast to the data on maintenance of cytokine production to mycobacterial ligands in HIV infection, a more subtle effect on monocytes was revealed from analysis of CD14 regulation. In HIV-uninfected individuals, there was an inverse correlation between the degree of CD14 shedding in LAM, LPS and BCG-stimulated cultures and cytokine responses to these TLR stimuli. In contrast, this relationship was disrupted in HIV-infected individuals, where no such correlation was observed. The degree of CD14 shedding from monocytes is important in regulating cytokine production upon monocyte activation. However, this association was lost during HIV infection. With LPS stimulation (TLR4 response), the relationship between the degree of CD14 shedding from monocytes and cytokine production may be impaired in HIV infection as a consequence of the enhanced production of cytokines that was

observed. However, with LAM stimulation, no cytokine differences were observed. The results of this study revealed that the degree of CD14 shedding from monocytes was reduced in response to LAM in HIV infection compared to HIV-uninfected individuals, leading to dysregulation of the relationship between CD14 shedding by monocytes and their ability to produce cytokines. Although this did not affect monocyte cytokine responses to TLR stimuli in these individuals as measured by the short-term assay (6 h of stimulation) employed in this study, it may be possible that there would be longer term functional consequences. Since shedding of CD14 from monocytes helps to control the level of monocyte cytokine response, the reduced degree of shedding may therefore not be able to modulate the cytokine response well. It is speculated that this may have long-term consequences on monocyte activation such that the cytokine response in monocytes would not be tightly regulated anymore and this may lead to a constant signaling of the TLR2/CD14 pathway, resulting into constitutive production of cytokines. Hence, it would be expected that HIV-infected individuals would have increased cytokine production to LAM compared to the healthy controls. The effects of reduced CD14 shedding on cytokine production may only be seen with longer stimulation. Therefore, it can only be speculated that if the cytokines were to be measured in a long-term whole blood assay, elevated levels of cytokine production from monocytes would be detected in HIV infection in response to LAM. It would thus be of interest to perform longer term assays to assess this. Hence, the tight regulation in the degree of CD14 shedding is an important mechanism for modulating monocyte cytokine responses. HIV may be targeting this mechanism leading to the prolonged production of pro-inflammatory cytokines and in turn a prolonged inflammation. On the other hand, the CD14 dysregulation observed for mycobacterial ligands may affect other monocyte functions or processes apart from cytokine production. Since monocytes are key regulators of immune response in TB infection, dysfunction of these cells could result in the cell being in a constant activated state and this may help to fuel the inflammatory environment in the lung that could lead to impaired containment and control of M.tb in HIV co-infection.

Taken together, these results suggest that during HIV infection, the relationship between CD14 loss and monocyte cytokine responses is disrupted. With LPS stimulation, this relationship may be disturbed due to the enhanced monocyte

cytokine production observed in HIV-infected individuals. However, with LAM stimulation, the reduced degree of CD14 loss observed in response to LAM in HIV-infected individuals might have a long-term consequences on cytokine production or other functions of monocytes. Further studies are required to confirm this model and assess the relationship between the degree of CD14 shedding and the ability of monocytes to produce cytokines.

Stimulation of monocytes with LPS, the non-mycobacterial TLR ligand that was included in these studies, revealed interesting results. HIV-infected individuals had enhanced monocyte cytokine responses to LPS. This observation extends and confirms published data regarding dysregulated LPS responses during HIV infection. Enhanced IL-1β, IL-6 and TNF-α levels in supernatants of monocyte cultures from HIV-infected individuals after stimulation with LPS from periodontal pathogens has been demonstrated (Baqui *et al*., 2000). Monocytes from healthy individuals produced significantly enhanced cytokine responses to LPS after prior stimulation with HIV-derived TLR ligands (inactivated HIV, containing single-stranded viral RNA; Mureith *et al*., 2010). HIV may thus have an indirect effect on monocyte functioning, whereby sustained stimulation of HIV-responsive TLRs may influence how monocytes respond to other TLRs, in a process termed "TLR cross talk" (Chang & Altfeld, 2009). Indeed, this cross talk could explain the enhanced monocyte responses to LPS observed in HIV-infected individuals in the present study. However, in contrast to these findings, Mir *et al.* (2012) reported no differences in TNF-α production from whole blood monocytes upon LPS stimulation between HIV-infected and uninfected individuals. This disparity in results may be due to differences in the assays or cohort recruited. The participants recruited in this study had CD4 counts ranging from 4 to 833 cells/mm^3 and were treatment naïve for at least one year. However, our study involved participants that were highly immune competent and had CD4 counts greater than 400 cells/mm^3 and who have never been on ARV treatment before. In addition, this study only used CD14 to characterise monocytes by flow cytometry, without including the markers HLA-DR, CD66 and CD56 to exclude granulocytes and NK cells, which could have confounded the results of the study.

In addition to HIV-encoded TLR ligands, it is hypothesised that microbial translocation through the gastrointestinal tract allows components of gut bacteria to enter the circulation, leading to monocyte activation (Brenchley *et al*., 2006b). High levels of plasma LPS in HIV-infected individuals were associated with higher levels of sCD14, indicating monocyte activation (Brenchley *et al.*, 2006a). Thus, the enhanced monocyte responses may be a major contributor to the heightened immune activation observed in HIV infection. In fact, plasma levels of sCD14 were associated with an increased risk of mortality in HIV infection (Sandler *et al.*, 2011).

In HIV infection, significantly reduced frequencies of CD14+ monocytes were observed compared to uninfected individuals, consistent with findings from a previous report (Han *et al.*, 2009). The reduced frequency of the CD14+ monocytes in HIV-infected individuals may be due to an elevated frequency of the CD16+ monocyte subsets that have previously been demonstrated to expand in HIV infection, and this may lead to lower proportion of the classical monocytes. Determining the absolute counts of the monocyte subsets in blood is therefore important to confirm whether the reduced frequency of CD14+ subsets are a result of reduced numbers of CD14+ monocytes during HIV infection or due to expansion of other monocyte subsets. The reduced frequency of CD14+ monocytes could also be due to shedding of CD14 *in vivo*, in response to circulating LPS in plasma, as a result of microbial translocation (Brenchley *et al*., 2006a). Triggering of TLR4 may lead to CD14 shedding and loss of detection of CD14+ monocytes, as was observed in the stimulated cultures in this study.

A number of additional experiments could extend these findings. Since M.tb infection occurs in the lungs, examining AMs in HIV co-infection is very important. Ongoing studies in our laboratory are examining the effects of HIV co-infection on transcriptional profiles of AM. Furthermore, HIV infection may alter the production of other cytokines and mediators than the three that were measured by flow cytometry in this study; they are unlikely to represent the total cytokine response of monocytes to TLR stimulation. Hence, multiplex cytokine assays on culture supernatants will be performed in future experiments to examine a wider range of cytokines, chemokines and other mediators released in response to mycobacterial TLR stimuli, such as IL-12, IL-10, NO and MCP-1. HIV infection may also affect

the function of the other monocyte subsets that this study was unable to characterise (those expressing CD16). Hence, it would be of interest to determine whether HIV alters TLR functioning and cytokine production from these subsets in response to mycobacteria-derived stimuli, which could be performed in PBMC that were stored from these participants.

Summary

Collectively, the data presented in this thesis demonstrate that monocytes from HIV-infected individuals released pro-inflammatory cytokines and preserved their innate ability to respond to mycobacterial-derived stimuli. In contrast, there was enhanced responsiveness of monocytes to LPS stimulation during HIV infection, that may reflect specific TLR4 cross talk from ongoing activation of the innate immune system by HIV-encoded TLR ligands or by the circulating products of microbial translocation from the gut. The results of this study also provide evidence for CD14 dysregulation in response to mycobacterial LAM in HIV-infected individuals, and propose a model for how HIV differentially affects TLR2 and TLR4 responses. The precise mechanism responsible for the CD14 dysregulation is yet to be elucidated. These data provide a framework for future experiments and an insight into how HIV affects innate immunity to M.tb, which is important for a better understanding of how a protective immune response develops against M.tb. This could improve our understanding of immunity against TB by highlighting deficiencies that lead to disease in the context of HIV infection and may ultimately lead to better TB vaccines and immunotherapies, thereby decreasing morbidity and mortality for both diseases. In addition, this study complements ongoing studies in our laboratory that are investigating how HIV affects both adaptive and innate immunity to M.tb in the lungs of HIV-infected individuals with latent TB infection. These studies promise to provide a more complete picture of how HIV co-infection affects immunity to M.tb.

Future work

Apart from the additional studies already suggested in this discussion, other avenues for further research include the following:

- The effect of HIV on the expression of M.tb-responsive TLRs on monocytes could be investigated by flow cytometry on the archived samples stored from the study. This would allow us to address whether HIV infection leads to altered expression of TLRs on monocytes that would affect how these cells respond to M.tb.
- In addition to TLRs, there are additional pattern recognition receptors on and within monocytes and AM that M.tb can stimulate, such as NOD-like receptors, CLRs (MR, DC-SIGN) and scavenger receptors. These receptors as well as downstream adaptor proteins and molecules (MyD88) all represent potential avenues for further research using archived clinical material that have been collected and stored in this study.
- Studies could also be performed to explore how HIV affects other monocyte functions, such as their differentiation potential into macrophages upon recruitment to the site of infection. *In vitro* studies could explore how the inflammatory environment of the lung during HIV-infection affects monocyte differentiation, by culturing monocytes from HIV-uninfected individuals to BAL fluid that we have collected from HIV-infected individuals and then determining how the macrophages subsequently respond to M.tb.

REFERENCES

Abeles, R.D., McPhail, M.J., Sowter, D., Antoniades, C.G., Vergis, N., Vijay, G.K.M., Xystrakis, E., Khamri, W., Shawcross, D.L., Ma, Y., Wendon, J.A. and Vergani, D. 2012. CD14, CD16 and HLA-DR reliably identifies human monocytes and their subsets in the context of pathologically reduced HLA-DR expression by $CD14^{hi}/CD16^{neg}$ monocytes: Expansion of $CD14^{hi}/CD16^{pos}$ and contraction of $CD14^{lo}/CD16^{pos}$ monocytes in acute liver failure. Cytometry A. 81, 823–34.

Aderka, D., Le, J.M. and Vilcek, J. 1989. IL-6 inhibits lipopolysaccharide-induced tumor necrosis factor production in cultured human monocytes, U937 cells, and in mice. J. Immunol. 143, 3517–23.

Akira, S. and Takeda, K. 2004. Toll-like receptor signalling. Nat. Rev. Immunol. 4, 499–511.

Akira, S., Takeda, K. and Kaisho, T. 2001. Toll-like receptors : critical proteins linking innate and acquired immunity. Nat. Immunol. 2, 675-680.

Alexaki, A. and Wigdahl, B. 2008. HIV-1 infection of bone marrow hematopoietic progenitor cells and their role in trafficking and viral dissemination. PLoS Pathog. 4, e1000215.

Ansari, A.W., Meyer-Olson, D. and Schmidt, R.E. 2013. Selective expansion of pro-inflammatory chemokine CCL2-loaded CD14+CD16+ monocytes subset in HIV-infected therapy naïve individuals. J. Clin. Immunol. 33, 302–6.

Ansari, A.W., Heiken, H., Meyer-Olson, D. and Schmidt, R.E. 2011. CCL2: a potential prognostic marker and target of anti-inflammatory strategy in HIV/AIDS pathogenesis. Eur. J. Immunol. 41, 3412–8.

Antal-Szalmás, P. 2000. Evaluation of CD14 in host defence. Eur. J. Clin. Invest. 30, 167–79.

Appleby, L.J., Nausch, N., Midzi, N., Mduluza, T., Allen, J.E. and Mutapi, F. 2013. Sources of heterogeneity in human monocyte subsets. Immunol. Lett. 152, 32–41.

Auffray, C., Sieweke, M.H. and Geissmann, F. 2009. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. Annu. Rev. Immunol. 27, 669–92.

Autissier, P., Soulas, C., Burdo, T.H. and Williams, K.C. 2010. Evaluation of a 12-color flow cytometry panel to study lymphocyte, monocyte, and dendritic cell subsets in humans. Cytometry A. 77, 410–9.

Awomoyi, A.A., Marchant, A., Howson, J.M.M., McAdam, K.P.W.J., Blackwell, J.M. and Newport, M.J. 2002. Interleukin-10, polymorphism in SLC11A1 (formerly NRAMP1), and susceptibility to tuberculosis. J. Infect. Dis. 186, 1808–14.

Bagchi, A., Herrup, E.A., Warren, H.S., Trigilio, J., Shin, H.-S., Valentine, C. and Hellman, J. 2007. MyD88-dependent and MyD88-independent pathways in synergy, priming, and tolerance between TLR agonists. J. Immunol. 178, 1164–71.

Bailly, S., Ferrua, B., Fay, M. and Gougerot-Pocidalo, M.A. 1990. Differential regulation of IL 6, IL-1α, IL-1β and TNF-α production in LPS-stimulated human monocytes: role of cyclic AMP. Cytokine. 2, 205–10.

Balboa, L., Romero, M., Laborde E., Garcia C.A.S., Baile J.I., Schierloh P., Yokobori N., Musella R.M., Castagnino J., Barrera S., Sasiain M.C. and Aleman M. 2013. Impaired dendritic cell differentiation of CD16-positive monocytes in tuberculosis : Role of p38 MAPK. Eur. J. Immunol. 43, 335–347.

Balboa, Romero, M., Basile, J.I., Sabio, C.A., Schierloh, P., Geffner, L., Musella, R.M., Castagnino, J., Abbate E., Barrera S., Sasiain M.C. and Aleman M. 2011. Paradoxical role of CD16+CCR2+CCR5+ monocytes in tuberculosis : efficient APC in pleural effusion but also mark disease severity in blood. J. Leukoc. Biol. 90, 69–75.

Baqui, A.A.M.A., Jabra-rizk, M.A., Kelley, J.I., Zhang, M., Falkler, W.A. and Meiller, T.F. 2000. Enhanced interleukin-1β, interleukin-6 and tumor necrosis factor-α production by LPS stimulated human monocytes isolated from HIV+ patients. Immunopharmocol. Immunotoxicol. 22, 401-421.

Bazil, V. and Strominger, J.L. 1991. Shedding as a mechanism of down-modulation of CD14 on stimulated human monocytes. J. Immunol. 147, 1567–74.

Beamer, G.L., Flaherty, D.K., Assogba, B.D., Stromberg, P., Gonzalez-Juarrero, M., de Waal Malefyt, R., Vesosky, B. and Turner, J. 2008. Interleukin-10 promotes *Mycobacterium tuberculosis* disease progression in CBA/J mice. J. Immunol. 181, 5545–50.

Belge, K.-U., Dayyani, F., Horelt, A., Siedlar, M., Frankenberger, M., Frankenberger, B., Espevik, T. and Ziegler-Heitbrock, L. 2002. The proinflammatory CD14+CD16+DR++ monocytes are a major source of TNF. J. Immunol. 168, 3536–42.

Bergamaschi, A. and Pancino, G. 2010. Host hindrance to HIV-1 replication in monocytes and macrophages. Retrovirology. 7, 31.

Berhanu, D., Mortari, F., De Rosa, S.C. and Roederer, M. 2003. Optimized lymphocyte isolation methods for analysis of chemokine receptor expression. J. Immunol. Methods. 279, 199–207.

Berrington, W.R. and Hawn, T.R. 2007. *Mycobacterium tuberculosis*, macrophages, and the innate immune response: does common variation matter? Immunol. Rev. 219, 167–86.

Borrow, P. 2011. Innate immunity in acute HIV-1 infection. Curr. Opin. HIV AIDS. 6, 353–63.

Brenchley, J.M., Knox, K.S., Asher, A I., Price, D. A, Kohli, L.M., Gostick, E., Hill, B.J., Hage, C. A, Brahmi, Z., Khoruts, A, Twigg, H.L., Schacker, T.W. and Douek, D.C. 2008. High frequencies of polyfunctional HIV-specific T cells are associated with preservation of mucosal CD4 T cells in bronchoalveolar lavage. Mucosal Immunol. 1, 49–58.

Brenchley, J.M., Price, D. a, Schacker, T.W., Asher, T.E., Silvestri, G., Rao, S., Kazzaz, Z., Bornstein, E., Lambotte, O., Altmann, D., Blazar, B.R., Rodriguez, B., Teixeira-Johnson, L., Landay, A., Martin, J.N., Hecht, F.M., Picker, L.J., Lederman, M.M., Deeks, S.G. and Douek, D.C. 2006a. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. Nat. Med. 12, 1365–71.

Brenchley, J.M., Price, D.A. and Douek, D.C. 2006b. HIV disease: fallout from a mucosal catastrophe? Nat. Immunol. 7, 235–9.

Brenchley, J.M., Schacker, T.W., Ruff, L.E., Price, D.A., Taylor, J.H., Beilman, G.J., Nguyen, P.L., Khoruts, A., Larson, M., Haase, A.T. and Douek, D.C. 2004. CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. J. Exp. Med. 200, 749–59.

Briken, V., Porcelli, S.A., Besra, G.S. and Kremer, L. 2004. Mycobacterial lipoarabinomannan and related lipoglycans: from biogenesis to modulation of the immune response. Mol. Microbiol. 53, 391–403.

Bruunsgaard, H., Pederson A.N., Schroll M., Skinhoj P. and Pederson B.K. 1999. Impaired production of proinflammatory cytokines in response to lipopolysaccharide (LPS) stimulation in elderly humans. Clin. Exp. Immunol. 118, 235–241.

Bufler, P., Stiegler, G., Schuchmann, M., Hess, S., Krüger, C., Stelter, F., Eckerskorn, C., Schütt, C. and Engelmann, H. 1995. Soluble lipopolysaccharide receptor (CD14) is released via two different mechanisms from human monocytes and CD14 transfectants. Eur. J. Immunol. 25, 604–10.

Bulut, Y., Faure, E., Thomas, L., Equils, O. and Arditi, M. 2001. Cooperation of Toll-like receptor 2 and 6 for cellular activation by soluble tuberculosis factor and Borrelia burgdorferi outer surface protein A lipoprotein: role of Toll-interacting protein and IL-1 receptor signaling molecules in Toll-like receptor 2 signaling. J. Immunol. 167, 987–94.

Burdo, T.H., Margaret R.L., Autissier P., Krishnan A., Halpern E., Letendre S., Rosenberg S., Ellis R.J. and Williams, K.C. 2011. Soluble CD163 made by monocyte/macrophages is a novel a marker of HIV activity in early and chronic infection prior to and after anti-retroviral therapy. J. Infect. Dis. 204, 154-63.

Burgmann, H., Winkler, S., Locker, G.J., Presterl, E., Laczika, K., Staudinger, T., Knapp, S., Thalhammer, F., Wenisch, C., Zedwitz-Liebenstein, K., Frass, M. and Graninger, W. 1996. Increased Serum Concentration of Soluble CD14 Is a Prognostic Marker in Gram-Positive Sepsis. Clin. Immunol. Immunopathol. 80, 307–310.

Casanova, J.-L. and Abel, L. 2002. Genetic dissection of immunity to mycobacteria: the human model. Annu. Rev. Immunol. 20, 581–620.

Chang, J.J. and Altfeld, M. 2009. TLR-mediated immune activation in HIV. Blood. 113, 269–70.

Chang, J.J., Lacas, A., Lindsay, R.J., Doyle, E.H., Axten, K.L., Pereyra, F., Rosenberg, E.S., Walker, B.D., Allen, T.M. and Altfeld, M. 2012. Differential regulation of TLR pathways in acute and chronic HIV-1 infection. AIDS. 26, 533–41.

Chattopadhyay, P.K., Gaylord, B., Palmer, A., Jiang, N., Raven, M.A, Lewis, G., Reuter, M. A, Nur-ur Rahman, A.K.M., Price, D. A, Betts, M.R. and Roederer, M. 2012. Brilliant violet fluorophores: a new class of ultrabright fluorescent compounds for immunofluorescence experiments. Cytometry A. 81, 456–66.

Cho, Y.S., Dobos, K.M., Prenni, J., Yang, H., Hess, A., Rosenkrands, I., Andersen, P., Ryoo, S.W., Bai, G.-H., Brennan, M.J., Izzo, A., Bielefeldt-Ohmann, H. and Belisle, J.T. 2012. Deciphering the proteome of the *in vivo* diagnostic reagent "purified protein derivative" from *Mycobacterium tuberculosis*. Proteomics. 12, 979–91.

Coleman, C.M. and Wu, L. 2009. HIV interactions with monocytes and dendritic cells: viral latency and reservoirs. Retrovirol. 6, 51.

Collini, P., Noursadeghi, M., Sabroe, I., Miller, R.F. and Dockrell, D.H. 2010. Monocyte and macrophage dysfunction as a cause of HIV-1 induced dysfunction of innate immunity. Curr. Mol. Med. 10, 727–40.

Cooper, A.M., Dalton, D.K., Stewart, T.A., Griffin, J.P., Russell, D.G. and Orme, I.M. 1993. Disseminated tuberculosis in interferon γ gene-disrupted mice. J. Exp. Med. 178, 2243–2247.

Cooper, A.M., Magram, J., Ferrante, J. and Orme, I.M. 1997. Interleukin 12 (IL-12) Is Crucial to the Development of Protective Immunity in Mice Intravenously Infected with *Mycobacterium tuberculosis*. J. Exp. Med. 186, 39–45.

Cooper, Mayer-barber, K.D. and Sher, A. 2011. Role of innate cytokines in mycobacterial infection. Mucosal Immunol. 4, 252–260.

Corbett, E.L., Watt, C.J., Walker, N., Maher, D., Williams, B.G., Raviglione, M.C. and Dye, C. 2003. The Growing Burden of Tuberculosis. Arch Intern Med (JAMA). 163, 1009–1021.

Crevel, R. Van, Ottenhoff, T.H.M. and Meer J.W.M Van der., Meer. 2002. Innate Immunity to *Mycobacterium tuberculosis*. Clin. Microbiol. Rev. 15, 294–309.

Crowe, S.M. and Ziegler-Heitbrock, L. 2010. Editorial: Monocyte subpopulations and lentiviral infection. J. Leukoc. Biol. 87, 541–3.

Cruz, A., Fraga, A.G., Fountain, J.J., Rangel-Moreno, J., Torrado, E., Saraiva, M., Pereira, D.R., Randall, T.D., Pedrosa, J., Cooper, A.M. and Castro, A.G. 2010. Pathological role of interleukin 17 in mice subjected to repeated BCG vaccination after infection with *Mycobacterium tuberculosis*. J. Exp. Med. 207, 1609–16.

Danis, V.A., Millington, M., Hyland, V.J. and Grennan, D. 2008. Cytokine production by normal human monocytes: inter-subject variation and relationship to an IL-1 receptor antagonist (IL-1Ra) gene polymorphism. Clin. Exp. Immunol. 99, 303–310.

Davis, C., Wu, X., Li, W., Fan, H. and Reddy, M. 2011. Stability of immunophenotypic markers in fixed peripheral blood for extended analysis using flow cytometry. J. Immunol. Methods. 363, 158–65.

De Chastellier, C., 2008. The many niches and strategies used by pathogenic mycobacteria for survival within host macrophages. Immunobiol. 214, 526–42.

De Waal Malefyt, R. 1991. Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J. Exp. Med. 174, 1209–1220.

Diedrich, C.R. and Flynn, J.L. 2011. HIV-1/*Mycobacterium tuberculosis* coinfection immunology: How does HIV-1 exacerbate Tuberculosis? Infect. Immun. 79, 1407–17.

Dieli, F., Troye-Blomberg, M., Ivanyi, J., Fournié, J.J., Krensky, A.M., Bonneville, M., Peyrat, M.A., Caccamo, N., Sireci, G. and Salerno, A. 2001. Granulysin-dependent killing of intracellular and extracellular *Mycobacterium tuberculosis* by Vgamma9/Vdelta2 T lymphocytes. J. Infect. Dis. 184, 1082–5.

Dinarello, C.A., Cannon, J.G., Wolff, S.M., Bernheim, H.A., Beutler, B., Cerami, A., Figari, I.S., Palladino, M.A. and O'Connor, J. V. 1986. Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1. J. Exp. Med. 163, 1433–50.

Divangahi, M., Mostowy, S., Coulombe, F., Kozak, R., Guillot, L., Veyrier, F., Kobayashi, K.S., Flavell, R.A., Gros, P. and Behr, M.A. 2008. NOD2-deficient mice have impaired resistance to *Mycobacterium tuberculosis* infection through defective innate and adaptive immunity. J. Immunol. 181, 7157–65.

Dobmeyer, T.S., Raffel, B., Dobmeyer, J.M., Findhammer, S., Klein, S.A., Kabelitz, D., Hoelzer, D., Helm, E.B. and Rossol, R. 1995. Decreased function of monocytes and granulocytes during HIV-1 infection correlates with CD4 cell counts. Eur. J. Med. Res. 1, 9–15.

Doherty, T.M. and Arditi, M. 2004. TB, or not TB: that is the question -- does TLR signaling hold the answer? J. Clin. Invest. 114, 1699–703.

Dorhoi, A., Reece, S.T. and Kaufmann, S.H.E. 2011. For better or for worse: the immune response against *Mycobacterium tuberculosis* balances pathology and protection. Immunol. Rev. 240, 235–51.

Dye, C., Scheele S., Dolin P., Pathania V. and Raviglione M. 1999. Global Burden of Tuberculosis: Estimated Incidence, Prevalence, and Mortality by Country. JAMA 282 (7): 677-686.

Ellery, P.J., Tippett, E., Chiu, Y., Paukovics, G., Cameron, P.U., Solomon, A., Lewin S.R., Gorry, P.R., Jaworowski, A., Greene W.C., Sonza, S. and Crowe, S.M. 2012. The CD16+ monocyte subset is more permissive to infection and Preferentially Harbors HIV-1 *In Vivo.* J. Immunol. 178, 6581-6589.

Ernst, J.D. 1998. Macrophage Receptors for *Mycobacterium tuberculosis.* Infect. Immun. 66, 1277–1281.

Ernst, J.D. 2012. The immunological life cycle of tuberculosis. Nat. Rev. Immunol. 12, 581–91.

Eum, S.-Y., Kong, J.-H., Hong, M.-S., Lee, Y.-J., Kim, J.-H., Hwang, S.-H., Cho, S.-N., Via, L.E. and Barry, C.E. 2010. Neutrophils are the predominant infected phagocytic cells in the airways of patients with active pulmonary TB. Chest 137, 122–8.

Fenton, M.J. and Vermeulen, M.W. 1996. Immunopathology of tuberculosis: roles of macrophages and monocytes. Infect. Immun. 64, 683–90.

Ferwerda, G., Girardin, S.E., Kullberg, B.-J., Le Bourhis, L., de Jong, D.J., Langenberg, D.M.L., van Crevel, R., Adema, G.J., Ottenhoff, T.H.M., Van der Meer, J.W.M. and Netea, M.G. 2005. NOD2 and toll-like receptors are nonredundant recognition systems of *Mycobacterium tuberculosis*. PLoS Pathog. 1, 279–85.

Ferwerda, G., Meyer-Wentrup, F., Kullberg, B.-J., Netea, M.G. and Adema, G.J. 2008. Dectin-1 synergizes with TLR2 and TLR4 for cytokine production in human primary monocytes and macrophages. Cell. Microbiol. 10, 2058–66.

Flores-Villanueva, P.O., Ruiz-Morales, J.A., Song, C.-H., Flores, L.M., Jo, E.-K., Montaño, M., Barnes, P.F., Selman, M. and Granados, J. 2005. A functional promoter polymorphism in monocyte chemoattractant protein-1 is associated with increased susceptibility to pulmonary tuberculosis. J. Exp. Med. 202, 1649–58.

Flynn, J. and Chan, J. 2003. Immune evasion by *Mycobacterium tuberculosis* : living with the enemy. Curr. Opin. Immunol. 15, 450–455.

Flynn, J.L. and Chan, J. 2001. Immunology of Tuberculosis. Annu. Rev. Immunol. 19, 93–129.

Flynn, J.L., Chan, J., Triebold, K.J., Dalton, D.K., Stewart, T.A. and Bloom, B.R. 1993. An essential role for interferon γ in resistance to *Mycobacterium tuberculosis* infection. J. Exp. Med. 178, 2249–54.

Flynn, J.L., Goldstein, M.M., Chan, J., Triebold, K.J., Pfeffer, K., Lowenstein, C.J., Schrelber, R., Mak, T.W. and Bloom, B.R. 1995. Tumor necrosis factor-α is required in the protective immune response against *Mycobacterium tuberculosis* in mice. Immunity. 2, 561–572.

Frankenberger, M., Sternsdorf, T., Pechumer, H., Pforte, A. and Ziegler-Heitbrock, H.W. 1996. Differential cytokine expression in human blood monocyte subpopulations: a polymerase chain reaction analysis. Blood 87, 373–377.

Geissmann, F., Manz, M.G., Jung, S., Sieweke, M.H., Merad, M. and Ley, K. 2010. Development of Monocytes, Macrophages, and Dendritic Cells. Science. 327, 656–662.

Gekonge, B., Giri, M.S., Kossenkov, A. V, Nebozyhn, M., Yousef, M., Mounzer, K., Showe, L. and Montaner, L.J. 2012. Constitutive gene expression in monocytes from chronic HIV-1 infection overlaps with acute Toll-like receptor induced monocyte activation profiles. PLoS One. 7, e41153.

Geldmacher, C., Schuetz, A., Ngwenyama, N., Casazza, J.P., Sanga, E., Saathoff, E., Boehme, C., Geis, S., Maboko, L., Singh, M., Minja, F., Meyerhans, A., Koup, R. A. and Hoelscher, M. 2008. Early depletion of *Mycobacterium tuberculosis*-specific T helper 1 cell responses after HIV-1 infection. J. Infect. Dis. 198, 1590–8.

Geldmacher, C., Schuetz, A., Ngwenyama, N., Scuetz A., Petrovas C., Reither K., Heeregrave E.J., Casazza J.P., Ambrozak D.R., Louder M., Ampofo W., Pollakis G., Hill B., Sanga E., Saathoff E., Maboko L., Roederer M., Paxton W.A., Hoelscher M. and Koup R.A. 2010. Preferential infection and depletion of *Mycobacterium tuberculosis*-specific CD4 T cells after HIV-1 infection. J.Exp Med. 207 , 2869-81.

Glynn, J.R., Murray, J., Bester, A., Nelson, G., Shearer, S. and Sonnenberg, P. 2008. Effects of duration of HIV infection and secondary tuberculosis transmission on tuberculosis incidence in the South African gold mines . AIDS. 22(14), 1859-67.

Han, J., Wang, B., Han, N., Zhao, Y., Song, C., Feng, X., Mao, Y., Zhang, F., Zhao, H. and Zeng, H. 2009. $CD14^{high}CD16^{+}$ rather than $CD14^{low}CD16^{+}$ monocytes correlate with disease progression in chronic HIV-infected patients. J. Acquir. Immune Defic. Syndr. 52, 553–9.

Heggelund, L., Müller, F., Lien, E., Yndestad, A., Ueland, T., Kristiansen, K.I., Espevik, T., Aukrust and P., Frøland, S.S. 2004. Increased expression of Toll-like receptor 2 on monocytes in HIV infection: possible roles in inflammation and viral replication. Clin. Infect. Dis. 39, 264–9.

Heimbeck, I., Hofer, T.P.J., Eder, C., Wright, A.K., Frankenberger, M., Marei, A., Boghdadi, G., Scherberich, J. and Ziegler-Heitbrock, L. 2010. Standardized single-platform assay for human monocyte subpopulations: Lower CD14+CD16++ monocytes in females. Cytometry A. 77, 823–30.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K. and Akira, S. 2000. A Toll-like receptor recognizes bacterial DNA. Nat. 408, 740–5.

Hernandez, J.C., Stevenson M., Latz E. and Urcuqui-Inchima S. 2012. HIV Type 1 Infection Up-Regulates TLR2 and TLR4 Expression and Function *in Vivo* and *in Vitro*. AIDS Res Hum Retroviruses. 28.

Hirsch, C.S., Ellner, J.J., Russell, D.G. and Rich, E.A. 1994. Complement receptor-mediated uptake and tumor necrosis factor-alpha-mediated growth inhibition of *Mycobacterium tuberculosis* by human alveolar macrophages. J. Immunol. 152, 743–53.

Hoebe, K., Janssen, E. and Beutler, B. 2004. The interface between innate and adaptive immunity. Nat. Immunol. 10, 971–975.

Hornung, V., Rothenfusser, S., Britsch, S., Krug, A., Jahrsdörfer, B., Giese, T., Endres, S. and Hartmann, G. 2002. Quantitative expression of Toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J. Immunol. 168, 4531–37.

Hossain, M.M. and Norazmi, M.-N. 2013. Pattern recognition receptors and cytokines in *Mycobacterium tuberculosis* infection--The double-edged sword? Biomed Res. Int. 2013, 179174.

Imperiali, F.G., Zaninoni, A., La Maestra, L., Tarsia, P., Blasi, F. and Barcellini, W. 2001. Increased *Mycobacterium tuberculosis* growth in HIV-1-infected human macrophages: role of tumour necrosis factor-alpha. Clin. Exp. Immunol. 123, 435–42.

Jacobs, M., Togbe, D., Fremond, C., Samarina, A., Allie, N., Botha, T., Carlos, D., Parida, S.K., Grivennikov, S., Nedospasov, S., Monteiro, A., Le Bert, M., Quesniaux, V. and Ryffel, B. 2007. Tumor necrosis factor is critical to control tuberculosis infection. Microbes Infect. 9, 623–8.

Jalbert, E., Crawford, T.Q., D'Antoni, M.L., Keating, S.M., Norris, P.J., Nakamoto, B.K., Seto, T., Parikh, N.I., Shikuma, C.M., Ndhlovu, L.C. and Barbour, J.D. 2013. IL-1B Enriched Monocytes Mount Massive IL-6 Responses to Common Inflammatory Triggers among Chronically HIV-1 Infected Adults on Stable Anti-Retroviral Therapy at Risk for Cardiovascular Disease. PLoS One. 8, e75500.

Jambo, K.C., Sepako, E., Fullerton, D.G., Mzinza, D., Glennie, S., Wright, A.K., Heyderman, R.S. and Gordon, S.B. 2011. Bronchoalveolar CD4+ T cell responses to respiratory antigens are impaired in HIV-infected adults. Thorax. 66, 375–82.

Jiang, W., Lederman, M.M., Hunt, P., Sieg, S.F., Haley, K., Rodriguez, B., Landay, A., Martin, J., Sinclair, E., Asher, A.I., Deeks, S.G., Douek, D.C., Brenchley, J.M. 2009. Plasma levels of bacterial DNA correlate with immune activation and the magnitude of immune restoration in persons with antiretroviral-treated HIV infection. J. Infect. Dis. 199, 1177–85.

Jiang, W., Lederman, M.M., Salkowitz, J.R., Rodriguez, B., Harding, C.V and Sieg, S.F. 2005. Impaired Monocyte Maturation in Response to CpG Oligodeoxynucleotide Is Related to Viral RNA Levels in Human Immunodeficiency Virus Disease and Is at Least Partially Mediated by Deficiencies in Alpha / Beta Interferon Responsiveness and Production. J. Virol. 79, 4109–4119.

Juarez, E., Nuñez, C., Sada, E., Ellner, J.J., Schwander, S.K. and Torres, M. 2010. Differential expression of Toll-like receptors on human alveolar macrophages and autologous peripheral monocytes. Respir. Res. 11, 2.

Kaneko, H., Yamada, H., Mizuno, S., Udagawa, T., Kazumi, Y., Sekikawa, K. and Sugawara, I. 1999. Role of tumor necrosis factor-alpha in Mycobacterium-induced granuloma formation in tumor necrosis factor-alpha-deficient mice. Lab. Invest. 79, 379–86.

Kang, P.B., Azad, A.K., Torrelles, J.B., Kaufman, T.M., Beharka, A., Tibesar, E., DesJardin, L.E. and Schlesinger, L.S. 2005. The human macrophage mannose receptor directs *Mycobacterium tuberculosis* lipoarabinomannan-mediated phagosome biogenesis. J. Exp. Med. 202, 987–99.

Kawai, T. and Akira, S. 2011. Toll-like Receptors and Their Crosstalk with Other Innate Receptors in Infection and Immunity. Immunity 34, 637–50.

Keane, J., Gershon, S., Wise, R.P., Mirabile-Levens, E., Kasznica, J., Schwieterman, W.D., Siegel, J.N. and Braun, M.M. 2001. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. N. Engl. J. Med. 345, 1098–104.

Khader, S.A. and Cooper, A.M. 2008. IL-23 and IL-17 in tuberculosis. Cytokine. 41, 79–83.

Khader, S.A, Partida-Sanchez, S., Bell, G., Jelley-Gibbs, D.M., Swain, S., Pearl, J.E., Ghilardi, N., Desauvage, F.J., Lund, F.E. and Cooper, A.M. 2006. Interleukin 12p40 is required for dendritic cell migration and T cell priming after *Mycobacterium tuberculosis* infection. J. Exp. Med. 203, 1805–15.

Khan, K.A., Abbas, W., Varin, A., Kumar, A., Di Martino, V., Dichamp, I. and Herbein, G. 2013. HIV-1 Nef Interacts with HCV Core, Recruits TRAF2, TRAF5 and TRAF6, and Stimulates HIV-1 Replication in Macrophages. J. Innate Immun. 5, 639–56.

Kim, W.-K., Sun, Y., Do, H., Autissier, P., Halpern, E.F., Piatak, M., Lifson, J.D., Burdo, T.H., McGrath, M.S. and Williams, K. 2010. Monocyte heterogeneity underlying phenotypic changes in monocytes according to SIV disease stage. J. Leukoc. Biol. 87, 557–67.

Kleinnijenhuis, J., Oosting, M., Joosten, L.A.B., Netea, M.G. and Van Crevel, R. 2011. Innate immune recognition of *Mycobacterium tuberculosis.* Clin. Dev. Immunol. 2011, 405310.

Knudsen, T.B., Gustafson, P., Kronborg, G., Kristiansen, T.B., Moestrup, S.K., Nielsen, J.O., Gomes, V., Aaby, P., Lisse, I., Møller, H.J. and Eugen-Olsen, J. 2005. Predictive value of soluble haemoglobin scavenger receptor CD163 serum levels for survival in verified tuberculosis patients. Clin. Microbiol. Infect. 11, 730–5.

Krutzik, S.R. and Modlin, R.L. 2004. The role of Toll-like receptors in combating mycobacteria. Semin. Immunol. 16, 35–41.

Krutzik, S.R., Tan, B., Li, H., Ochoa, M.T., Liu, P.T., Sharfstein, S.E., Graeber, T.G., Sieling, P. A, Liu, Y., Rea, T.H., Bloom, B.R. and Modlin, R.L. 2005. TLR activation triggers the rapid differentiation of monocytes into macrophages and dendritic cells. Nat. Med. 11, 653–60.

Kumawat, K., Pathak, S.K., Spetz, A.-L., Kundu, M. and Basu, J. 2010. Exogenous Nef is an inhibitor of *Mycobacterium tuberculosis*-induced tumor necrosis factor-alpha production and macrophage apoptosis. J. Biol. Chem. 285, 12629–37.

Ladel, C.H., Blum, C., Dreher, A., Reifenberg, K., Kopf and M., Kaufmann, S.H.E. 1997. Lethal tuberculosis in interleukin-6-deficient mutant mice. Infect. Immun. 65, 4843-4849.

Lambotte, O., Taoufik Y., de Goer M.G., Wallon. C., Goujard C. and Delfraissy J.F. 2000. Detection of Infectious HIV in Circulating Monocytes from patoents on prolonged Highly Active Antiretroviral Therapy. JAIDS 23, 114-119.

Lancaster, G.I., Khan, Q., Drysdale, P., Wallace, F., Jeukendrup, A.E., Drayson, M.T. and Gleeson, M. 2005. The physiological regulation of toll-like receptor expression and function in humans. J. Physiol. 563, 945–55.

Landmann, Knopf, H., Link, S., Sansano, S., Schumann, R. and Zimmerli, W. 1996. Human monocyte CD14 is upregulated by lipopolysaccharide. Infect. Immun. 64, 1762-1769.

Landmann, R., Müller, B. and Zimmerli, W. 2000. CD14, new aspects of ligand and signal diversity. Microbes Infect. 2, 295–304.

Landmann, R., Zimmerli, W., Sansano, S., Link, S., Hahn, A., Glauser, M.P. and Calandra, T. 1995. Increased Circulating Soluble CD14 Is Associated With High Mortality In Gram-Negative Septic Shock. J. Infect. Dis. 171, 639–644.

Lankford, C.S.R. and Frucht, D.M. 2003. A unique role for IL-23 in promoting cellular immunity. J. Leukoc. Biol. 73, 49–56.

Lathey J.L., Kanangat S., Rouse, B.T., Agosti, M. and Spector S.A. 1994. Dysregulation of cytokine expression in monocytes from HIV-positive individuals. J. Leukoc. Biol. 56, 347–352.

Lazarevic, V. and Flynn, J. 2002. CD8+ T cells in tuberculosis. Am. J. Respir. Crit. Care Med. 166, 1116–21.

Lee, A.W., Sharp, E.R., Mahony, A.O., Rosenberg, M.G., Israelski, D.M., Nolan, G.P. and Nixon, D.F. 2008. Single-Cell, Phosphoepitope-Specific Analysis Demonstrates Cell Type- and Pathway-Specific Dysregulation of Jak / STAT and MAPK Signaling Associated with *In Vivo* Human Immunodeficiency Virus Type 1 Infection. J. Virol. 82, 3702-3712.

Lewin, S.R., Kirihara, J., Sonza, S., Irving, L., Mills, J. and Crowe, S.M. 1998. HIV-1 DNA and mRNA concentrations are similar in peripheral blood monocytes and alveolar macrophages in HIV-1-infected individuals. AIDS. 12, 719-727.

Lewinsohn, D.A., Heinzel, A.S., Gardner, J.M., Zhu, L., Alderson, M.R. and Lweinsohn D.M. 2003. *Mycobacterium tuberculosis*-specific CD8+ T cells preferentially recognize heavily infected cells. Am. J. Respir. Crit. Care Med. 168, 1346–52.

Lien, E., Aukrust, P., Sundan, a, Müller, F., Frøland, S.S. and Espevik, T. 1998. Elevated levels of serum-soluble CD14 in human immunodeficiency virus type 1 (HIV-1) infection: Correlation to disease progression and clinical events. Blood. 92, 2084–92.

Lin, Y., Gong, J., Zhang, M., Xue, W. and Barnes, P.F. 1998. Production of monocyte chemoattractant protein 1 in tuberculosis patients. Infect. Immun. 66, 2319–22.

Lin, J., Yao, Y., Yu, Y., Chai, J., Huang, Z., Dong, N. and Sheng, Z. 2007. Effects of CD14-159 C/T polymorphism on CD14 Expression and the balance between proinflammatory and anti-inflammatory cytokines in whole blood culture. Shock. 28, 148–153.

Lin, P.L., Flynn, J.L. 2010. Understanding latent tuberculosis: A moving target. J. Immunol. 185, 15–22.

Liu, P.T., Stenger, S., Tang, D.H. and Modlin, R.L. 2007. Cutting edge: Vitamin D-mediated human antimicrobial activity against *Mycobacterium tuberculosis* is dependent on the induction of cathelicidin. J. Immunol. 179, 2060–3.

Lockhart, E., Green, A.M. and Flynn, J.L. 2006. IL-17 production is dominated by γδ T cells rather than CD4 T cells during *Mycobacterium tuberculosis* infection. J. Immunol. 177, 4662–9.

Louis, E., Franchimont, D., Piron, a, Gevaert, Y., Schaaf-Lafontaine, N., Roland, S., Mahieu, P., Malaise, M., De Groote, D., Louis, R. and Belaiche, J. 1998. Tumour necrosis factor (TNF) gene polymorphism influences TNF-alpha production in lipopolysaccharide (LPS)-stimulated whole blood cell culture in healthy humans. Clin. Exp. Immunol. 113, 401–6.

Mahnke, Y.D. and Roederer, M. 2007. Optimizing a multicolor immunophenotyping assay. Clin. Lab. Med. 27, 469–85.

Mäkelä, S.M., Strengell, M., Pietilä, T.E., Osterlund, P. and Julkunen, I. 2009. Multiple signaling pathways contribute to synergistic TLR ligand-dependent cytokine gene expression in human monocyte-derived macrophages and dendritic cells. J. Leukoc. Biol. 85, 664–72.

Manuzak, Dillon, S.M., Lee, E.J., Dong, Z.M., Hecht, D.K. and Wilson, C.C. 2013. Increased *Escherichia coli*-Induced Interleukin-23 Production by CD16+ Monocytes Correlates with Systemic Immune Activation in Untreated HIV-1-Infected Individuals. J. Virol. 87, 13252–13262.

Marchetti, G., Tincati, C. and Silvestri, G. 2013. Microbial translocation in the pathogenesis of HIV infection and AIDS. Clin. Microbiol. Rev. 26, 2–18.

McCarthy, D.A., Macey, M.G., Cahill, M.R. and Newland, A.C. 1994. Effect of fixation on quantification of the expression of leucocyte function-associated surface antigens. Cytometry. 17, 39–49.

Means, T.K., Wang, S., Lien, E., Yoshimura, A., Golenbock, D.T. and Fenton, M.J. 1999a. Human toll-like receptors mediate cellular activation by *Mycobacterium tuberculosis*. J. Immunol. 163, 3920–7.

Means, T.K., Lien, E., Yoshimura, A., Wang, S., Golenbock, D.T. and Fenton, M.J. 1999b. The CD14 ligands lipoarabinomannan and lipopolysaccharide differ in their requirement for Toll-like receptors. J. Immunol. 163, 6748-55.

Mege, J.-L., Meghari, S., Honstettre, A., Capo, C. and Raoult, D. 2006. The two faces of interleukin 10 in human infectious diseases. Lancet Infect. Dis. 6, 557–69.

Meier, A., Alter, G., Frahm, N., Sidhu, H., Li, B., Bagchi, A., Teigen, N., Streeck, H., Stellbrink, H.-J., Hellman, J., van Lunzen, J. and Altfeld, M. 2007. MyD88-dependent immune activation mediated by human immunodeficiency virus type 1-encoded Toll-like receptor ligands. J. Virol. 81, 8180–91.

Mir K.D., Bosinger S.E., Gasper M., Ho O., Else J.G., Brenchley J.M., Kelvin D.J., Silverstri G., Hu SL. and Sodora D.L. 2012. Simian Immunodeficiency Virus-induced alterations in monocyte production of TNF-α contribute to reduced immune activation in sooty mangabeys. J. Virol. 86, 7605-7615.

Mortellaro, A., Robinson, L. and Ricciardi-castagnoli, P. 2009. Spotlight on mycobacteria and dendritic cells : will novel targets to fight tuberculosis emerge ? EMBO Mol. Med. 1, 19–29.

Müller, F., Rollag, H. and Froland, S.S. 1990. Reduced oxidative burst responses in monocytes and monocyte-derived macrophages from HIV-infected subjects. Clin. Exp. Immunol. 82, 10–15.

Mureith, M.W., Chang, J.J., Lifson, J.D., Ndung'u, T. and Altfeld, M. 2010. Exposure to HIV-1-encoded Toll-like receptor 8 ligands enhances monocyte response to microbial encoded Toll-like receptor 2/4 ligands. AIDS. 24, 1841–8.

Muzio, M., Bosisio, D., Polentarutti, N., D'amico, G., Stoppacciaro A., Mancinelli, R., Veer, C., Penton-Rol, G., Ruco, L.P., Allavena, P. and Mantovani, A. 2000. Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. J. Immunol. 164, 5998–6004.

Mwandumba, H.C., Russell, D.G., Nyirenda, M.H., Anderson, J., White, S. A, Molyneux, M.E. and Squire, S.B. 2004. *Mycobacterium tuberculosis* resides in nonacidified vacuoles in endocytically competent alveolar macrophages from patients with tuberculosis and HIV infection. J. Immunol. 172, 4592–8.

Napolitani, G., Rinaldi, A., Bertoni, F., Sallusto, F. and Lanzavecchia, A. 2005. Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. Nat. Immunol. 6, 769–76.

Nicol, M.Q., Mathys, J.-M., Pereira, A., Ollington, K., Ieong, M.H. and Skolnik, P.R. 2008. Human immunodeficiency virus infection alters tumor necrosis factor alpha production via Toll-like receptor-dependent pathways in alveolar macrophages and U1 cells. J. Virol. 82, 7790–8.

Nnoaham, K.E. and Clarke, A. 2008. Low serum vitamin D levels and tuberculosis: a systematic review and meta-analysis. Int. J. Epidemiol. 37, 113–9.

North, R.J. 1998. Mice incapable of making IL-4 or IL-10 display normal resistance to infection with *Mycobacterium tuberculosis.* Clin. Exp. Immunol. 113, 55–8.

Noursadeghi, M., Katz, D.R. and Miller, R.F. 2006. HIV-1 infection of mononuclear phagocytic cells : the case for bacterial innate immune deficiency in AIDS. Lancet Infect Dis. 6, 794-804.

O'Garra, A., Redford, P.S., McNab, F.W., Bloom, C.I., Wilkinson, R.J. and Berry, M.P.R. 2013. The immune response in tuberculosis. Annu. Rev. Immunol. 31, 475-527.

O'Mahony, D.S., Pham, U., Iyer, R., Hawn, T.R. and Liles, W.C. 2008. Differential constitutive and cytokine-modulated expression of human Toll-like receptors in primary neutrophils, monocytes, and macrophages. Int. J. Med. Sci. 5, 1–8.

Oesterreicher, C., Pfeffel, F., Petermann, D. and Müller, C. 1995. Increased *in vitro* production and serum levels of the soluble lipopolysaccharide receptor sCD14 in liver disease. J. Hepatol. 23, 396–402.

Orr, S.L. and Tobias, P. 2000. LPS and LAM activation of the U373 astrocytoma cell line: Differential requirement for CD14. J. Endotoxin Res. 6, 215–222.

Ottenhoff, T.H., Kumararatne, D. and Casanova, J.-L. 1998. Novel human immunodeficiencies reveal the essential role of type-1 cytokines in immunity to intracellular bacteria. Immunol. Today. 19, 491–494.

Paiardini M., Müller-Trutwin, M. 2013. HIV-associated chronic immune activation. Immunol. Rev. 254, 78–101.

Patel, N.R., Zhu, J., Tachado, S.D., Zhang, J., Wan, Z., Saukkonen, J and, Koziel, H., 2007. HIV impairs TNF-alpha mediated macrophage apoptotic response to *Mycobacterium tuberculosis*. J. Immunol. 179, 6973–80.

Pathak, S., Souza, G.A.De., Salte T., Wiker H.G., and Asjo B. 2009. HIV Induces both a Down-Regulation of IRAK-4 that Impairs TLR Signalling and an Up-regulation of the Antibiotic Peptide Dermcidin in Monocytic Cells. Scand J. Immunol. 70, 264–276.

Pathak, S., Wentzel-Larsen, T. and Asjö, B. 2010. Effects of *in vitro* HIV-1 infection on mycobacterial growth in peripheral blood monocyte-derived macrophages. Infect. Immun. 78, 4022–32.

Perfetto, S.P., Chattopadhyay, P.K., Lamoreaux, L., Nguyen, R., Ambrozak, D., Koup, R. A and Roederer, M. 2006. Amine reactive dyes: An effective tool to discriminate live and dead cells in polychromatic flow cytometry. J. Immunol. Methods. 313, 199–208.

Perfetto, S.P., Chattopadhyay, P.K. and Roederer, M. 2004. Seventeen-colour flow cytometry: unravelling the immune system. Nat. Rev. Immunol. 4, 648–55.

Philips, J.A. and Ernst, J.D. 2012. Tuberculosis pathogenesis and immunity. Annu. Rev. Pathol. 7, 353–84.

Pieters, J. 2008. *Mycobacterium tuberculosis* and the macrophage: maintaining a balance. Cell Host & Microbe. 3, 399–407.

Pollice, A.A., McCoy, J.P., Shackney, S.E., Smith, C.A., Agarwal, J., Burholt, D.R., Janocko, L.E., Hornicek, F.J., Singh, S.G. and Hartsock, R.J. 1992. Sequential paraformaldehyde and methanol fixation for simultaneous flow cytometric analysis of DNA, cell surface proteins, and intracellular proteins. Cytometry. 13, 432–44.

Prasad, T.S.K., Verma, R., Kumar, S., Nirujogi, R.S., Sathe, G.J., Madugundu, A.K., Sharma, J., Puttamallesh, V.N., Ganjiwale, A., Myneedu, V.P., Chatterjee, A., Pandey, A., Harsha, H. and Narayana, J. 2013. Proteomic analysis of purified protein derivative of *Mycobacterium tuberculosis.* Clin. Proteomics. 10, 8.

Pugin, J., Heumann, I.D., Tomasz, a, Kravchenko, V. V, Akamatsu, Y., Nishijima, M., Glauser, M.P., Tobias, P.S. and Ulevitch, R.J. 1994. CD14 is a pattern recognition receptor. Immunity 1, 509–16.

Quesniaux, V., Fremond, C., Jacobs, M., Parida, S., Nicolle, D., Yeremeev, V., Bihl, F., Erard, F., Botha, T., Drennan, M., Soler, M.-N., Le Bert, M., Schnyder, B. and Ryffel, B. 2004. Toll-like receptor pathways in the immune responses to mycobacteria. Microbes Infect. 6, 946–59.

Ragno, S., Romano, M., Howell, S., Pappin, D.J., Jenner, P.J. and Colston, M.J. 2001. Changes in gene expression in macrophages infected with *Mycobacterium tuberculosis:* a combined transcriptomic and proteomic approach. Immunology 104, 99–108.

Rasaiyaah, J., Tan, C.P., Fletcher, A.J., Price, A.J., Blondeau, C., Hilditch, L., Jacques, D. A, Selwood, D.L., James, L.C., Noursadeghi, M. and Towers, G.J. 2013. HIV-1 evades innate immune recognition through specific cofactor recruitment. Nat. 503, 402–5.

Roach, D.R., Bean, A.G.D., Demangel, C., France, M.P., Briscoe, H. and Britton, W.J. 2002. TNF regulates chemokine induction essential for cell recruitment, granuloma formation, and clearance of mycobacterial infection. J. Immunol. 168, 4620–7.

Roederer, M., Nozzi, J. and Nason, M. 2011. SPICE: Exploration and analysis of post-cytometric complex multivariate datasets. Cytometry A. 79, 167–74.

Russell, D.G. 2007. Who puts the tubercle in tuberculosis? Nat. Rev. Microbiol. 5, 39–47.

Sandler, N.G., Wand, H., Roque, A., Law, M., Nason, M.C., Nixon, D.E., Pedersen, C., Ruxrungtham, K., Lewin, S.R., Emery, S., Neaton, J.D., Brenchley, J.M., Deeks, S.G., Sereti, I. and Douek, D.C. 2011. Plasma levels of soluble CD14 independently predict mortality in HIV infection. J. Infect. Dis. 203, 780–90.

Saraiva, M. and O'Garra, A. 2010. The regulation of IL-10 production by immune cells. Nat. Rev. Immunol. 10, 170–81.

Sasindran, S.J. and Torrelles, J.B. 2011. *Mycobacterium Tuberculosis* Infection and Inflammation: what is beneficial for the host and for the bacterium? Front. Microbiol. 2, (2).

Sato, S., Nomura, F., Kawai, T., Takeuchi, O., Mühlradt, P.F., Takeda, K. and Akira, S., 2000. Synergy and cross-tolerance between toll-like receptor (TLR) 2-and TLR4-mediated signaling pathways. J. Immunol. 165, 7096–101.

Saunders, B.M. and Cooper, A.M. 2000. Restraining mycobacteria: Role of granulomas in mycobacterial infections. Immunol. Cell Biol. 78, 334–41.

Savedra, R., Delude, R., Ingalls, R.R., Fenton, M.J. and Golenbock D.T. 1996. Mycobacterial lipoarabinomannan recognition requires a receptor that shares components of the endotoxin signaling system. J. Immunol. 157, 2549-2554.

Schröder, N.W.J., Morath, S., Alexander, C., Hamann, L., Hartung, T., Zähringer, U., Göbel, U.B., Weber, J.R. and Schumann, R.R. 2003. Lipoteichoic acid (LTA) of *Streptococcus pneumoniae* and *Staphylococcus aureus* activates immune cells via Toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved. J. Biol. Chem. 278, 15587–94.

Shey, M.S., Hughes, E.J., de Kock, M., Barnard, C., Stone, L., Kollmann, T.R., Hanekom, W. A., Scriba, T.J. 2012. Optimization of a whole blood intracellular cytokine assay for measuring innate cell responses to mycobacteria. J. Immunol. Methods. 376, 79–88.

Shi, C., Jia, T., Mendez-Ferrer, S., Hohl, T.M., Serbina, N. V, Lipuma, L., Leiner, I., Li, M.O., Frenette, P.S. and Pamer, E.G. 2011. Bone marrow mesenchymal stem and progenitor cells induce monocyte emigration in response to circulating toll-like receptor ligands. Immunity 34, 590–601.

Sims, J.E. and Smith, D.E. 2010. The IL-1 family: regulators of immunity. Nat. Rev. Immunol. 10, 89–102.

Sonnenberg, P., Glynn, J.R., Fielding, K., Murray, J., Godfrey-Faussett, P., Shearer, S. 2005. How soon after infection with HIV does the risk of tuberculosis start to increase? A retrospective cohort study in South African gold miners. J. Infect. Dis. 191, 150–8.

Sonza, S., Mutimer, H.P., Oelrichs, R., Jardine, D., Harvey, K., Dunne, A., Purcell, D.F., Birch, C. and Crowe, S.M. 2001. Monocyte harbour replication-competent, non-latent HIV-1 in patients on highly active antiretroviral therapy. AIDS. 15, 17–22.

Spear, G.T., Kessler, H.A., Rothberg, L., Phair, J. and Landay, A.L. 1990. Decreased oxidative burst activity of monocytes from asymptomatic HIV-infected individuals. Clin. Immunol. Immunopathol. 54, 184–191.

Stenger, S. and Modlin, R.L. 2002. Control of *Mycobacterium tuberculosis* through mammalian Toll-like receptors. Curr. Opin. Immunol. 14, 452–7.

Stenger, S., Rosat, J.-P., Bloom, B.R., Krensky, A.M. and Modlin, R.L. 1999. Granulysin: a lethal weapon of cytolytic T cells. Immunol. Today 20, 390–394.

Stewart, J.C., Villasmil, M.L. and Frampton, M.W. 2007. Changes in fluorescence intensity of selected leukocyte surface markers following fixation. Cytometry A. 71, 379–85.

Tachado, S.D., Li, X., Bole, M., Swan, K., Anandaiah, A., Patel, N.R., Dc, W. and Koziel, H. 2010. MyD88-dependent TLR4 signaling is selectively impaired in alveolar macrophages from asymptomatic HIV+ persons. Blood 115, 3606–15.

Tacke, F. and Randolph, G.J. 2006. Migratory fate and differentiation of blood monocyte subsets. Immunobiol. 211, 609–18.

Takeuchi, O., Sato, S., Horiuchi, T., Hoshino, K., Takeda, K., Dong, Z., Modlin, R.L. and Akira, S. 2002. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J. Immunol. 169, 10–4.

Thieblemont, N., Weiss, L., Sadeghi, H.M., Estcourt, C. and Haeffner-cavaillon, N. 1995. $CD14^{low}CD16^{high}$: a cytokine-producing monocyte subset which expands during human immunodeficiency virus infection. Eur. J. Immunol. 25, 3418–3424.

Tilton, J.C., Johnson, A.J., Luskin, M.R., Manion, M.M., Yang, J., Adelsberger, J.W., Lempicki, R. A, Hallahan, C.W., McLaughlin, M., Mican, J.M., Metcalf, J. A, Iyasere, C. and Connors, M. 2006. Diminished production of monocyte proinflammatory cytokines during human immunodeficiency virus viremia is mediated by type I interferon. J. Virol. 80, 11486–97.

Torrado, E. and Cooper, A.M. 2010. IL-17 and Th17 cells in tuberculosis. Cytokine Growth Factor Rev. 21, 455–62.

Tosato, G. and Jones, K.D. 1990. Interleukin-1 induces interleukin-6 production in peripheral blood monocytes. Blood 75, 1305–10.

Triantafilou, M. and Triantafilou, K. 2002. Lipopolysaccharide recognition: CD14, TLRs and the LPS-activation cluster. Trends Immunol. 23, 301–4.

Trinchieri, G. and Sher, A. 2007. Cooperation of Toll-like receptor signals in innate immune defence. Nat. Rev. Immunol. 7, 179–90.

Turner, D.M., Williams, D.M., Sankaran, D., Lazarus, M., Sinnott, P.J. and Hutchinson, I. V. 1997. An investigation of polymorphism in the interleukin-10 gene promoter. Eur. J. Immunogenet. 24, 1–8.

Turner, Gonzalez-Juarrero, M., Ellis, D.L., Basaraba, R.J., Kipnis, A., Orme, I.M. and Cooper, A.M. 2002. *In vivo* IL-10 production reactivates chronic pulmonary tuberculosis in C57BL/6 mice. J. Immunol. 169, 6343–51.

Twigg, H.L. and Knox, K.S. 2007. HIV-Related Lung Disorders. Drug Discov. Today. Dis. Mech. 4, 95–101.

Twigg III, H.L., Weiden, M., Valentine, F., Schnizlein-Bick, C.T., Bassett, R., Zheng, L., Wheat, J., Day, R.B., Rominger, H., Collman, R.G., Fox, L., Brizz, B., Dragavon, J., Coombs, R.W. and Bucy, R.P. 2008. Effect of highly active antiretroviral therapy on viral burden in the lungs of HIV-infected subjects. J. Infect. Dis. 197, 109–16.

Underhill, D.M., Ozinsky, A., Smith, K.D. and Aderem, A. 1999. Toll-like receptor-2 mediates mycobacteria-induced proinflammatory signaling in macrophages. PNAS. 96, 14459–14463.

Urdahl, K.B., Shafiani, S. and Ernst, J.D. 2011. Initiation and regulation of T-cell responses in tuberculosis. Mucosal Immunol. 4, 288–93.

Vanheyningen, T.K., Collins H.L. and Russell, D.G. 1997. 1L-6 Produced by macrophages infected with *Mycobacterium* species suppresses T cell responses. J. Immunol. 158, 330-337.

Wahl, S., Allen J.B., Gartner S., Orenstein J.M., Popovic M., Chenoweth, D.E., Arthur, L.O., Farrar, W.L. and Wahl, L.M. 1989. HIV-1 and its envelope glycoprotein down-regulate chemotactic ligand receptors and chemotactic function of peripheral blood monocytes. J. Immunol. 142, 3553–9.

Walker, N.F., Meintjes, M., Wilkinson, R.J. 2013. HIV-1 and the Immune response to TB. Future Virol. 8, 57–80.

Weaver, L.K., Hintz-goldstein, K.A., Pioli, P.A., Wardwell, K., Qureshi, N., Vogel, S.N. and Guyre, P.M. 2006. Pivotal advance: activation of cell surface Toll-like receptors causes shedding of the hemoglobin scavenger receptor CD163. J. Leukoc. Biol. 80, 26–35.

Weaver, L.K., Pioli, P. A, Wardwell, K., Vogel, S.N. and Guyre, P.M. 2007. Up-regulation of human monocyte CD163 upon activation of cell-surface Toll-like receptors. J. Leukoc. Biol. 81, 663–71.

WHO Global Tuberculosis Report 2013a, accessed from www.who.org in January 2014.

WHO HIV-Associated TB Facts 2013b, accessed from www.who.org in January 2014.

Wieland, C.W., Knapp, S., Florquin, S., de Vos, A.F., Takeda, K., Akira, S., Golenbock, D.T., Verbon, A. and van der Poll, T. 2004. Non-mannose-capped lipoarabinomannan induces lung inflammation via toll-like receptor 2. Am. J. Respir. Crit. Care Med. 170, 1367–74.

Wong, K.L., Tai, J.J., Wong, W., Han, H., Sem, X., Yeap, W., Kourilsky, P. and Wong, S. 2011. Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. Blood. 118, 16-31.

Wong, K.L., Yeap, W.H., Tai, J.J.Y., Ong, S.M., Dang, T.M. and Wong, S.C. 2012. The three human monocyte subsets: implications for health and disease. Immunol. Res. 53, 41–57.

Wood, K.L., Chaiyarit P., Day R.B, Wang Y., Schnizlein-Bick C.T., Gregory R.L. and Twigg III H.L. 2003. Measurements of HIV Viral Loads From Different Levels of the Respiratory Tract. Chest 124, 536–542.

Woodworth, J.S.M. and Behar, S.M. 2006. *Mycobacterium tuberculosis*-Specific CD8+ T Cells and Their Role in Immunity. Crit. Rev. Immunol. 26, 317–352.

Wright, S., Ramos, R., Tobias, P., Ulevitch, R. and Mathison, J. 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science. 249, 1431–1433.

Wu, J.Q., Ruth Sassé, T., Wolkenstein, G., Conceicao, V., Miranda Saksena, M., Soedjono, M., Perera, S.S., Wang, B., Dwyer, D.E. and Saksena, N.K. 2012. Transcriptome analysis of primary monocytes shows global down-regulation of genetic networks in HIV viremic patients versus long-term non-progressors. Virology.

Yamada, H., Mizumo, S., Horai, R., Iwakura, Y. and Sugawara, I. 2000. Protective Role of Interleukin-1 in Mycobacterial Infection in IL-1α/β Double-Knockout Mice. Lab. Investig. 80, 759–767.

Yoshida Y.O., Umemura, M., Yahagi, A., O'Brien, R.L., Ikuta, K., Kishihara, K., Hara, H., Nakae, S., Iwakura, Y. and Matsuzaki, G. 2010. Essential role of IL-17A in the formation of a mycobacterial infection-induced granuloma in the lung. J. Immunol. 184, 4414–22.

Yu, W., Soprana, E., Cosentino, G., Volta, M., Lichenstein, H.S., Viale, G. and Vercelli, D. 1998. Soluble CD14(1-152) confers responsiveness to both lipoarabinomannan and lipopolysaccharide in a novel HL-60 cell bioassay. J. Immunol. 161, 4244–51.

Zhang, Y., Doerfler, M., Lee, T.C., Guillemin, B. and Rom, W.N. 1993. Mechanisms of Stimulation of Interleukin-1β and Tumor Necrosis Factor-α by *Mycobacterium tuberculosis* Components. 91, 2076–2083.

Zhu, T. 2002. HIV-1 in peripheral blood monocytes: an underrated viral source. J. Antimicrob. Chemother. 50, 309–311.

Zhu, T., Muthui, D., Holte, S., Nickle, D., Feng, F., Brodie, S., Hwangbo, Y., Mullins, J.I. and Corey, L., 2002. Evidence for Human Immunodeficiency Virus Type 1 Replication *In Vivo* in CD14 + Monocytes and Its Potential Role as a Source of Virus in Patients on Highly Active Antiretroviral Therapy. J. Virol. 76: 707.

Ziegler-Heitbrock, L. 2000. Definition of human blood monocytes. J. Leukoc. Biol. 67, 603–6.

Ziegler-heitbrock, L. 2007. The CD14+ CD16+ blood monocytes: their role in infection and inflammation. J. Leukoc. Biol. 81, 584–92.

Ziegler-Heitbrock, L., Ancuta, P., Crowe, S., Dalod, M., Grau, V., Hart, D.N., Leenen, P.J.M., Liu, Y.-J., MacPherson, G., Randolph, G.J., Scherberich, J., Schmitz, J., Shortman, K., Sozzani, S., Strobl, H., Zembala, M., Austyn, J.M. and Lutz, M.B. 2010. Nomenclature of monocytes and dendritic cells in blood. Blood. 116, 74–80.