The Role of Innate Immunity In HIV-1 Transmission and Pathogenesis: A Study of NK cells and Monocytes

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Abstract

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This dissertation investigates two key innate immune cell subsets, monocytes and NK cells, with regard to their ability to influence HIV transmission and disease progression, including their role in defense against opportunistic pathogens.

Evaluation of HIV transmission focused on mother-to-child transmission (MTCT). We performed a case-control study to evaluate cord blood cells from HIV-1 exposed infants who acquired infection by one month (cases) to those who remained uninfected for 1 year (controls). Compared to controls, cord blood from cases displayed altered proportions of NK cell subsets, fewer activated non-HIV-target NK and CD8+ T cells and a higher proportion of HIV target effector memory CD4+ T cells. Subsequent studies were then performed to determine the role of BCG vaccine administration as a potential

source of immune activation (which correlates with the risk of HIV acquisition) in HIV-exposed infants. BCG vaccination induced an increase in activated CD4+ T cells, which have potential to serve as a pool of HIV-1 targets, and this occurred independently of innate cell activation. Together, these studies identify altered cellular phenotypes that are associated with increased target cell activation and subsequent HIV-1 acquisition, providing insights into immune factors associated with MTCT.

In the majority of patients that progress to AIDS, disease is accompanied by opportunistic infections (like *Mycobacteria*). To better understand HIV pathogenesis and increased susceptibility to opportunistic pathogens, we exposed peripheral blood cells from SIV+ sooty mangabey monkeys (that do not develop AIDS) and HIV-infected humans to *Mycobacteria* species and evaluated NK cell and monocyte responses. This study demonstrated that HIV+ patients have significantly altered NK cell gene expression profiles and deficient monocyte IL-12 production in response to the opportunistic pathogen BCG compared to HIV-negative donors. In contrast, SIV+ mangabeys maintain similar NK cell gene expression profiles and an increase in the production of monocyte-derived TNF-alpha and IL-12 (compared to uninfected controls). As these cytokines serve key roles in mycobacteria defense and are critical for activating NK cells, these studies provide tangible targets for immunotherapy designed to augment monocyte and NK cell function during pathogenic HIV infection to prevent acquisition of opportunistic infections.

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CHAPTER 1:

INTRODUCTION AND LITERATURE REVIEW

OVERVIEW

This chapter contains an overview of the concepts and literature relevant to this dissertation. Topics include the epidemiology of HIV-1, the natural history of SIV and HIV infections; HIV-1 transmission, with a focus on mother to child transmission; the HIV-1 disease course, including the progression to AIDS and opportunistic infections; and the innate immune system. As the studies described in this dissertation highlight the role of monocytes and NK cells and their involvement in the human immunodeficiency virus type 1 (HIV-1) and *M. tuberculosis* (Mtb) infections, these topics are therefore emphasized in this introductory chapter. Additionally, this chapter contains an outline of the overall goals of the dissertation.

INTRODUCTION

Aggressive and cooperative efforts to achieve United Nations' Millennium Development Goal 6 (to combat HIV/AIDS, malaria, and other diseases) have resulted in a decline in the incidence of HIV infection and an increase in access to life-saving antiretroviral therapy across the globe. However, as of 2012 over 35 million people worldwide were still living with HIV. Despite the increased availability of ART, only about a third of HIV-infected individuals received ART in 2012 [6], indicating that over 20 million HIV-infected people are still without treatment and the majority will therefore develop AIDS.

Following HIV acquisition, disease progression to AIDS is associated with increased viral replication, CD4+ T cell depletion, and a subsequent generalized immune dysregulation that becomes manifested through a number of opportunistic infections or malignancies. *Mycobacteria* species, in particular, are responsible for significant morbidity and mortaility in HIV/AIDS patients around the globe. Innate immunity is critical for the initial recognition of pathogens, like HIV and *Mycobacteria,* as well as for dictating the quantity and quality of a long-lasting adaptive immune response to those pathogens. The overall goal of this dissertation is to yield new insight into mechansims by which innate immunity influences HIV-1 transmission and impairs innate immune cell responses to *Mycobacteria* species during the pathogenesis phase of infection. Through a clearer perspective of HIV's interplay with innate immunity, vaccines and new therapeutic approaches to combat opportunistic infections, like *Mycobacteria* species, which exploit the immunocompromised host, can be developed.

HIV ORIGIN AND DISEASE COURSE

The Ancient Origin of HIV Infections

Several species of African monkeys can be found in the wild infected with their own species-specific strains of the simian immunodeficiency virus (SIV). It has been recently determined through a detailed phylogenetic analysis that these monkeys, known as "natural hosts" of SIV infections, have been infected with SIV for several thousands of years [7]. Natural hosts rarely progress to AIDS despite high levels of replicating virus as a result of co-evolution with their species-specific viruses over thousands of years and a resultant harmonious coexistence of host and virus that ultimately does not result in host progression to AIDS.

In contrast to non-pathogenic infections of natural hosts, pathogenic, progressive lentiviral infections, like HIV-1 and HIV-2 have only relatively recently flourished. Both viruses originated from cross-species transmissions from non-human primates, in which they are referred to as simian immunodeficiency viruses (SIVs), to humans. HIV-1 arose as a result of consecutive cross-species transmission events from African natural hosts, through chimpanzee intermediates, into human hosts. The chimpanzee was likely a critical factor in creating a virus with pandemic potential like HIV-1, as it provided the virus the chance to adapt to a new host extremely similar in genetic composition to humans. Further because chimpanzees are routinely hunted for bush meat in Africa, chimpanzees ultimately brought human and virus together for repeated exposures needed for a successful transmission event. In contrast, HIV-2 has arisen from direct viral transmission from sooty mangabeys into humans. Like HIV-1, HIV-2 was likely transmitted to humans through the butchering of infected sooty mangabeys for bush meat. Additionally, experimental passage of SIVs from sooty mangabeys into Asian Rhesus macaques results in an extremely pathogenic disease course that mirrors HIV-1 infection of humans, including an increased susceptibility to opportunistic infections [8]. Rhesus macaques are therefore commonly used as an experimental model of HIV-1 infection for laboratory-based

studies. Collectively, this suggests that transmission of an SIV from a "natural" to a "non-natural" host results in pathogenic disease and AIDS.

Although separate viruses, HIV-1 and HIV-2 share many similarities, including modes of transmission, intracellular viral replication, and similar clinical AIDS manifestations with disease progression. Unlike HIV-1, which has reached pandemic proportions, HIV-2 has remained relatively confined to the Western Africa, and its prevalence is declining (reviewed in [9]). An in-depth discussion as to the differences between these viruses that lead to their different pandemic-causing potential is beyond the scope of this dissertation, but may be due to the more infectious nature of HIV-1, which is ultimately attributable to a higher level of viral fitness (reviewed in [10]). Despite these differences, HIV-1 and although less frequently, HIV-2 infections both have potential to cause characteristic AIDS pathology, including severe immune system dysfunction and the acquisition of opportunistic infections (OIs) (reviewed in [11]). Following disease progression of either HIV-1 or HIV-2, the major cause of death for AIDS patients is tuberculosis, either pulmonary or meningitis [12].

The Contribution of Natural Host Species to Informing HIV/AIDS Research

Two natural hosts species, sooty mangabeys and African green monkeys, have been the subjects of intensive scientific research in order to better understand their non-progressive phenotypes; These natural hosts have been critical to our understanding of AIDS pathogenesis. Despite similarities to hosts of pathogenic infections, including high levels of innate and adaptive immune activation during acute infection and intact adaptive antiviral immune responses that are unable to control viral replication [13-16], several factors unique to the natural hosts may play a role in keeping them free of simian AIDS (reviewed in [17]). It was initially thought that the preservation of peripheral CD4+ T cell levels was the reason for lack of disease progression in these hosts. However, data from our laboratory and others have demonstrated that sooty mangabeys can remain free of AIDS and opportunistic infections even in the face of dramatic

CD4+ T cell depletion [18, 19]. This suggests that other immune cell types, like innate immune cells, may be important in keeping natural hosts free of opportunistic infections during chronic viral infection. Additionally, in contrast to hosts of pathogenic infections, natural hosts maintain normal lymph node architecture and function as well as mucosal immunity, ultimately resulting in a lack of microbial translocation that contributes to chronic, generalized immune activation in pathogenic infections [19-21].

One key difference between hosts of pathogenic and non-pathogenic infections includes the presence of chronic, generalized immune activation in hosts of pathogenic infections and lack thereof in hosts of non-pathogenic infections. Chronic immune activation is defined by the presence of persistent, increased plasma proinflammatory cytokines and chronic stimulation of immune cells, ultimately leading to a high turnover of adaptive immune cells (reviewed in [22]). This persistent activation in hosts of pathogenic infection likely drives immune anergy seen in pathogenic infections, and is a probable contributing factor to the inability of immune cells from hosts of pathogenic infections to respond to foreign pathogens [23, 24].

There are a number of hypothesized sources of immune activation, including direct stimulation by virus, the dysfunction or depletion of immunoregulatory cells, the leaking of microbial byproducts from the gut lumen into systemic circulation following dramatic gut CD4+ T cell depletion, and the presence of opportunistic pathogens. A number of studies have identified that the level of immune activation may be a stronger correlate of disease progression than viral load [25-27]. Ultimately, the resolution of immune activation during the acute-to-chronic transition phase of the non-pathogenic SIV disease course likely contributes to proper immune system functioning in the face of chronic viral infection and replication (reviewed in [28]).

HIV-1 Transmission

There are three main modes of HIV transmission: through blood, sexual intercourse, or from mother to child (in utero, during labor and delivery, via breastfeeding, Table 1-1). While the risk of HIV acquisition is highest through blood products, including blood transfusions or needle sharing for injection drug use, sexual transmission remains, by far, the most common mode of HIV-1 transmission. Overall, however, HIV-1 transmission rates through sexual intercourse are comparatively low in relation to transmission through blood.

Exposure Route	Rate of transmission (%)	Reference
BLOOD		
Blood transfusion	90	[29]
Needle sharing/IDU	0-7.2	[30]
SEXUAL		
Anal	0.04-3.0	[31, 32]
Vaginal	0.04-0.3	[31, 33]
Oral	0-0.04	[32, 34, 35]
VERTICAL		
Peripartum	13-30	[36-38]
Breastfeeding	14-50	[38-40]

Table 1-1. Routes and their associated rates of HIV-1 transmission. While the rate of HIV-1 transmission is the highest through blood and blood products, HIV-1 infections worldwide occur predominately through sexual exposure and via mother to child transmission.

Without a doubt, successful introduction of and compliance with antiretroviral therapy has been a critical factor in preventing mother to child transmission of HIV-1 (MTCT) [41, 42]. Despite a decline of over 50% since 2001, however, MTCT still accounted for over 250,000 new HIV-1 infections in 2012 [6]. One category of factors influencing MTCT includes maternal behavioral practices, such as health-seeking behavior and compliance with ART regimen. In addition to behavioral factors, several virologic and immunologic factors influence the likelihood of a transmission event. For example, maternal viral RNA load remains the single strongest predictor of transmitting HIV from mother to child [43]. In accordance with this, other virologic factors that may ultimately increase the maternal viral burden, such as the presence of ART-resistant viruses or viral replicative fitness may also impact MTCT [44]. Finally, maternal and fetal immunologic factors, such as the presence of maternal neutralizing antibodies and MHC concordance, and the presence of immune activation in either mother or infant may impact MTCT. As the umbilical cord is attached to the placenta, which serves as a critical interface between the mother and the developing fetus, cord blood cells are affected by maternal factors, which may therefore influence infant susceptibility to HIV-1. Chapter 2 of this dissertation examines the role of innate and adaptive immune cell phenotypes and activation status in the cord blood of infants, independent of maternal viral load, in influencing the risk of MTCT.

Postnatally, additional factors that result in inflammation and therefore in an immune microenvironment conducive to productive HIV-1 infection are also risk factors for an MTCT event through breastfeeding. Breast inflammation, including nipple lesions or mastitis in the mother or oral infections in the infant, such as oral thrush, have all been identified as risk factors for postnatal MTCT [45]. While the mechanism for this increased transmission has yet to be clearly defined, these infections likely result in inflammation, including the recruitment and increased frequency of HIV target CD4+ T cells, macrophages, and dendritic cells to the

immune microenvironment sites of exposure, ultimately increasing the likelihood of a successful transmission event.

An additional source of immune activation during infancy may arise from the administration of vaccinations. Infants worldwide are routinely vaccinated against several communicable diseases, such as rotavirus, diphtheria, pertussis, and others at several time points. In addition to the universal vaccine schedule, infants in most developing countries receive the live-attenuated Bacillus Calmette-Guérin (BCG) vaccine, which is effective in preventing childhood and extra-pulmonary tuberculosis. While vaccines provide millions of children with life-saving immune education, they are (by design) highly immunogenic. Therefore, one effect of these vaccination schedules is the repeated introduction of a high level of systemic immune activation. While this has little or no negative effect on healthy children, it may have unintended consequences for the HIV-exposed infant, due to the previously described potential for increased HIV-1 acquisition in the context of immune activation. Chapter 3 of this dissertation, performed in collaboration with Dr. Heather Jaspan, explores the potential for BCG vaccination to induce immune activation in a cohort of HIV-exposed infants.

The Course of HIV-1 Disease Progression

The natural course of HIV-1 disease progression (without drug treatment) is typically divided into three major stages: acute infection, chronic infection, and AIDS (figure 1-1). During acute infection, which immediately follows establishement of the infection, HIV rapidly infects and replicates in CD4+ T cells, and disseminates into systemic tissues. During acute infection, a high viral titer, averaging around 10^6 to 10^7 viral RNA copies/ml can be found in peripheral blood (reviewed in [46]). Previous studies suggest that the gut-associated lymphoid tissue (GALT) is the major site of HIV viral replication during the acute infection phase [47-49]. Destruction caused by this initial replication, and subsequent leaking of commensal microbes

into systemic circulation, termed “microbial translocation,” is likely a major driver of chronic, generalized immune activation throughout infection [50].

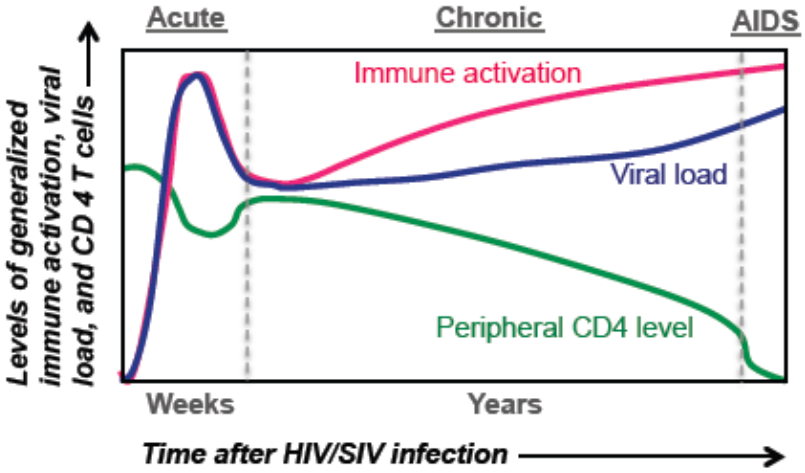

Figure 1-1. Course of HIV-1 Disease Progression. HIV disease progression can be broadly characterized into three disctinct stages: acute infection, chronic infection, and AIDS. Each stage of infection is characterized by unique immunologic (CD4 count, greenn line and immune activation, pink line) and virologic parameters (viral load, blue line).

Following the acute phase of infection, CD4 T cell levels begin to replenish and viral load drops, indicative of initial immune control over viral replication and a balance between CD4 T cell renewal and death [51-53]. During this phase of HIV-1 infection, a balancing act of immune control and virus replication result in the establishment of a viral set point. This phase of the HIV-1 infection stage can range from weeks to years, and is typically progressive yet asymptomatic. Peripheral CD4+ T cell levels decline as viral replication ultimately exhausts T cell regeneration. As a result of chronic stimulation by virus/viral products, continuing translocated commensals from the destroyed gut, or direct depletion of immunoregulatory cells following their direct HIV-1 infection, this stage is also characterized by sustained, and perhaps increasing immune activation.

The third phase of infection, AIDS, is characterized by clinical immunodeficiency due to a complete destruction and/or dysregulation of several arms of the immune system, including innate NK cells, monocytes, macrophages and adaptive CD4+ T cells, CD8+ T cells, and B cells, among others. In some HIV-infected patients, disease progression is associated with the emergence of HIV species that utilize additional co-receptors for entry [54, 55] further depleting the pool of remaining T cells. As HIV-infected patients eventually progress to AIDS (clinically defined as CD4 counts below 200 cells per μl blood), opportunistic infections caused by fungal, bacterial, and parasitic pathogens, as well as certain types of cancers (such as Kaposi sarcoma) become life threatening as a result of immune system failure. Mycobacterial species, in particular, remain a common source of opportunistic infections even in the United States (Table 1-2), and these numbers are undoubtedly higher in areas with poor access to antiretroviral therapy.

Opportunistic Infections		2003-2007
	n	Rate (95%CI)
Esophageal candidiasis	67	5.2 (4.1-6.7)
Pneumocystis pneumonia	46	3.9 (2.9-5.2)
Cervical Cancer	10	3.5 (1.7-6.5)
***Mycobacterium avium* complex**	**32**	**2.5 (1.7-3.5)**
Cytomegalovirus	23	1.8 (1.1-2.7)
Non-Hodgkin's Lymphoma	21	1.6 (1.0-2.4)
HIV encephalopathy	18	1.4 (0.8-2.2)
Kaposi's sarcoma	16	1.2 (0.7-2.0)
Chronic herpes simplex disease	13	1.0 (0.5-1.7)
Mycobacterium tuberculosis	**11**	**0.8 (0.4-1.5)**
Cryptococcosis	11	0.8 (0.4-1.5)
Cryptosporidiosis	10	0.8 (0.4-1.4)
Progressive multifocal leukoencephalopathy	9	0.7 (0.3-1.3)
Toxoplasmosis	6	0.5 (0.2-1.0)
CNS-lymphoma	3	0.2 (0.0-0.7)
Atypical mycobacteriosis	**2**	**0.2 (0.0-0.5)**
Pulmonary candidiasis	2	0.2 (0.0-0.5)
TOTAL	*300*	

Table 1-2. Absolute Numbers and Rates Per 1000 Person-Years of AIDS-Defining Opportunistic Illnesses in US patients, the HIV Outpatient Cohort Sudy, 1994-2007. Opportunistic illnesses are ordered by frequency during 2003-2007, and *Mycobacterial* disesae incidence is in bold. Modified from [1].

TUBERCULOSIS AND OTHER MYCOBACTERIAL DISEASES IN AIDS

Overview

It has been well established that HIV-1 dramatically alters the ability to contain and control Mycobacterial infections [56-59], leading to not only an increase in Mycobacterial infections, but also an increase in death from those infections [60, 61]. While a healthy individual has a 5-10% *lifetime* risk of developing active TB, HIV-infected individuals have a 10% *annual* risk of developing the disease [62-65]. Further, several species of *Mycobacteria* are able to cause disease in immunocompromised patients. Each of these species presents unique clinical challenges in diagnosis and treatment. For many of these species, molecular and epidemiologic synergism with the HIV-1 pandemic, together with the emergence of drug-resistant bacteria have resulted in the emergence and reemergence of disease outbreaks that spread faster than current public health measures can abate. This introduction will focus on two *Mycobacteria*, spp, whose presence and pathogenesis has been significantly escalated by the HIV-1 epidemic, and that cause significant morbidity and mortality during HIV-1 infection: *M. tuberculosis* and the live-attenuated vaccine strain administered against *M. tuberculosis, M. bovis* BCG.

HIV-1 and Tuberculosis Epidemiology

The HIV/TB syndemic can be attributed to pathogen and disease interactions on several levels. As described below and shown in figure 1-2, the global distribution of these two diseases significantly overlaps. Whether this overlap is a cause or consequence of the syndemic is complex. However there is no doubt that this epidemiological overlap of the two diseases contributes to fueling the syndemic.

In 2012, an estimated 35 million people were living with HIV-1, with nearly 90% of these HIV+ people living in either South/South-East Asia or Sub-Saharan Africa, where the country-specific prevalence of HIV-1 has been reported to be as high as 28% (Swaziland; [6]). Further, although the number of AIDS deaths has dropped by nearly 30% since 2005, still over one million deaths were attributed to AIDS in 2012 [6]. The leading cause of death among people living with HIV/AIDS is infection with Mtb and resultant tuberculosis disease. In 2011, there were approximately 8.7 million total incident TB cases [66], with nearly 12-14% of these cases specifically occurring among HIV-infected persons. Nearly mirroring the distribution of HIV-1 prevalence, the regions with the highest estimated rates of TB disease include Sub-Saharan Africa and South/South-East Asia [66]. Indeed, the highest coinfection burden (79% of the total HIV/TB coinfection cases worldwide) can be found in the WHO-designated African region [66]. Because HIV-1 infection increases not only the likelihood of incident case acquisition, but also mortality from infection, it is not surprising that these same regions are where TB is responsible for causing a significant proportion of total deaths [67].

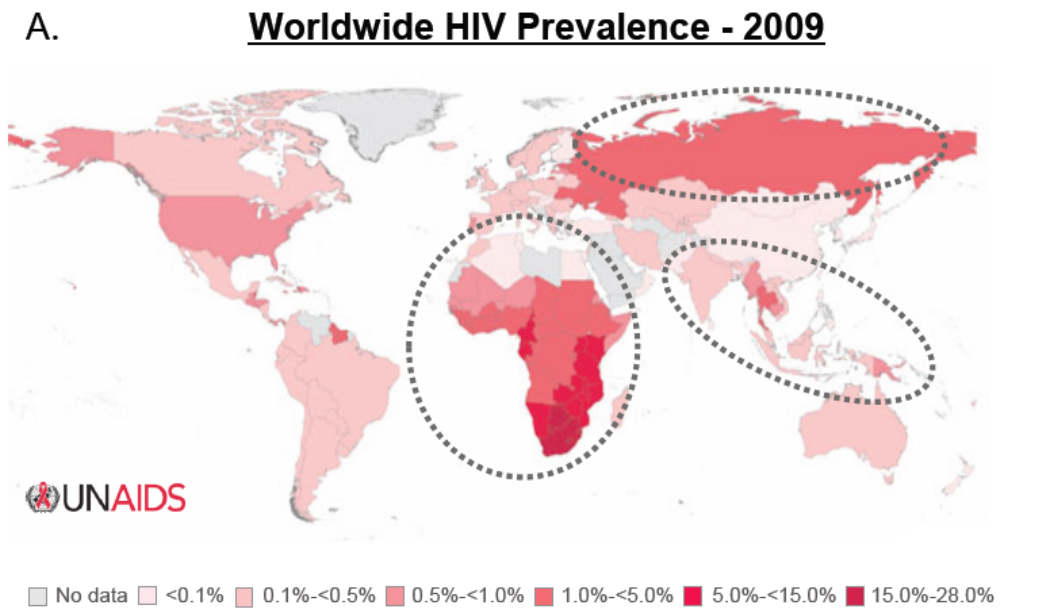

Figure 1-2. Overlap of the HIV-1 and Tuberculosis Epidemics. Regions of the world with a significant prevalence of HIV-1 in 2009 (A.) also have a high estimate of incident tuberculosis disease in 2010 (B). Regions with significant overlap are highlighted with gray circles.

Despite the enormous burden of HIV/TB coinfection, it has remained a relatively understudied area in the HIV/AIDS field, due largely in part to technical and spatial constraints of working with the two (BSL-3 level) pathogens, but also due to the inherent complexity of studying pathogen interactions. However, available data in the field of coinfection suggest that HIV-1 and Mtb synergism can be found at both cellular and molecular levels.

As an aerosol transmissible pathogen, Mtb initially infects a host through the respiratory tract before ultimately transiting to the lung. In the lung, several types of immune cells including macrophages, lymphocytes, dendritic cells, neutrophils and NK cells and others coalesce to capture the bacteria, ultimately forming a structure called a granuloma. It was initially hypothesized that the granuloma serves to contain the bacteria and as an immune microenvironment for cellular interactions to limit Mycobacterial growth. However, recent data suggests that Mtb and other *Mycobacteria* may actually hijack the granuloma's composition of closely-positioned cells to facilitate cell-cell spread [68, 69].

At a cellular level, HIV-mediated depletion of CD4 T cells is a likely contributor to increased susceptibility to tuberculosis disease, as it is important for several components of the immune response to TB, including its initial containment and sequestration in a granuloma. Indeed, although tuberculosis infections can occur even before HIV-mediated CD4+ T cell depletion occurs, HIV-induced decreases in peripheral CD4+ T cells correlates with susceptibility to Mtb [5, 70]. Further, evidence exists that HIV-1 may selectively deplete Mtb-specific CD4+ T cells [71, 72], ultimately impairing the finely-tuned adaptive immune response to Mtb. As previously mentioned, HIV-1 pathogenesis alters the phenotypic and functional parameters of not only T cells, but also innate cells, which are also critical players in mounting and maintaining an effective immune response to Mtb. Chapters 4, and 5 of this dissertation examine the how the innate immune response to Mtb, particularly by monocytes and NK cells, is altered in the context of chronic pathogenic HIV-1 infection.

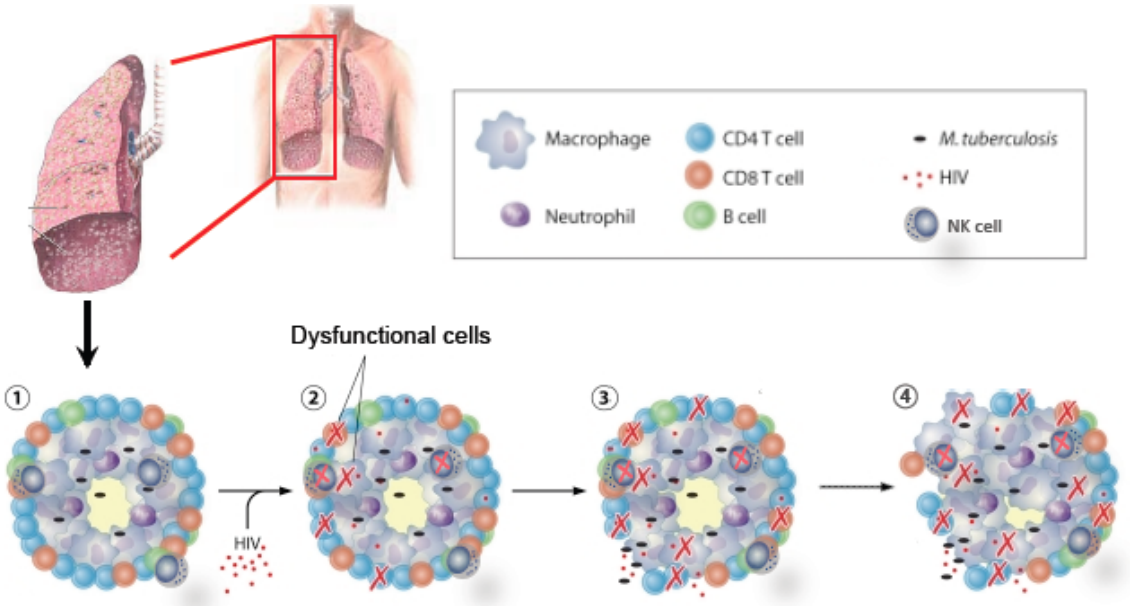

Figure 1-3. HIV-induced Cellular Alterations Lead to the Failure to Contain and Clear Mycobacterial Infections. A "normal" granuloma functioning to limit the spread of the *Mtb* is shown in stage 1. As HIV impacts a wide variety of cellular types, including the preferential destruction of Mtb-specific CD4+T cells (stage 2), the granuloma becomes both structurally and functionally impaired (stage 3). This ultimately leads to the dissemination and/or reactivation of Mtb (stage 4). Adapted from [5].

The live-attenuated vaccine, *M. bovis* Bacillus Calmette-Guérin (BCG), is used throughout the world as a vaccine against Mtb. Although BCG is rarely pathogenic in immunocompetent persons, HIV-infected infants given the BCG vaccine at birth show an increased risk of disseminated BCG disease [73], indicating that BCG has the pathogenic potential in immunocompromised persons. In response to this observation, the WHO has made infant HIV infection (if known) a contraindication to BCG vaccination. However, residual MTCT transmission rates are high in some areas, and infant HIV status is unable to be confirmed until 4 to 6 weeks of age due to the presence of passively transferred maternal antibodies at earlier timepoints, which can result in false-positive HIV anitbody test results for the infant. As a result, millions of children in Sub-Saharan Africa (SSA) are vaccinated at birth with BCG before HIV

status is known. Data generated using Simian Immuno-deficiency Virus (SIV)-infected Rhesus macaques (an animal model of HIV-1) show that BCG can drive rapid viral dissemination and SIV pathogenesis [74, 75]. As HIV/SIV primarily infects activated CD4+ T cells, this suggests that BCG-driven T cell activation can accelerate HIV-1 disease progression and by similar mechanisms, may enhance HIV-1 infection. Knowing the risks and benefits of administering BCG to HIV-exposed (and possibly infected) infants is vital for policy makers in high TB burdened settings. Chapter 3 of this dissertation examines the role of innate immune cell responses to BCG vaccination in altering the HIV-exposed infant's risk of acquiring HIV-1.

INNATE IMMUNITY

Toll-like Receptors and the Induction of Innate Immunity

In addition to defining the quality and quantity of the adaptive immune response, innate immunity is key to the initial containment of pathogens through mechanisms that do not generate specific, long-lasting protective immunity. It is, however, critical in keeping pathogen replication ”in check” until the development of a long-lasting adaptive response. Innate immune defenses mediate the killing of pathogens within minutes to hours of initial infection [76]. The first line of innate defense is composed of mechanical barriers, such as epithelial cells, cilia in the lungs and nose, and tears in the eye. Once a pathogen crosses the mechanical barriers and enters into tissues, phagocytes like monocytes and macrophages (discussed in detail in the next section), neutrophils, or NK cells can recognize, ingest, and/or destroy them or the infected cell without the aid of adaptive immunity. Following cell-mediated recognition of pathogens, an anti-inflammatory response is mounted, mediated largely through the NOD and TOLL-like receptors as well as cytokines and chemokines.

Although innate immunity functions without the finely tuned antigen-specificity of the adaptive immune system, it is still exquisitely able to distinguish self from non-self, primarily through pattern recognition receptors (PRRs), such as toll like receptors (TLRs) that recognize unique pathogen-associated molecular patterns (PAMPs) on pathogens. Toll-like receptors are a set of cell-surface or intracellular (endosomal) molecules that are critical to sensing the presence of pathogens and/or pathogen products. To date, 11 TLRs have been recognized in humans (TLRs 1-11; reviewed in [77]). Each TLR is able to recognize a specific PAMP; examples of PAMPs include: components of cellular walls, such as lipopolysaccharide (gram-negative bacteria) or lipoteichoic acid (gram-positive bacteria), nucleic acids, and others (Figure 1-4). The innate response to HIV-1 is mediated primarily through interactions with TLRs 7/9, although it has recently been shown to also interact with TLRs 2, 4, and 8 [78-80]. Meanwhile, TLRs 1/2, 2/6 (heterodimers), 4, and 9 likely play the most prominent roles in initiating the

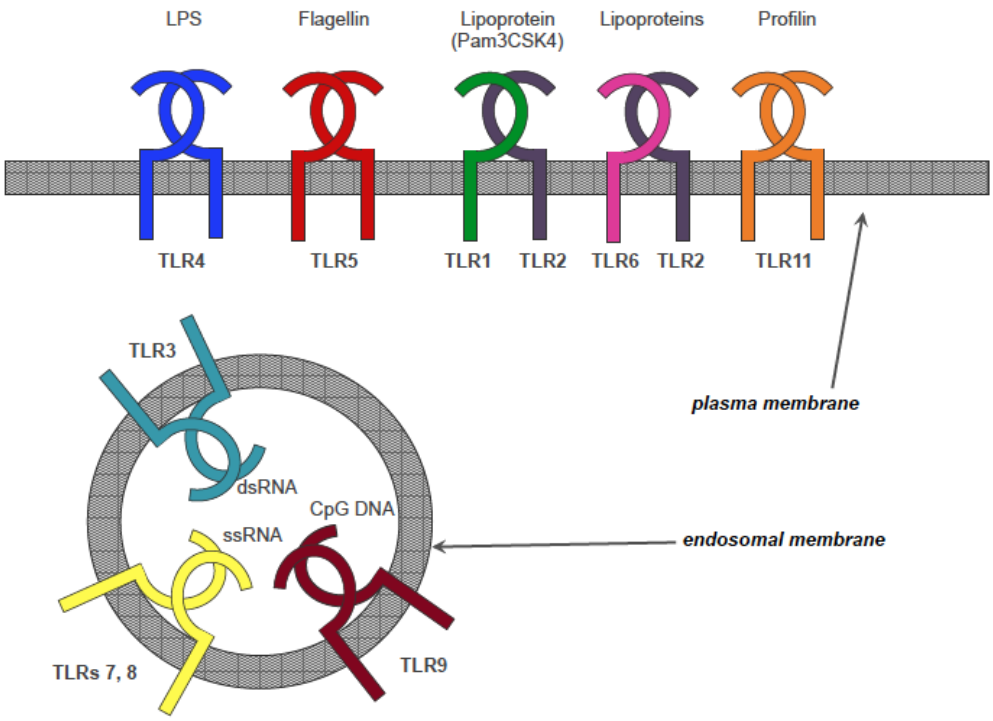

Figure 1-4. Known Human Toll-Like Receptors and Their Ligands. A set of six plasma membrane-bound and three endosomal TLRs recognize a diverse array of PAMPS to initiate the cellular inflammatory and antiviral responses. While TLRs 1/2 and 2/6 occur in the form of heterodimers, other TLRs for homodimers on the cell surface or in endosomal compartments. *Adapted from [2].*

response to *Mycobacteria* spp. and are triggered by components of the complex, lipid rich *Mycobacterial* cell wall (reviewed in [81]). Activation of TLRs by PAMPS ultimately results in the activation of either the inflammatory cascade, mediated by NFkB and MAPK or the antiviral cascade, mediated by the interferon response factors (IRFs).

The magnitude of the inflammatory response generated by TLR stimulation is dependent upon several factors, including the expression level of TLRs on/inside of the cell as well as which TLRs are stimulated and in what order. The diverse array of TLR expression particularly on monocytes and macrophages may be a critical factor in governing the physiological complexity of responses to pathogens, and may therefore likely play an important role in the context of coinfection (reviewed in [77] and [82]).

In addition to fast-acting control of invading pathogens, innate immunity is also largely responsible for initiating the adaptive arm of the immune system. Through mechanisms such as cytokine and chemokine signaling, resulting in the recruitment of lymphocytes and other cells to lymph nodes and/or secondary lymphoid structures for antigen presentation, as well as antigen presentation by macrophages and dendritic cells, a finely tuned, adaptive response mediated by T and B lymphocytes is established.

Monocytes and macrophages

Monocytes are circulating precursors to mature tissue-resident macrophages. They are derived from hematopoietic stem cells in bone marrow, and compose approximately 4-10% of circulating leukocytes in humans. Upon inflammatory conditions, monocytes migrate to inflamed tissue and mature into tissue-resident macrophages, where they take place in both host defenses and tissue repair. This migration into tissue is largely controlled by a set of chemokines, most notably CCL2, or monocyte chemotactic protein-1. In addition to receptors that mediate chemotaxis to inflamed tissue, monocytes express a diverse array of PRRs and surface molecules, to initiate the response to pathogens. For example, circulating monocytes have been shown to be somewhat promiscuous with regard to TLR expression, and express relatively high levels of all known TLRs, with the exception of TLR3 (reviewed in [77], [82]), but upon migration to tissues, the TLR repertoire becomes more refined and tissue-specific. Additionally, both monocytes and macrophages express the lineage marker CD14, a cell surface receptor involved in the recognition and binding of LPS.

Following their activation, monocytes and macrophages carry out several critical effector functions for pathogen clearance following pathogen invasion including initial phagocytosis of the pathogen, the generation of reactive oxygen and nitrogen intermediates to mediate intracellular killing, antigen presentation, and the production of a wealth of cytokines and chemokines to mediate inflammation and the ensuing immune response. Importantly, all of

these functions are critical in the response to Mycobacterial infections. Through these responses, monocytes and macrophages are not only able to engulf and destroy the bacteria, but are also able to initiate formation of the granuloma [83, 84]. Because monocytes and macrophages exhibit such a diverse array of functional properties, they also play an important role in communicating with and educating several other cell types, including granulocytes, other monocytes and macrophages, T and B lymphocytes, and NK cells.

While monocytes are rarely found to be directly infected with HIV-1 (reviewed in [85]) several of their functional capacities, which are all important for intracellular bacterial killing, become altered during HIV-1 infection. These functions include phagocytosis [86-88], the production of reactive oxygen species [89], and cytokine production [90-93]. The dysregulation of these functions during HIV-1 infection likely all contribute to the observed increase in opportunistic Mycobacterial diseases in HIV-infected patients. Although the exact source of this dysfunction in the absence of direct cellular infection is complex and has not been elucidated, one potential explanation includes the presence of generalized, chronic immune activation during the chronic phase of HIV-1 infection. Interestingly, monocytes themselves are well known for being highly proinflammatory, and secrete an abundance of proinflammatory cytokines. Therefore, potentially through TLR and other surface molecule recognition of HIV-1, LPS (following microbial translocation), and other PAMPs, monocytes may also be key contributors to chronic immune activation during pathogenic infections if their proinflammatory cytokine responses are not tightly regulated.

NK Cells

Natural killer (NK) cells are a population of granular lymphocytes and normally compose approximately 10% of the total lymphocyte population in human peripheral blood (reviewed in [94]). Despite gaining much attention due to their recently-discovered potential for possessing immune memory [95, 96], NK cells are canonically regarded as a component of the innate

immune system. They are perhaps best known for their capacity to identify and kill virally-infected or otherwise stressed cells, without previous licensing by an antigen presenting cell, through the induction of perforin and granzyme-B-mediated cytotoxicity or antibody-dependent cellular cytotoxicity (ADCC). Additionally, NK cells are exquisite producers of interferon gamma, and TNF-alpha, which significantly influence recruitment and subsequent function of other immune cells.

Phenotypically, NK cells are classically defined as cells that are lacking the T cell receptor CD3, and expressing the NCAM marker (CD56) alone or in combination with the FcγIII receptor (CD16), which is able to mediate ADCC. In addition, NK cells express high levels of TLR1, and moderate/low levels of TLRs 2, 3, 5, and 6 [82] as well as a diverse and complex array of activating and inhibitory receptors that mediate NK cell activity (reviewed in [94]).

The major targets of NK-mediated cytotoxicity are cells that have either downmodulated the MHC class I ligand, which is a common trait of tumor or virally-infected cells, or that have upregulated stress-induced ligands for activating receptors found on the NK cells. The presence and relative levels of these activating receptors on NK cells play a key role in guiding NK cell activity (reviewed in [94]). Additionally, through both contact-dependent (ligands for NK cell receptors) and soluble mechanisms (IL-12 and IL-18 cytokines, in particular), innate "accessory cells," like monocytes and macrophages, are thought to be critical for NK cell activation (reviewed [97]). Chapter 5 of this dissertation explores the role of monocyte and NK cell interactions in controlling the immune response to *Mycobacteria* in the context of HIV-1 infection. A summary of known interactions regarding this interplay is depicted in figure 1-5.

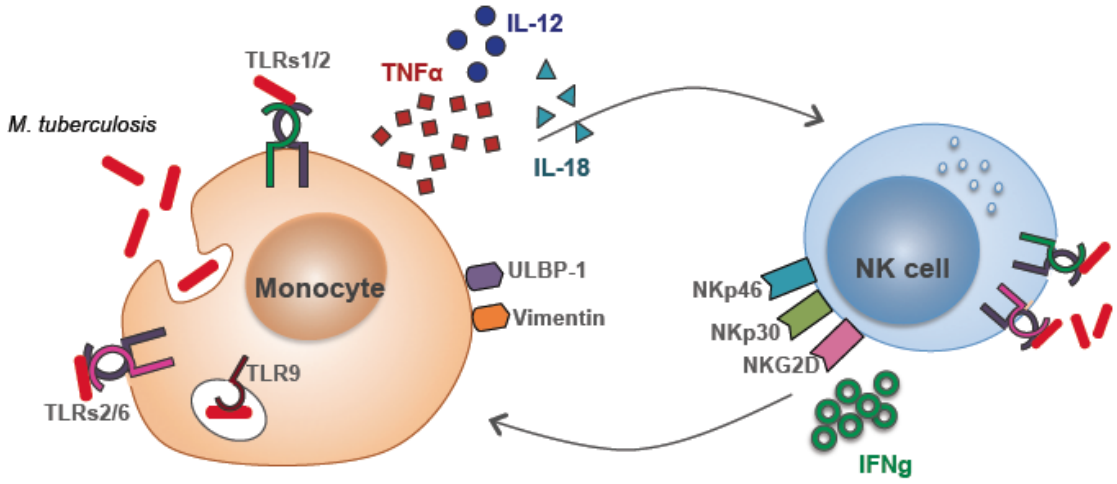

Figure 1-5. Monocyte and NK Cells Interact to Control *M. tuberculosis* Infections. Monocytes and NK cells initially recognize Mycobacteria through TLR-mediated recognition. Activated monocytes produce proimflammatory cytokines (IL-12, IL-18, and TNFα) necessary for recruiting and activating NK cells. Activated NK cells can then mediate killing of Mtb-infected monocytes through both soluble (IFNγ) and contact-dependent mechanisms.

NK cells are likely important players in the immune response to HIV-1, and can contribute to a reduction in rates of infection or slower rates of disease progression (reviewed in [98]). Further, like monocytes, NK cells are not typically infected with HIV, but exhibit altered phenotypic and functional properties during an HIV-1 infection, which may subvert their ability to protect against opportunistic pathogens. For example, HIV-1 infection results in increased levels of an anergic NK cell population (classified as CD56-CD16+) that are impaired in not only their capacity to mediate cellular cytotoxicity but also in their ability to produce cytokines [99-101]. While HIV-1 nucleic acids have the potential to impact NK cell function directly [102], chronic stimulation during HIV-1-induced chronic immune activation also likely contributes to NK cell dysfunction.

While the role of NK cells in the control of Mycobacterial infections currently remains unclear, several lines of evidence argue that they may indeed be important. First, *in vitro* data have shown that in healthy donors, NK cells respond to mycobacterial products via TLR 2 [103].

Second, NK cells provide efficient control of mycobacterial replication in monocytes/macrophages [104-107] potentially through the interaction of NKp46 on NK cells with ULBP1 expressed on infected monocytes [108]. Finally, NK cells have been shown to localize to the site of *Mycobacteria* infection; *In-vivo* experiments using a mouse model have found that activated NK cells accumulate in the lungs of BCG-infected mice [109]. Additionally, in recent studies of humans, NK cells have been identified within granulomas and *ex-vivo*, human NK cells have been shown to have the ability to produce IFNγ and TNFα following stimulation with BCG or Mtb in the presence of the cytokine IL-2 [110]. Interestingly, NK cells in the human granuloma were located preferentially in close proximity to blood vessels, suggesting recent extravasation [110]. NK cell localization to granulomas underscores their ability to serve as a link between innate and adaptive immunity through interactions with macrophages and monocytes as well as T and B cells, which are also part of a granuloma.

GOALS OF THIS DISSERTATION

The goals of this dissertation are to examine the role of innate immune cells and immune activation in the context of MTCT and to better understand HIV-1 immunopathogenesis, with an emphasis on innate immune responses to opportunistic and pathogenic *Mycobacteria*. Chapters 2 and 3 highlight the roles of innate immunity in influencing HIV-1 transmission. While chapter 2 describes the dichotomous roles of innate (NK cell) and adaptive (T cell) cord blood cell activation in MTCT, chapter 3 further explores sources of cellular activation in infants, including the potential for innate immune cells (NK cells and monocytes) to contribute to increased CD4+ T cell activation observed following BCG vaccination of HIV-exposed infants. Later chapters (chapters 4 and 5) focus on understanding innate cells in HIV-1 pathogenesis and acquisition of OIs. Chapter 4 describes how altered innate immune responses as a result of chronic, pathogenic HIV-1 infection may lead to increased susceptibility to Mycobacterial infections. Through a comparison of pathogenic HIV-1 and non-pathogenic SIV infections, this study highlights the utility of using model monkey species that do not exhibit chronic immune activation, thereby maintaining immune health, which may otherwise become altered in the face of HIV-1-induced chronic immune activation. Using models that do not progress to AIDS (and therefore remain free of opportunistic infections) may ultimately allow the discovery of immune correlates of immunocompetence maintained by these hosts in the face of high levels of replicating virus. Finally, chapter 5 examines functional consequences of chronic HIV-1 infection on innate immune cell crosstalk in response to *Mycobacteria.* Together, this dissertation contributes novel work to the fields of innate cell contributions to HIV-1 transmission as well as HIV-1/Mtb coinfection.

CHAPTER 2:

NK CELL AND T CELL SUBSET DISTRIBUTIONS INFLUENCE SUSCEPTIBILITY TO PERINATAL HIV-1 INFECTION

OVERVIEW

To determine the role of innate and adaptive lymphocyte activation on mother-to-child-transmission of HIV-1, we designed a case-control study comparing cord blood mononuclear cells (CBMC) from HIV-1 exposed infants who subsequently acquired infection by one month (cases) to those who remained uninfected by 1 year of life (controls). For this, we employed multiparameter flow cytometry to detect NK and T cell subsets and their activation status. Additionally, the functionality of these cells was tested in a viral suppression assay to evaluate NK cell inhibition of HIV-1 replication in autologous CD4+ T cells. The data from this study demonstrate that CBMC from cases contained a skewed NK cell repertoire, characterized by an increased proportion of CD16-CD56+ NK cells. Additionally, cases displayed less-activated CD16-CD56+ NK cells and CD8+ T cells, based on HLA-DR+CD38+ co-staining. In addition, the ability of NK cells to suppress HIV-1 replication *ex-vivo* correlated with the proportion of acutely activated CD69+ CD16-CD56+ NK cells. Finally, we detected a higher proportion of CD27-CD45RA- effector memory CD4+ and CD8+ T cells in cord blood from cases compared to controls. Together, these data provide novel evidence to suggest that MTCT may be influenced by the phenotypic ratio and activation status of innate and adaptive immune cells in infant CBMC.

INTRODUCTION

While HIV-1 transmission events predominantly occur at a mucosal site, repeated exposures are typically required for the establishment of an infection, making mucosal HIV-1 transmission a relatively rare event (reviewed in [32]). Transmitter infectiousness and host protective mechanisms lead to differential susceptibility of the host, and ultimately influence the likelihood of a productive HIV-1 infection. Therefore, a better understanding of host immune factors associated with HIV-1 transmission or protection from transmission is needed to inform vaccine design. The mother-to-child HIV-1 transmission (MTCT) model provides a unique opportunity to explore cellular immune cofactors of HIV-1 transmission due to systematic access to transmitter viral load data and relative precision regarding timing of infection.

Evidence exists that infant acquisition of HIV-1 infection is influenced by the status and location of HIV-1 target cells (reviewed in [34]). For example, clinical conditions associated with enhanced inflammation lead to increased HIV-1 susceptibility [45, 111, 112]. Further, cord blood lymphocyte activation in response to maternal helminth coinfection has been correlated with increased MTCT [113]. On the other hand, activation of immune cells and the presence of immune modulators that can inhibit HIV-1 replication, such as interferons or alpha- and beta-defensins, may protect infants from becoming HIV-1 infected [114, 115].

NK cells are a component of the innate immune response and are capable of identifying and killing virally infected cells. Three functionally distinct populations of NK cells can be defined based on differential cell surface expression of CD16 and CD56. CD16+CD56+ cells make up to 90% of adult NK cells and contain high concentrations of perforin, reflecting their cytotoxic nature. NK cells that do not express CD16, including $CD56^{bright}$ and CD56+ NK cells, make up approximately 10% of adult NK cells. This subset is poorly cytotoxic and $CD56^{bright}$ NK cells, in particular, are capable of producing large amounts of cytokines (reviewed in [116]). Finally, CD16+CD56- NK cells are thought to reflect an anergic or progenitor state of NK cells that are

poorly cytotoxic and expanded in HIV-1- or hepatitis C-infected adults [100, 117, 118]. To date, few studies have focused on the role of NK cells in HIV-1 transmission. Studies of highly HIV-1-exposed seronegative adults (HESNs) showed that NK cells from HESNs had increased cytolytic activity and increased IFNγ, TNFα, and β-chemokine secretion compared to both unexposed controls and seroconverters [119]. Additionally, maternal and infant HIV-1 peptide-specific NK cell responses have been associated with reduced risk of MTCT [120]. Together, these findings suggest a role for NK cell functionality in protecting against HIV-1 infection.

In contrast to the beneficial, antiviral effects associated with NK cell activation, an activated or mature T cell repertoire likely enhances susceptibility to HIV-1, potentially through the generation of HIV-1 target cells, and maintaining a dormant or quiescent T cell phenotype is critical in resisting HIV-1 infection [113, 121-124]. Thus, immune activation may serve as a "double-edged sword" in the context of HIV-1 infection, where maintaining an optimal balance between activated cells with antiviral capacity and activated HIV-1 target cells may be critical for preventing HIV-1 infection.

Here we conducted a case-control study to compare NK cells and T cells in cord blood of infants who were HIV-1-uninfected at birth but acquired HIV-1-infection by 1 month to those who remained uninfected. Because maternal viral load (VL) is highly correlated with transmission [125], we controlled for maternal VL to evaluate the contribution of cord blood NK cell and T cell phenotypic subsets and activation status to infant HIV-1 transmission.

MATERIALS AND METHODS

Cohort and Specimen Selection

This study used specimens from an MTCT cohort collected between 1999 and 2005 in Nairobi, Kenya, depicted in Table 2-1 and described previously [3, 4]. Women were recruited during pregnancy, provided written informed consent for participation and storage of specimens, and received zidovudine (ZDV) prophylaxis for preventing MTCT [126]. Maternal peripheral blood was collected at 32 weeks gestation, prior to initiation of ZDV, for baseline viral load (VL). Infants were examined at birth, cord blood was collected at delivery, and peripheral blood was collected within 48 hours of life and at 1 month, 3 months, and quarterly thereafter to test for HIV-1 infection status.

We used a case-control design based on proportional representation across quartiles of maternal VL because maternal baseline VL is the most significant risk factor associated with vertical transmission [127]. Selected cases were HIV-1-uninfected at birth but HIV-1-infected by 1 month of age, had multiple cryopreserved vials of cord blood mononuclear cells (CBMC), and CBMC viability >40% upon thaw (n = 7). Approximately 4 controls, which were HIV-1-uninfected at birth and by 1 year of age, were identified per case (n=24). Controls were selected to match maternal VL quartile of each case, while also meeting sample quality criteria above. Selection criteria and VL quartile cutoffs are detailed in the participant flow chart (supplemental figure 1). All components of this study were approved by the Kenyatta National Hospital Ethics and Research Committee and the University of Washington Institutional Review Board.

Cord Blood Collection and Preservation

Approximately 40 mL of umbilical cord blood was collected by venipuncture after clamping the cord in two places. Cord blood mononuclear cells (CBMC) were isolated by density gradient purification, washed in RPMI-1640 medium, lymphocytes were enumerated by

morphology, and were then cryopreserved in freezing media containing 10% dimethyl sulfoxide-90% Fetal Calf Serum (FCS, all Sigma-Aldrich).

Infant HIV-1 Diagnosis

Infants were diagnosed with HIV-1 infection as previously described [3]. Briefly, an infant was considered HIV-1-infected if either HIV-1 gag DNA was detected from blood spotted onto filter papers by PCR [128] or HIV-1 RNA was detected in plasma with the Gen-Probe HIV-1 Viral Load Assay (Gen-Probe Inc) [129]. Infection was considered peripartum if the birth specimen collected within 48 hours of life had undetectable HIV-1 DNA or RNA and the one-month specimen was HIV-1 DNA or RNA positive. All peripartum infections were later confirmed by re-testing the birth specimens using a real-time transcription-mediated amplification HIV-1 viral load assay under development by Gen-Probe.

CBMC Sample Preparation and Multiparameter Flow Cytometric Analysis

CBMC were thawed according to the HIV-1 Vaccine Trials Network standard operating procedure [130]. Cell number and viability was determined using trypan blue (Cellgro Mediatech) exclusion, and samples with greater than 40% viability were used for further analysis. Dead cells were identified using LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen). All antibodies were from BD Bioscience unless otherwise noted. The gating strategy for both NK and T cell subsets first selected singlets and viable cells. NK cells were then identified using anti-CD16 AlexaFluor647 (clone 3G8) and anti-CD56 PE-Cy5 (clone B159) while not expressing CD20 (anti-CD20 PerCPCy5.5, clone 2H7) or CD3 (anti-CD3 ECD, clone UCHT1, Beckman Coulter). T cells were identified via anti-CD3 ECD (clone UCHT1, Beckman Coulter), anti-CD4 PE-Cy5 (clone RPA-T4), and anti-CD8 APC (clone RPA-T8). Anti-CD27 APC-Cy7 (clone O323, Biolegend), and anti-CD45RA PE (clone 5H9) were used to distinguish effector and memory populations. Anti-CD38 FITC (clone AT-1, StemCell Technologies), anti-CD69 PE (clone L78), and anti-HLA-DR PE-Cy7 (clone L243) were used to identify activated NK cells and T cells.

Positivity gates were established using results from fluorescence-minus-one (FMO) staining for each panel. Cells were stained for 30 min on ice prior to being fixed in 2% paraformaldehyde and acquired on a BD-LSRII.

Viral Suppression Assay (VSA)

NK cell suppression of viral replication in autologous, activated $CD4^+$ T cells was performed as previously described for CD8+ T cells [131]. Briefly, CBMC with viability >66% were thawed and enriched by negative selection using Human $CD4^+$ T cell Enrichment Kit (EasySep, StemCell Technologies). Selected cells (2×10^6 cells/mL) were stimulated with phytohemagglutinin (PHA; 2µg/mL) and cultured in R10 supplemented with recombinant IL-2 (50U/mL). After 3 days, cells were washed in R10 and infected with HIV-$1CSF_{JR}$ at an MOI of 0.01 using Viromag-magnetofection (OZ Biosciences). On day 3, autologous CBMC were thawed for NK effector cell enrichment using the Human NK cell enrichment Kit (EasySep, StemCell Technologies). A minimum of triplicate cultures were plated in the presence of autologous effector NK cells at an effector:target ratio of 5:1 in 96-well plates. Uninfected $CD4^+$ T cells alone served as a negative control. The level of HIV-1 p24 in the day 7 supernatants was quantified using an HIV-1 p24 Antigen ELISA Kit (Perkin Elmer) and the ability of NK cells to suppress viral replication was calculated as follows:

$$\frac{(\text{p24 from infected target cells}) - (\text{p24 from infected target cells with NK cells}) \times 100}{(\text{p24 from infected target cells})}$$

Data Analysis

Flow cytometry data were analyzed using Flowjo (Treestar v. 8.8.6) and statistical analyses were performed using GraphPad Prism. The Mann-Whitney U test was used to compare proportions of NK cell and T cell population and NK cell subset populations were modeled as continuous variables (p-values are two-tailed at $\alpha=0.05$).

RESULTS

Cohort Description

Our case-control cohort (n=31) was selected based on proportional matching of maternal VL measured during pregnancy and included 7 infants who acquired HIV-1 by one month of life (cases) and 24 infants who remained uninfected during the first year of life (controls). To confirm that cases were truly uninfected at birth, samples from these infants were re-tested using ultrasensitive PCR. The mothers of infants selected into the cohort were young, HIV-1-infected, and their infants were of normal birth weight and maturity (Table 2-1). As a result of proportional maternal VL selection, baseline maternal VL measured at 32 weeks gestation did not differ significantly between cases and controls (5.0 vs. 5.1 $\log_{10}$ copies HIV-1 RNA/ml, p=0.5). There were no other significant differences in maternal or infant characteristics (Table 2-1).

	Cases Median (IQR) or N (%)	Controls Median (IQR) or N (%)	p-value
MATERNAL CHARACTERISTICS	N=7	N=24	
Age, yrs	21 (19-28)	27 (24-29)	0.1
Parity	1 (1-2)	1 (1-2)	0.7
Pregnancy CD4 count	365 (236-732)	345 (242-495)	0.8
Pregnancy CD4 percent	24 (10-33)	22 (16-28)	0.9
Pregnancy HIV viral load, log_{10} copies/ml plasma	5.0 (4.7 -5.7)	5.1 (4.8-5.4)	0.5
Vaginal Delivery	6 (86%)	19 (79%)	1.0
INFANT CHARACTERISTICS			
Birthweight, kg	3.5 (2.9-3.8)	3.2 (2.9-3.4)	0.3
Dubowitz / Ballard score	55 (48-57)	56 (52-60)	0.3
Estimated maturity, weeks	39 (38-40)	39 (39-40)	0.3
Bw04 HLA type	4 (57%)	16 (66%)*	0.6
Month 1 plasma viral load, log_{10} copies/ml plasma	6.5 (5.7 -7.2)	ND**	

Table 2-1. Cohort Maternal and Infant Characteristics. This study used specimens from an MTCT cohort collected between 1999 and 2005 in Nairobi, Kenya and described previously [3, 4]. *P values determined using Kruskal-Wallis for 2 independent comparisons or Fishers exact test for mode of delivery; **ND: none detected.

Cord Blood NK Subsets and Activation Marker Expression

Cord blood from cases had a lower proportion of total NK cells, defined by the percentage of CD3-CD20- cells expressing CD16 and/or CD56 (median 8.5% compared to 40% for controls; p=0.03; Figure 1b). The NK cell subset distribution also differed between cases and controls (Figure 1c), with infants who went on to acquire HIV-1 showing a significant over-representation of CD16-CD56+ NK cells (80.5% vs. 21.5%; p=0.04, Figure 1d), a phenotype associated with cytokine secretion. In addition, cases exhibited a trend toward under-representation of CD16+CD56+ NK cells (16.5% vs. 49.1%; p=0.09), a phenotype associated with cytotoxicity. Interestingly, infants who acquired infection also had a trend toward under-representation of CD16+CD56- NK cells (4.1% and 14.1%; p=0.07;) compared to controls. This

population has been previously described as dysfunctional or immature [99, 100, 118, 132], suggesting a potential protective effect of having a larger pool of NK cells able to undergo differentiation into mature NK cells associated with effector functions.

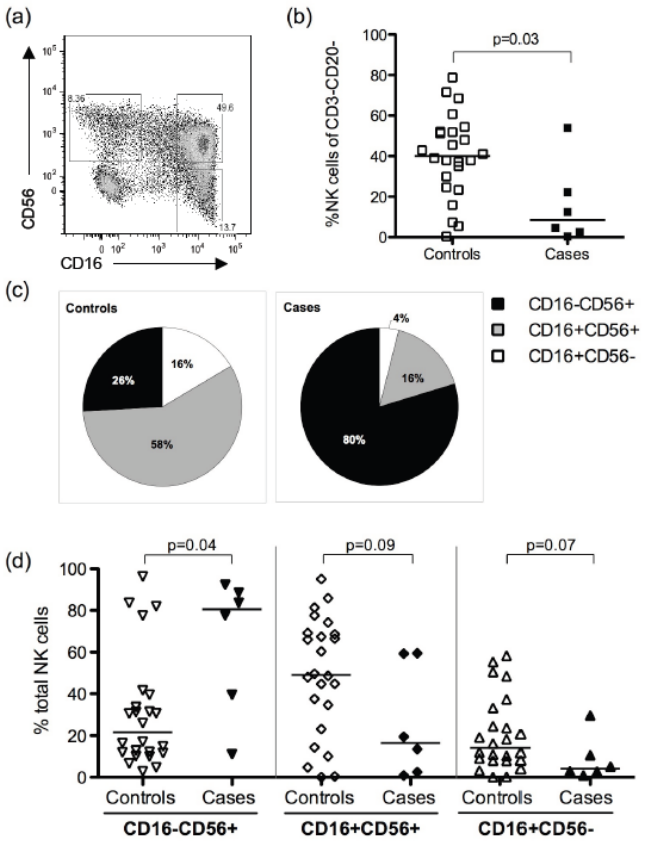

Figure 2-1. Natural Killer Cell Frequency and Subset Distribution in Cord Blood of Infants Grouped by Subsequent HIV-1 Acquisition. Cord blood mononuclear cells were gated on singlets and live cells prior to exclusion of CD3+ and CD20+ lymphocytes. NK cells subsets were defined by the expression of surface markers CD56 and/or CD16 (a.) Proportion of CD3-CD20- cells defined as NK cells in cases and controls (b.), and the phenotypic distribution of NK cell subsets as defined by the percentage of total NK cells (c). Lines represent the medians for a given subset. Statistics were generated via a Mann-Whitney test (*n.s. p>0.1*).

We also evaluated cord blood NK cell subsets for expression of surface markers associated with chronic activation, HLA-DR and CD38 (Figure 2a). While we observed a consistent trend toward decreased activation for all NK cell subsets in cases compared to controls, this reached statistical significance only in the CD16-CD56+ (activated/cytokine-secreting) NK subset (0.07% cases vs. 0.8% controls; p=0.01; Figure 2b). NK cell expression of the acute activation marker, CD69, was not different between cases and controls (data not shown).

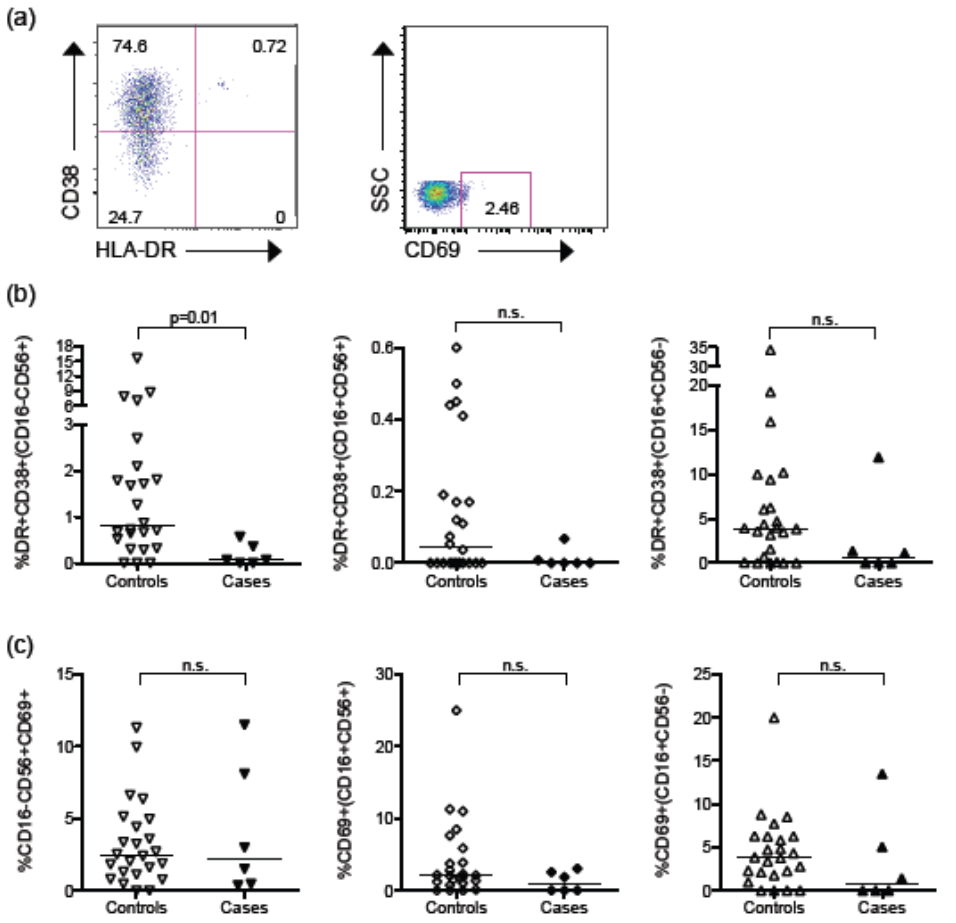

Figure 2-2. NK Cell Activation Status By Subset. Representative flow plot of either CD38 and HLA-DR expression (a; left panel) or CD69 (a; right panel) as indicators of chronic and acute activation, respecitvely. Activation status of NK cell subsets in cases and controls as measured by HLA-DR+CD38+ NK cells (b) or CD69+ NK cells (c). Lines represent medians. Statistics were generated via a Mann-Whitney test (*n.s. p>0.1*).

Cord Blood NK Cell Suppression of HIV-1 Replication in Autologous CD4+ T Cells

We performed a functional analysis to measure the ability of infant cord blood NK cells to suppress HIV-1 replication in autologous CD4+ T cells infected with HIV-1 *in vitro*. Bulk NK suppression of HIV-1 replication ranged from 0-99% in our cohort. A requirement for a large number of cells and high cell viability limited our power to detect differences between cases and controls. We were, however, able to detect a correlation between the suppressive capacity of bulk NK cells and the percentage of acutely activated (CD69+) CD16-CD56+ NK cells

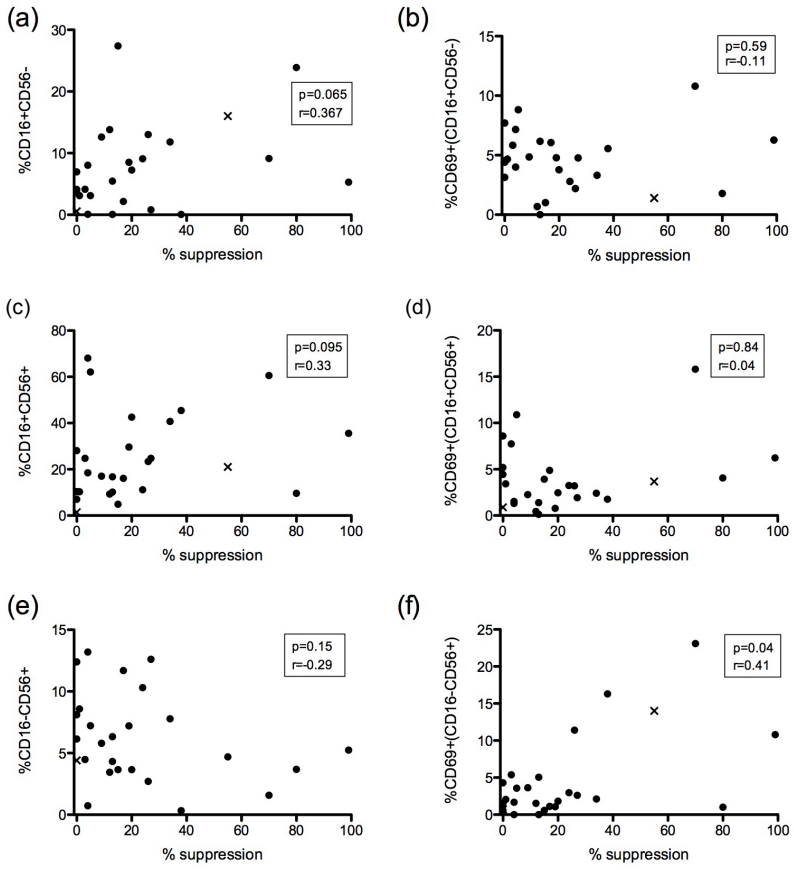

Figure 2-3. Viral Suppressive Capacity of Cord Blood NK Cells. NK cells were assessed for their ability to suppress HIV-1 replication in autologous CD4+ T cells (n=26). Suppressive capacity was then correlated with NK phenotype (a,c, e) and activation (b, d, f). Each point represents the average suppressive capacity of NK cells from each cord blood sample's replicates. Solid dots represent infants who remained HIV-1-uninfected while '×'s represent infants who subsequently became HIV-1 infected. Statistics were generated using a Spearman correlation.

(r=0.41;p=0.04; Figure 3). We did not detect any correlation between the capacity of NK cells to suppress HIV-1 replication in autologous CD4+ T cells and the proportion or activation status of other NK subsets (figure 2-3).

Cord Blood T Cell Activation and Differentiation

We were also interested in determining the relationship between T cell maturation status, activation, and HIV-1 acquisition. Infants who acquired HIV-1 infection displayed similar proportions of bulk CD3+CD4+CD8- T cells (median 69.7% vs. 68.4%, respectively) and CD3+CD4-CD8+ T cells (24.8% vs. 27.2%, respectively) compared to those that remain uninfected (data not shown). Our assessment of T cell activation identified a very high percentage of cord blood T cells expressing CD38 (HLA-DR-) in all cord blood samples examined (supplemental figure 3a,b). As CD38 can also serve as a marker of immaturity, this likely reflects the overall immature status of T cells in cord blood [133, 134]. While the proportion of CD4+ T cells expressing both chronic activation markers HLA-DR and CD38 was similar between the groups, cases exhibited a significantly lower proportion of activated (HLA-DR+CD38+) CD8 T cells compared to controls (0.095 vs. 0.63, respectively; p=0.02; Figure 4b, right panel). In addition, cases exhibited a lower proportion of CD8+CD38+ T cells compared to controls (median 74% vs. 87%, p=0.02) and a similar trend toward an increased percentage of CD38+(HLA-DR-) CD4+ T cells in controls compared to cases (medians 95% vs. 91%, respectively; p=0.08; supplemental figure 3), suggesting that cases have fewer immature CD4+ and CD8+ T cells compared to controls. The proportion of T cells expressing only HLA-DR was small and was similar between the groups (Figure 2-4).

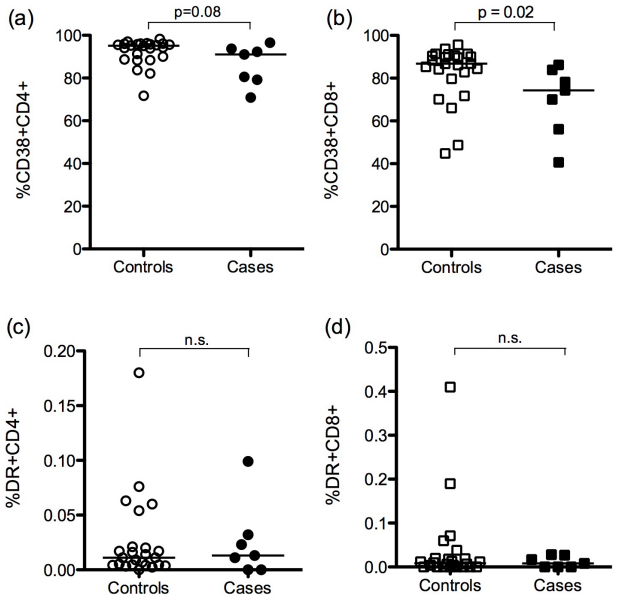

Figure 2-4. CD38 and HLA-DR Single Expression on CD4+ and CD8+ T cells in CBMC by Subsequent HIV-1 Acquisition. CD4+ or CD8+ lymphocytes were assessed for activation and maturation status by expression of CD38 (a. CD4+, circles; b. CD8+, squares) and HLA-DR (c. CD4+, circles; d. CD8+ squares). Lines represent the median of each group. Statistcis were generated using a Mann-Whitney test (n.s. p>0.1)

CD4+ and CD8+ T cell populations were also assessed for perturbation in naïve and memory phenotypes. Consistent with the finding that they had a lower proportion of CD38+ (immature) T cells, cases had an increased frequency of effector memory (T_{em}) (CD27-CD45RA-) CD4+ T cells (27.2% vs. 6.3%; p=0.02; Figure 2-4c) and CD8+ T cells(28.4% vs. 5.3%; p=0.01; Figure 4d, left panels), and a concomitant lower frequency of naïve (CD27+CD45RA+) CD8+ T cells (34.1% vs. 61%; figure 4d, second panel from left) compared to controls. While cases also displayed a trend toward decreased CD4+ naïve T cells, this did not reach statistical significance (Figure 4c). The median frequencies of central memory (T_{cm}) (CD27+CD45RA-) and effector (T_{eff}) (CD27-CD45RA+) phenotypes were similar between groups for both CD4+ and CD8+ T cells (Figures 2-4c and d).

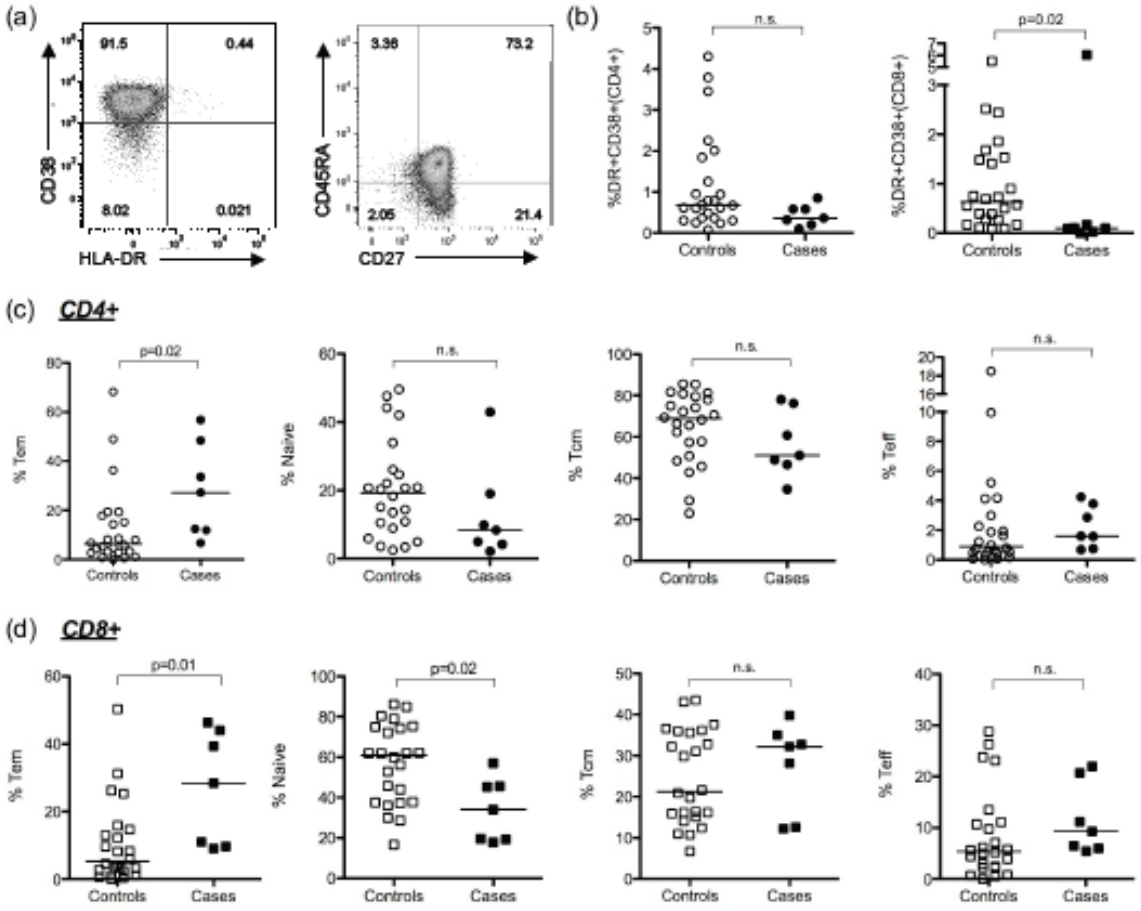

Figure 2-5. CD4+ and CD8+ T Cell Activation and Maturation Status in CBMC by Subsequent HIV-1 Acquisition. CD3+ lymphocytes were assessed for expression of CD4 and CD8 (a, left panel). CD4+ or CD8+ subsets were assessed for maturation status by expression of CD27 and/or CD45RA (a, right panel). Overall proportions of CD4+ (b, left panel) and CD8+ (b, right panel) T cells between cases and controls as well as the proportion of naive (CD27+CD45RA+), central memory (CD27+CD45RA-), effector (CD27-CD45RA+) or effector memory (CD27-CD45RA-) subset on CD4+ T cells (c) or CD8+ T cells (d) were assessed. Lines represent the medians, and statistics were generated using a Mann-Whitney test (*n.s. p>0.1*).

Because maternal viral load is the most significant risk factor associated with MTCT [125], we were also interesting evaluating the effect of maternal viral load on maturation subsets of CD4+ and CD8+ T cells in all HIV-1 exposed infants here, regardless of infection outcome (Figure 2-6). We observed no effect of maternal viral load on bulk proportions of CD4+ and CD8+ T cell populations in HIV-1 exposed infants. However, we observed a significant inversely proportional relationship between the frequencies of central memory CD4+ and CD8+T cells and increasing maternal viral load (p=0.034 between Q2 and Q4 for CD4 T cells; Figure 2.6a p=0.037 between Q2 and Q4 for CD8 T cells; Figure 2.6), with a concomitant directly proportional relationship between effector CD4+ and CD8+ T cells, (p=0.003 between Q2 and Q4 for CD4 T cells; Figure 2.6b and p=0.075 between Q2 and Q4 for CD8 T cells; Figure 2.6d). Maternal viral load quartile did not affect levels of naïve and effector memory CD4+ and CD8+ T cell populations.

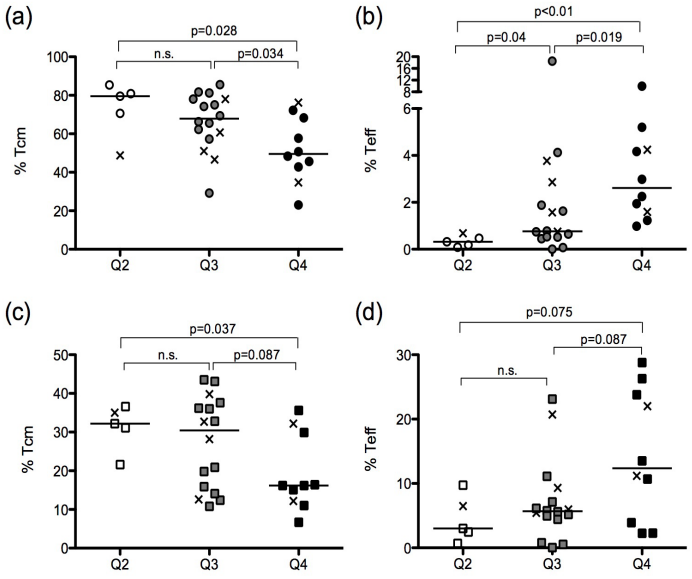

Figure 2-6. CD4+ and CD8+ T Cell Subset Distribution in CBMC by Maternal Viral Load Quartile. The proportions of central memory (CD27+CD45RA-) and effector (CD27-CD45RA+) T cells were assessed by maternal load viral quartile. Viral load quartile Q2 corresponded to viral load between ≥4.2 log and <4.7 log copies/mL, Q3 ≥ 4.7 and <5.3 copies/mL, and Q4 ≥ 5.3 copies/mL. Circles (a and b) represent CD4+ T cells and squares (c and d) represent CD8+ T cells. X's represent infants from each group that subsequently acuired HIV-1 infection. Lines represent the medians percentage of each subset, and statistics were generated using a Mann-Whitney test (*n.s.=p>0.1*).

DISCUSSION

MTCT provides a unique setting for identifying factors critical for HIV-1 transmission as it permits sampling of HIV-1-exposed individuals before transmission occurs, often within a month of the transmission event. This precision of pre-infection sampling is less possible using adult HIV-1 transmission models. Further, this setting provides systematic access to the transmitter (mother) viral load. Using a historical MTCT cohort, we identified several cellular immune correlates of HIV-1 transmission. We found that cord blood from cases contained an elevated proportion of CD16-CD56+ NK cells, less activated (HLA-DR+CD38+) CD16-CD56+ NK cells and CD8+ T cells and, finally, a higher proportion of T_{em} (CD27-CD45RA-) CD4+ and CD8+ T cells compared to cord blood from controls. These data provide new insights into cellular immune correlates of HIV-1 transmission.

CD16-CD56+ NK cells play a key role in the production of NK-derived cytokines (reviewed in [116]). The expansion of this cytokine-producing NK cell subset (to nearly 80% of NK cells, Fig. 1) in infants who go on to become HIV-1-infected may reflect the propensity of NK cell-derived proinflammatory cytokines, like interferon-gamma and TNF-alpha, to activate HIV-1 target cells, ultimately facilitating HIV-1 transmission. Further, this expansion was also associated with a contraction in the overall proportion of cytotoxic (CD16+CD56+) NK cells in cord blood of the case infants who went on to acquire HIV-1 infection, consistent with the hypothesis that NK cell cytotoxicity may be critical in defending against HIV-1 acquisition in the context of repeated exposures, such as MTCT. Further, our data argues that the relative frequency of NK cell subsets may be important in defending against HIV-1, with an advantageous outcome among infants with an expanded population of mature, cytotoxic, NK cells able to directly lyse virally-infected target cells to limit cellular infection and viral dissemination.

Because infants in our cohort acquired HIV-1 by one month of life, acquisition likely occurred during delivery or through breastfeeding (via breast milk). HIV-specific antibodies

commonly found in breast milk have been shown to mediate antibody-dependent cellular cytotoxicity (ADCC) and are associated with a reduced risk of MTCT [135]. Interestingly, in our study both subsets of NK cells expressing the Fc-gamma receptor CD16 and therefore capable of mediating ADCC (CD16+CD56+, CD16+CD56-) displayed trends toward underrepresentation in infants who went on to become infected compared to control infants. As NK cells are found at mucosal sites, including the gut mucosa [136], the capacity for NK cells to mediate ADCC in the context of exposure to HIV through breast milk may represent an important component of protection from infection.

The identification of a lower proportion of activated (HLA-DR+CD38+) NK cells and CD8+ T cells in cord blood from infants who went on to become HIV-1-infected suggests that maintaining an activated repertoire of NK and CD8+ T cells, both non-HIV-1-targets, may be protective in the context of MTCT. While we found a consistent trend toward lower activation of all NK cell subsets in infants who later acquired HIV-1, this only reached statistical significance in the cytokine-secreting (CD16-CD56+) NK population. Consistent with this observation, enhanced cytokine secretion and cytotoxicity by NK cells has previously been shown to correlate with protective immunity against HIV-1 transmission in adults [137]. Therefore, while cord blood from infants in our cohort had an expanded population of cytokine-secreting (CD16-CD56+) NK cells, they were less activated than NK cells in cord blood from controls, suggesting that activation of these cells, rather than solely their presence, may be important for protection from HIV-1 transmission. Together, these results suggest a beneficial effect of activated NK and CD8+ T cells in the context of repeated HIV-1 exposures.

Through the viral suppression assays, we found that cord blood NK cells from different individuals display a marked variation in the ability to suppress HIV-1 replication in autologous CD4+ T cells (Figure 4; min 0% suppression-max 99% suppression), which is similar to a previous report of neonate and adult NK cells [138]. We also observed a modest correlation between the suppressive capacity of bulk NK cells and the percentage of acutely activated

(CD69+) CD16-CD56+ NK cells from all infants in our cohort. This finding again suggests that activation of these cytokine-producing NK cells may be an important component for suppressing viral replication in this assay, in agreement with previous studies [100, 138]. Further studies are warranted to examine the direct role of NK cell-derived cytokines in HIV-1 transmission.

Consistent with previous work [122], our assessment of T cell subsets identified a significant increase in the proportion of CD4 T_{em} cells in CBMC from infants who went on to become HIV-1-infected. As the HIV-1 co-receptor, CCR5, is primarily expressed on T_{em} CD4+ cells, our data suggests that a protective effect exists in having a lower proportion of mature, HIV-1-vulnerable T cells. This may be because there are fewer target cells that can contribute to initial cellular infection as well as the spread of infection to draining lymph nodes in the periphery [139]. While the underlying mechanism for alterations in these different immune cell parameters is not known, the maternal cytokine milieu may drive or support HIV-1 replication [140], or the activation or development of neonatal NK and T cells. Improved antenatal care to address underlying maternal health issues that induce an activated cell phenotype may prove beneficial role in reducing fetal T cell activation and, thus, alter the risk of MTCT. Finally, we observed an inversely proportional relationship between the frequencies of central memory CD4+ and CD8+T cells and increasing maternal viral load with a concomitant directly proportional relationship between effector CD4+ and CD8+ T cells and maternal VL. As HIV preferentially infects effector cells, this suggests that maternal viral load may influence HIV-1 transmission not only through a direct consequence of the quantity/concentration of virus present in the mother, but also indirectly by inducing infant immune cells to take on a more mature, infectable phenotype.

Perinatal transmission of HIV-1 is influenced by virologic and immunologic factors in both infant and the mother. To our knowledge, our study is the first to characterize the role of both infant NK cell and T cell immune status in MTCT. We have identified three immune phenotypes in CBMC associated with an increased risk of MTCT during the first month of life,

including an increased frequency of CD16-CD56+ NK cells, decreased activation of non-HIV-1-target NK and CD8+ T cells, and an increased frequency of T_{em} CD4+ cells, which may serve as HIV-1 target cells. Together, these findings provide evidence that the HIV-1 acquisition in infants is influenced by both innate and adaptive immune cell phenotypes and activation status.

CHAPTER 3:

CAUSES AND CONSEQUENCES OF BCG VACCINE-INDUCED IMMUNE ACTIVATION IN HIV-1 EXPOSED SOUTH AFRICAN INFANTS

OVERVIEW

The success of prevention of mother-to-child transmission of HIV (pMTCT) programs has contributed to the almost universal decrease of incident pediatric HIV-1 infections worldwide. However, preventing MTCT remains a challenge in countries with a high HIV prevalence where breast-feeding is widespread, as formula feeding is neither safe nor affordable, leading to HIV transmission through breast milk. This chapter examines the implications of administering the live, attenuated Bacillus Calmette-Guérin (BCG) vaccine, routinely administered to infants at birth to protect against tuberculosis, on immune activation and subsequent potential increase in infant susceptibility to HIV acquisition. In a controlled trial of 149 HIV-exposed infants in Khayelitsha, South Africa, randomized to receive either conventional BCG vaccination (at birth) or delayed BCG vaccination (at 8 weeks of life), we found that BCG vaccination leads to increased activation of HIV target CD4+ T cells (CCR5+HLA-DR+CD38+). Interestingly, however, we were unable to determine the source of T cell activation, as activation of innate monocytes and NK cells and generalized immune activation (measured through soluble and cell-associated inflammatory mediators) was not evident in either group following BCG vaccination. Regardless, because T cell activation has been previously linked to increased HIV transmission, these results may ultimately have important clinical implications regarding vaccine-induced immune activation in general, but specifically regarding the timing of BCG administration in HEU infants.

INTRODUCTION

As a result of a scale-up in prevention of mother to child HIV-1 transmission (pMTCT) programs, a 52% reduction in new pediatric HIV-1 infections has been achieved worldwide between 2001-2012 [6]. However, preventing MTCT remains a challenge in developing countries with a high HIV prevalence and where breast-feeding is common, making formula feeding neither safe nor affordable, and leading to HIV transmission through breast milk. In these populations MTCT still occurs, leading to 250,000 new pediatric infections in 2012 (UNAIDS). Additionally, while lower rates of MTCT mean that the prevalence of pediatric HIV is decreasing, it also means that the prevalence of HIV-1-exposed uninfected infants (HEU) is rising. Because of a unique set of social, environmental, and immunological factors that arise as a consequence of maternal HIV infection, HEU infants experience higher morbidity and mortality than unexposed infants (reviewed in [141]). Immunologically, HEU have shown to have fewer naïve CD4+ T cells compared to unexposed infants [141] as well as abnormalities in cytokine production following exposure to a wide array of stimuli, including HIV or *Mycobacteria* peptides, *S. aureus*, and Bacillus Calmette Guérin (BCG; [141]). Despite these known differences, a relatively limited amount of information is available regarding the care of HEU infants, presenting clinicians with unique challenges when providing care for these infants.

Currently, limited information exists regarding vaccine efficacy in HEU infants, which may be dramatically altered as a result of the aforementioned immune alterations in HEU infants. BCG is a live-attenuated vaccine administered around the world against tuberculosis. In HIV-uninfected children, BCG is safe, efficacious and cost-effective against disseminated tuberculosis [142-144]. The safety and efficacy of the BCG vaccine in HEU infants, however is unknown. For example, BCG has shown to be less immunogenic in HEU, and elicits reduced T cell proliferation and IFNγ production responses compared to unexposed infants, [145]. While

there is no clear correlate of protection from TB, reduced immunogenicity may translate into lower vaccine efficacy.

Through a complex interplay of cellular and immunological mechanisms, BCG and HIV each dramatically impact susceptibility and disease progression of the other. Thus, vaccinating HEU infants may also provide unique concerns regarding safety. For example, the vast majority of HEU infants are consistently challenged with the risk of acquiring HIV infection through exposure to virus through breast milk. Administering a live-attenuated vaccine, like BCG, which has the potential to cause disease in HIV-infected infants [73], may put infants at risk for disseminated BCG disease in the context of a successful MTCT event.

Importantly, not only does HIV increase an infant's risk of disseminated BCG disease, but BCG may also affect an infant's susceptibility to HIV. At a cellular level, and in the context of infant HIV exposure, BCG has the potential to lead to cellular activation and, therefore, the generation of HIV target cells, increasing an infant's risk of HIV acquisition. Further, data generated using simian immunodeficiency virus (SIV)-infected Rhesus macaques (a model of HIV) show that BCG can drive rapid viral dissemination and SIV pathogenesis [74, 75]. As HIV/SIV primarily infects activated CD4+ T cells, this suggests T cell activation driven by BCG exposure can accelerate also HIV/SIV disease progression. Thus, while vaccines undeniably provide life-saving immune education and protection from infectious diseases throughout the world, special consideration must be given to vaccinating HEU infants. A thorough knowledge of the risks and benefits of administering BCG to HEU (and possibly infected) infants is vital for policy makers in high TB burdened settings.

As described in chapter 1, innate immunity may be key to initiating an effective BCG vaccine response, as they are not only some of the first and primary responders to *Mycobacteria*, but they are also responsible for shaping the quality of the adaptive immune response. Data using a rabbit model demonstrated that two weeks following aerosolized

infection, innate cell gene networks including macrophages, NK cells, and dendritic cells were highly upregulated [146]. Because of the importance of innate immunity in not only shaping adaptive immune responses, but also in the potential to generate inflammation and immune activation, we included an analysis of monocyte and NK cell activation caused by BCG vaccination in the present study.

Monocytes are known to be highly inflammatory and are exquisite producers of potent, pleiotropic inflammatory cytokines, like TNF-α and IL-6. While monocyte responses are critical to mounting an effective immune response against Mycobacterial infections, monocyte activation can also result in the downstream activation of CD4+ cells, increasing their likelihood of becoming HIV targets, providing a direct link between monocyte activation and T cell activation in BCG-vaccinated infants.

NK cells, like monocytes, function as a part of the innate immune system. While currently little is known about NK cell responses to *Mycobacteria*, they have been shown to respond to *Mycobacteria* through TLR 2 stimulation [103], and to localize to the site of *Mycobacteria* infection [109, 110]. Further, NK cells are exquisite producers of IFNγ, a cytokine responsible for driving T cell polarization to a Th1 bias (a key feature of BCG vaccination). Finally, unlike their CD8+ T cell counterparts, NK cells are able to secrete IFNγ prior to the establishment of any immune memory, and therefore likely are the major source of IFNγ just hours to days after infection. Together, these data suggest that NK cells may be an important driver of the initial immune response to BCG vaccination.

Here we conducted a randmized controlled trial of HIV-exposed infants in Khayelitsha, South Africa, randomized to receive either conventional BCG vaccination (at birth) or delayed BCG vaccination (at 8 weeks of life) in order to address the hypothesis that BCG vaccination leads to immune activation in HEU infants, which may ultimately increase their susceptibility to HIV infection.

MATERIALS AND METHODS

Cohort and specimen selection

A total of 149 infants were enrolled within 20 hours postpartum from a maternal obstetric unit in Khayelitsha, an informal settlement in Cape Town, South Africa, for which the antenatal HIV prevalence is approximately 30% [147]. Due to a relatively strong pMTCT program, a residual transmission rate of approximately 4% existed during the period that this study was conducted (June 2010 - December 2011). Informed consent was obtained from the mothers prior to screening. Eligibility requirements for inclusion into the study included HIV exposure and lack of infection (negative HIV DNA PCR), the presence of a legal guardian willing and able to give informed consent, gestational age >36 weeks and birth weight >2.5 kg, no presence of acute illness, and no known current maternal TB or household TB contacts.

Blood collection, Vaccination, PBMC isolation and sample preservation

Infant blood was obtained at enrollment for HIV DNA PCR. Due to the World Health Organization recommendations, infants who tested positive for HIV DNA via PCR were not BCG vaccinated and were referred immediately for antiretroviral therapy. If the HIV DNA PCR was negative, infants were randomized to receive conventional/early (age 2-3 days) or delayed (8 weeks) BCG vaccination (randomization was performed by an independent statistician). For vaccination, Danish strain BCG 1331 (Statens Serum Institute) was administered intradermally. Peripheral blood mononuclear cells (PBMC) were extracted from blood obtained in sodium heparin tubes at birth, 2, 6, 8, and 14 weeks of age. Plasma and an aliquot of PBMC were stored in RNA Protect (Qiagen) at -80, and PBMC were cryopreserved in liquid nitrogen.

PBMC sample preparation and multiparameter flow cytometric analysis

For this study, frozen PBMC from 50 infants total (25 conventional/early and 25 delayed) were thawed according to the HVTN protocol (detailed in Chapter 2) and enumerated via a Guava automated cell counter (EMD Millipore, Darmstadt, Germany). Approximately 300,000-500,000 cells were added to one well of a 96-well plate, pelleted at 2100rpm for 3 minutes, and resuspended in 47.5ul PBS. 2.5ul LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen) diluted 1:50 in DI H2O was added to each well and samples were incubated for 20 minutes at RT in the dark. Cells were washed twice with 150ul PBS and pelleted at 2100rpm for 3 minutes. Cells were then resuspended in 50ul extracellular master mix containing a cocktail of the following antibodies (note: all antibodies were from BD Biosciences [San Jose, CA, USA] unless otherwise noted): Anti CD3 PE-Cy5 (clone UCHT1), Anti CD14 qDot605 (clone TÜK4, Life Technologies, Grand Island, NY, USA), Anti CD16 V450 (clone 3G8), Anti CD19 PE-Cy5 (Clone H1B19), Anti CD56 PE-Cy7 (clone NCAM16.2), Anti HLA-DR FITC (clone G46-6), Anti Tim3 PE (clone 344823, R&D Systems, Minneapolis, MN, USA), and Anti CD279 APC (clone MIH4). Following the addition of the extracellular mastermix, cells were washed 2x in 150ul FACS Wash buffer PBS+1% FCS), resuspended in 150ul CellFix, BD Bioscience), transferred to polystyrene FACS tubes, and acquired using a BD LSRII. Positivity gates were established using results from fluorescence-minus-one (FMO) staining.

Measurement of cytokine and chemokine transcripts in PBMC via Fluidigm

Total RNA was reverse-transcribed into first-strand complementary DNA (cDNA) using a QuantiTect Reverse Transcription Kit (Qiagen). cDNA was mixed with Taqman Preamp Master Mix (Applied Biosystems) and STA Multiplex Primer Pool. Subsequently, cDNA was pre-amplified using sequence-specific primers by denaturation at 95°C for 15s and annealing and amplification at 60°C for 4 min for 14 cycles. These preamplified products were then diluted 5-fold prior to analysis with Fast Start Taq-man Probe Master Mix and inventoried Taqman gene

expression assays (Applied Biosystems) in 48.48 Dynamic Arrays on a BioMark System (Fluidigm). Ct values were calculated from the system software [BioMark Real-Time PCR (polymerase chain reaction) Analysis, Fluidigm]. The expression value of each gene (targets listed in table 3-1) relative to the endogenous control genes GAPDH and HPRT were also calculated using the soft and ΔΔCt methods

Fluidigm Analysis Targets			
ARG1	CXCL10	IFNg	IRF4
CCL2	CXCL13	IL-10	ITGAM
CCL5	CXCL9	IL-12a	ITGAX
CCR2	CXCR3	IL-12b	KLF4
CD14	EMR1	IL-12Rb1	MARCO
CD209	FCGR3A/B	IL-18	NOS2
CD86	GAPDH	IL-18BP	PTGS2
CD8a	HPRT	IL-1a	SOCS2
CIITA	IDO1	IL-1b	SOCS3
CLEC4F	IFNa1	IL-23a	TNFa
CSF-1r	IFNaR2	IL-29	TRAIL
CX3CR1	IFNb	IL-6	TNFSF9

Table 3-1. List of targets for fluidigm analysis of PBMC from BCG vaccinated infants at 2 weeks of age.

Measurement of plasma cytokines/chemokines

Plasma samples from 0, 2 and 6-week time points were thawed on ice. Prior to cytokine measurements, plasma was centrifuged at 10 000xg for 10 minutes to remove debris. GMCSF, IFNα, IFNγ, IL-8, IP-10, MCP-1, MIP1b, TNFα, and IL-10 concentrations in plasma samples were determined using Milliplex™ Human Cytokine kits (Millipore). Data was collected using a Bio-Plex™ Suspension Array Reader (Bio-Rad Laboratories Inc) and a 5 PL regression formula was used to calculate sample concentrations from the standard curves. Data was analysed using BIO-plex manager software (version 4). Cytokine levels that were below the lower limit of detection of the assay were reported as the mid-point between the lowest concentrations measured for each cytokine and zero.

sCD163 ELISA

Plasma samples from the 8-week time point were thawed on ice. Prior to sCD163 analysis, plasma was centrifuged at 10 000xg for 10 minutes to remove debris. The sCD163 ELISA (R&D systems) was performed according to the manufacturer's instructions. Briefly, 100ul assay diluent (provided) was added to each precoated (anti-CD163) well of a 96-well microplate. 50ul plasma of each sample (diluted 1:10 in manufacturer provided calibration buffer) or standard (provided) were added to duplicate wells of the plate and incubated for 2h. The wells were then each washed 4x with 100ul provided wash buffer prior to the addition of 200ul conjugate to each well. Following a 2h incubation, wells were again washed 4x with was buffer prior to the addition of 200ul substrate solution to each well. After 30 minutes of incubation in the dark, 50ul of stop solution was added and the optical density at 450nm was meaured using a plate reader. The concentration of sCD163 in each sample was measured by averaging the optical density of the duplicate samples and plotting against a standard curve.

Statistical Analyses

The designated primary outcome of this study was the difference in CD4+HLADR+ expression between BCG-vaccinated versus unvaccinated infants at 6 weeks of age. The sample size and power calculations are based on the comparison of the median number of CD4+HLADR+ cells/µl in in routinely vaccinated HEU infants from previous data. Through these methods, a sample size of 48 infants in each arm was calculated to provide 90% power to detect a 0.5% difference in the number of CD4+ HLADR+ cells per µl at 6 weeks (2-tailed test at α=0.05).

Flow cytometry data were analyzed using Flowjo (Treestar v. 8.8.6) and statistical analyses were performed using GraphPad Prism. The Mann-Whitney U test was used to compare proportions of monocyte and NK cell subsets and activation (p-values are two-tailed at α=0.05). The relationships between immune activation and BCG vaccination status were

analyzed using Stata version 11 (Statcorp, College Station, TX). Adjustments for multiple comparisons were performed using the Holm step-down approach (Columb). Generalized estimation equation population-averaged models were used to determine the difference between groups through time.

RESULTS

Cohort

Of the 149 infants enrolled, five infants tested HIV positive through DNA PCR, four of these were at birth. There were two deaths and two protocol violations (infants given BCG vaccination at birth by the maternity unit staff), leaving 140 infants for randomization. Of these, 7 relocated to distant communities, one withdrew, and 9 were lost-to-follow-up for unknown reasons (untraceable), leaving 63 infants in the early BCG arm and 59 in the delayed. The baseline characteristics of these 122 infants are shown in Table 3-1. With the exception of age at BCG vaccination, there were no significant differences in characteristics between the two arms.

Characteristic	Early (n=63)	Delayed (n=59)	P value
Median age of BCG vaccination (days) (IQR)	3 (2-4)	58 (56-62)	<0.001
Median maternal CD4 count, cells/mm^3 (IQR)	371 (276-477)	358 (254-548)	0.674
Mean birth weight, kg (s.d.)	3.12 (0.39)	3.15 (0.41)	0.443
Mean gestational age (weeks)			
Male gender n (%)	28 (44)	29 (49)	0.331
Breastfed n (%)	12 (19)	8 (14)	0.464

Table 3-1. Cohort characteristics. This used specimen from HIV-exposed infants collected between June 2010 and December 2011 in Khayelitsha, South Africa. P-values were determined using two sample Wilcoxon (Mann-Whitney) rank-sum test for continuous variables, Chi2 for binary variables (gender and feeding).

T-lymphocyte Activation

We first measured T cell activation, through the expression of CCR5 (HIV co-receptor), HLA-DR, CD38 (T cell activation markers), and Ki67 (proliferation marker), on CD3+CD4+ and CD8+ cells via flow cytometry. At birth and at 2 weeks of age, there was no statistically significant difference in the proportion of any activation marker on CD4+ or CD8+ T cells, alone

or in combination (Fig 3-1a). However, by week six, the infants who received early BCG vaccination had significantly higher levels of CD4+ T cells co-expressing the HIV coreceptor CCR5, CD38, and HLA-DR (Figure 3-1a). This difference in activation persisted at 8 weeks of age, the final time point before the delayed group received BCG. Using a generalized estimation equation (GEE) population averaged model of time through 8 weeks, we observed that early/conventional BCG was significantly associated with CD4+CCR5+CD38+HLA-DR+ cell frequency. This was true after adjusting for maternal CD4 count, gender, and birth weight. There were no differences in CD8+ T cell activation marker expression at any time point.

Evaluation of T cell activation at 14 weeks of age (6 weeks after the delayed group received their BCG vaccination) revealed that infants in the delayed BCG arm had significantly higher frequency of CCR5+CD38+HLA-DR+CD4+ T cells than observed at pre-vaccination at 8 weeks (median 0.14% at week 8 vs. 0.41% week 14; p=0.0008), which ultimately became similar to CD4+ T cell activation in the early group (Fig 3-1b, week 14). CD4+ T cell activation in the infants from the early BCG arm at 14 weeks remained elevated, similar to the 8 week time point.

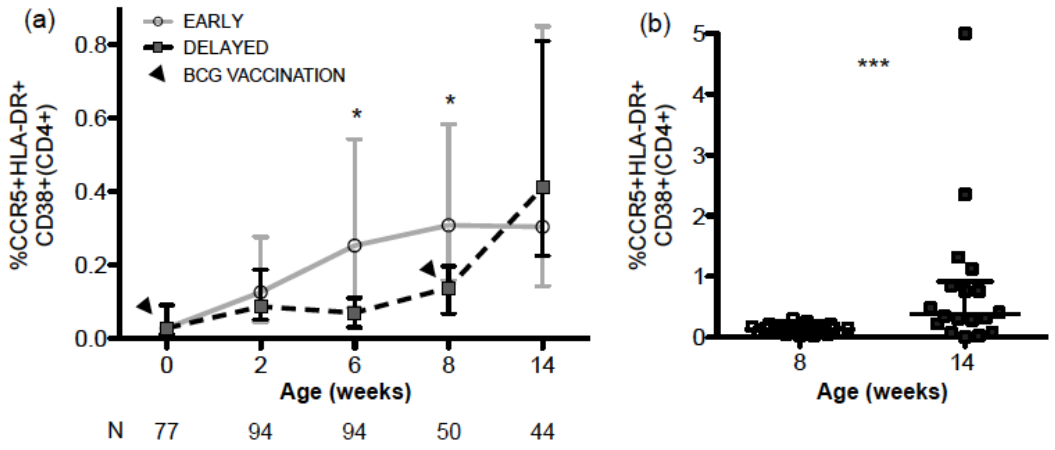

Figure 3-1. CD4+ T Cell Activation in HIV-exposed Infants Who Received BCG Vaccination. CD4+ T cell activation (CCR5+HLA-DR+CD38+) was measured via flow cytometric analysis at 0, 2, 6, 8, and 14 weeks of age in the delayed versus early vaccination groups (a). CD4+ T cell activation in the delayed group of infants at 8 (pre-vaccination) and 14 weeks (post-vaccination) (b.). Statistics were generated via a generalised estimation equation population-averaged model (a) or a paired t-test (b); *p<0.05, ***p<0.001.

Monocyte subset distribution and activation status

In order to determine a potential mechanism of increased CD4+ T cell activation following BCG vaccination, we undertook an analysis of innate cell activation, including monocytes and NK cells. CD3-CD19-HLA-DR+SSCmid cells were analyzed for the expression of CD14 and CD16 (Figure 3-2a), as differential expression/co-expression of these markers has been correlated with different inflammatory capacities. Overall, we found that the percentage of monocyte subsets were similar between infants who received conventional or delayed BCG vaccination, including classical CD14++ or inflammatory CD14+CD16+ and CD14dimCD16+ monocytes, with the highest proportion of cells falling into the CD14++ gate for both groups (approximately 78%; 3-2b).

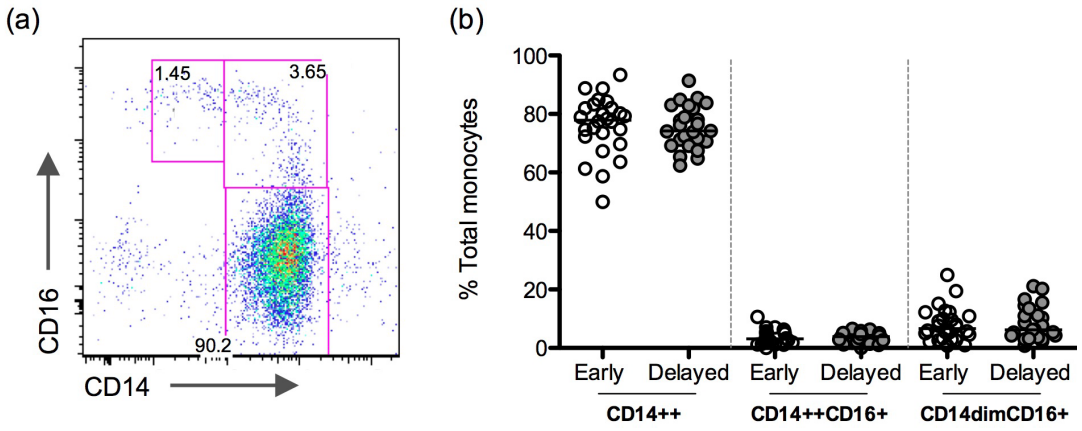

Figure 3-2. Monocyte Phenotypic Subset Distributions at Week 8 in HIV-exposed Infants Randomized to Receive Conventional or Delayed BCG Vaccination. Cells were first gated on singlets and live cells prior to the exclusion of CD3+ and CD19+ lymphocytes. Monocytes were subsequently gated on SSC-mid, HLA-DR+, and further classified by the presence of CD14 and CD16 (a). Proportion of monocyte subsets in infants who were BCG vaccinated at birth (early) or at 8 weeks (delayed)(b). Statistics were generated using a Mann-Whitney Test. If no p-value is reported, p>0.1

In addition to measuring the effect of BCG on monocyte phenotype, we were interested in examining the activation status of infant monocytes following BCG vaccination. We found no difference in monocyte activation at 8 weeks of age in the early or delayed BCG groups, as measured by the proportion expressing the markers Tim3 and PD1 (Figure 3-3a and b). Interestingly, the expression of Tim3 differed between the different subsets analyzed in both groups, with nearly all CD14++, CD14+CD16+, and CD14dimCD16+ cells expressing Tim3, but only roughly 50% of CD14dim (CD16 negative) monocytes were found to be Tim3+. Overall, a similar proportion of all monocyte subsets expressed PD-1 in both groups (range 2-5.3%; figure 3-3b). To confirm these findings suggesting limited differential monocyte activation between the groups, we measured the concentration of sCD163 in 8 week PBMC-matched plasma samples. We found no significant difference in the concentration of sCD163, with a median concentration of 68.6ng/mL and 67.6 ng/mL in conventional and delayed BCG vaccinated infants, respectively (figure 3-3c). The mean fluorescence intensity (MFI) of all activation markers on monocytes was also similar between groups (not shown).

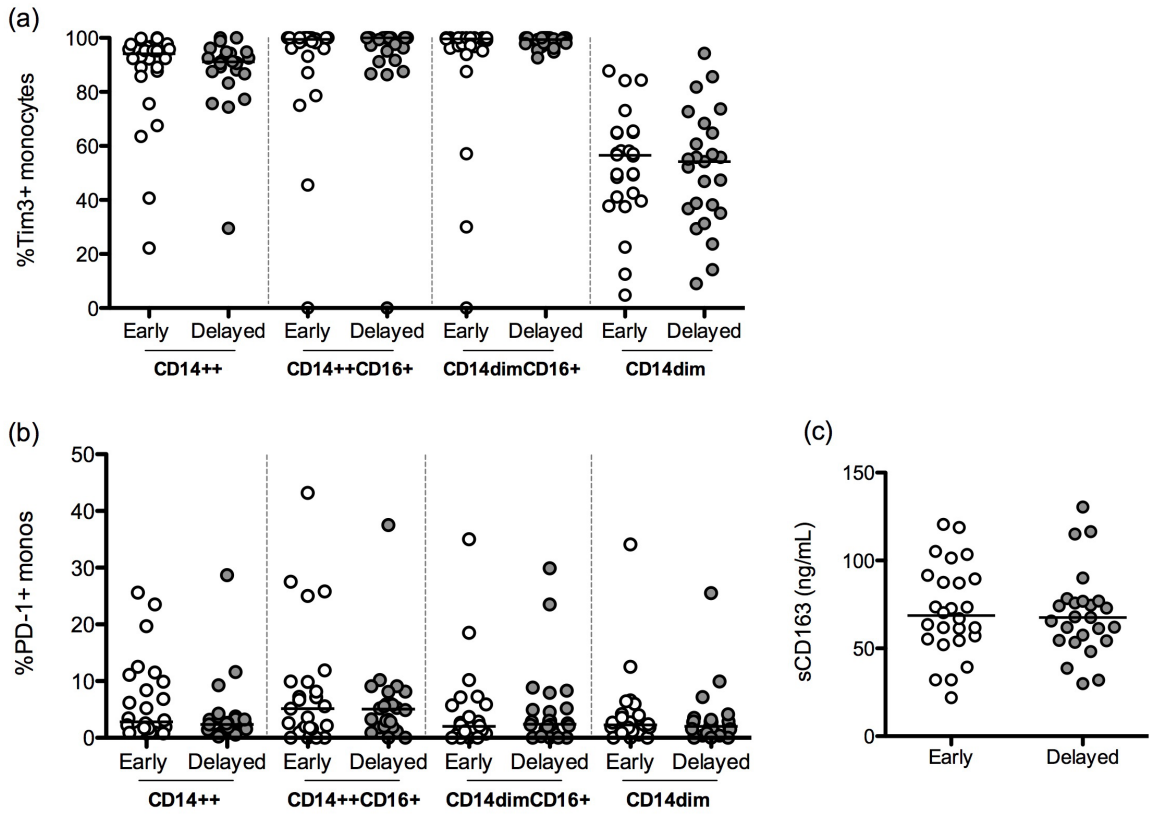

Figure 3-3. Monocyte Activation in 8 Week Old HIV-exposed Infants Randomized to Receive Conventional or Delayed BCG Vaccination. Monocyte activation was measured in PBMC through the percentage of monocytes expresisng Tim3 (a) or PD-1 (b) and by the presence of soluble CD163 in plasma (c). Statistics were generated using a Mann-Whitney Test. If no p-value is reported, p>0.1.

NK cell subset distribution

We also evaluated NK cell activation following BCG vaccination in week 8 samples from infants who received conventional or delayed BCG vacination. We first examined the phenotypic subset distribution of NK cells in each group and found that NK cell subset distributions were similar between infants that received conventional or delayed BCG vaccination, with the highest proportion of cells falling into the CD16+CD56+ (cytolytic NK cells, 36.8% and 40.1% for the early and delayed groups, respectively) and CD16-CD56+ (cytokine-

producing NK cells; 51% early 35% delayed) and the lowest proportion of NK cells falling into the CD16+CD56- (progenitor or dysfunctional) gate for both groups.

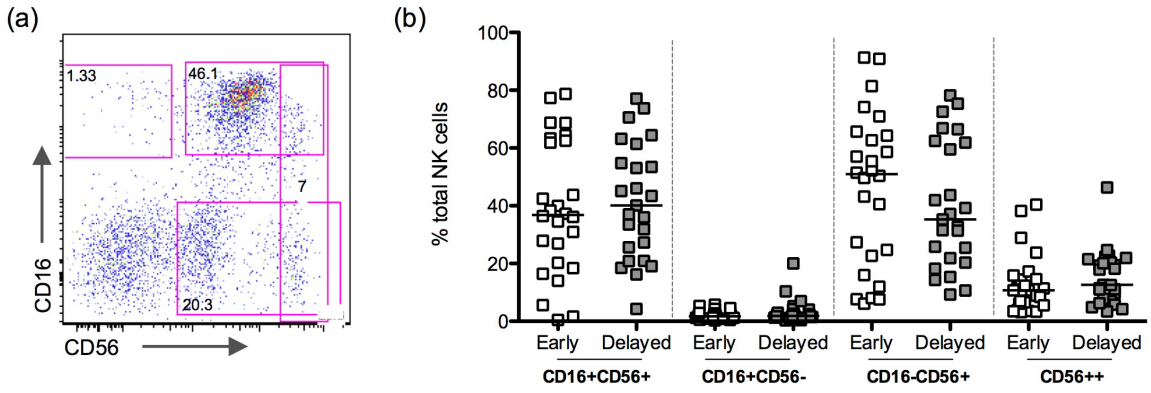

Figure 3-4. NK Cell Phenotypic Subset Distribution at 6 Weeks in HIV-exposed Infants Randomized to Receive Conventional or Delayed BCG Vaccination. Cells were first gated on singlets and live cells prior to the exclusion of CD3+ and CD19+ lymphocytes and CD14+ monocytes. NK cells were subsequently gated on the presence of CD56 and/or CD16 (a). Proportion of NK cell subsets in infants who were BCG vaccinated at birth (early) or at 8 weeks (delayed)(b). Statistics were generated using a Mann-Whitney Test. If no p-value is reported, $p>0.1$

In addition to NK phenotypic distribution, we were interested in examining NK cell activation and/or exhaustion through the percentage of NK cells expressing Tim3, HLA-DR and PD-1 expression in infants who had or had not received BCG vaccination. Similar to monocytes, we found NK cell activation to be similar between the early and delayed BCG groups. Specifically, NK cells expressed similar levels of Tim3 (Figure 3-5a), HLA-DR (Figure 3-5b) and PD-1 (not shown) regardless of BCG vaccination status. We did observe marked variation in the percentage of NK cells expressing Tim3 between subsets with nearly all CD16+ NK cells (including the CD16+CD56- and CD16+CD56+ subsets) and a lower proportion of CD16- NK cells (including the CD16-CD56+ and CD16-CD56++ subsets) expressing Tim3. The mean fluorescence intensity of all activation markers was similar.

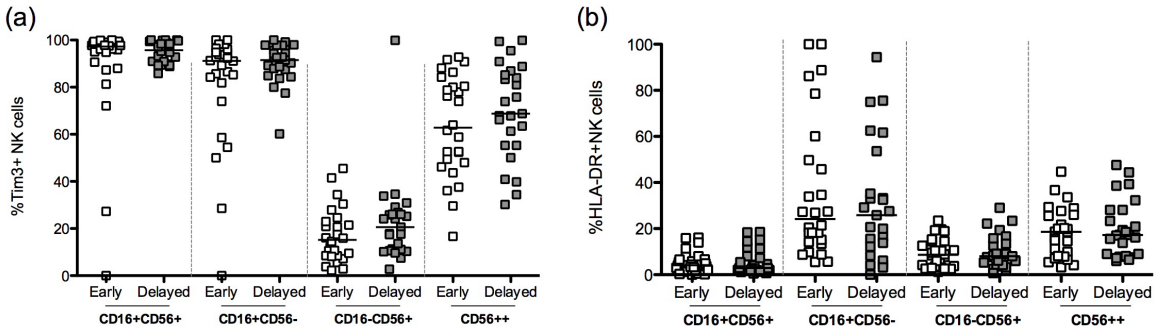

Figure 3-5. NK Cell Activation in HIV-exposed Infants Randomized to Receive Conventional or Delayed BCG Vaccination. NK cell activation was measured in PBMC through the percentage of NK cells expresisng Tim3 (a) or HLA-DR (b). Statistics were generated using a Mann-Whitney Test. If no p-value is reported, p>0.1

Levels of Inflammatory Mediators in Plasma and PBMC

Because systemic immune activation could also influence CD4+ T cell activation, we undertook an assessment of cytokine levels in plasma and PBMC of infants randomized to receive early versus delayed BCG. Because we found no significant differences in innate immune cell activation in week 8 samples, we performed an analysis of inflammatory markers at ealier time points (2 and 6 weeks of age), consistent with the idea that innate immune cell activation would likely happen at an earlier time point post vaccination and could potentially be transient. We observed via Luminex analysis no significant differences in plasma levels of GMCSF, IFNα, IFNγ, IP-10, MCP1, TNFα, MIP-1β or IL-8 at week two or at week six following adjustment for multiple comparisons (n=58) (not shown). In addition to Luminex, we performed Fludigm analysis on mRNA from week 2 PBMC samples (a full list of the targets analyzed can be found in Table 3-1 of the methods section). We found no significant differences in transcript levels of inflammatory mediators in PBMC of infants that had received early or conventional BCG vaccination via real time PCR.

DISCUSSION

BCG remains one of the most widely used vaccines, and it is administered routinely in developing countries where TB is endemic, as it is safe and efficacious against childhood and disseminated TB in healthy infants. However, special consideration needs to be given BCG vaccination of HIV-exposed infants. While BCG (and vaccines in general) provide life-saving immune education and subsequent protection from infectious diseases, its highly immunogenic nature can induce a state of inflammation and immune activation that has the potential to alter the infant's susceptibility to HIV infection in the context of repeated exposures. Here we have examined the role of BCG vaccination in influencing infant immunity in a randomized trial of conventional or delayed BCG vaccination. We found that BCG does indeed induce a state of increased T cell activation that is unique to CD4+ T cells and includes CCR5-expressing cells (HIV target cells), and that happens independently of innate cell activation or increased levels of inflammatory mediators in either plasma or whole PBMC.

Increased T cell activation has been linked to HIV-1 infection through several independent observations. A study of SIV infected non-human primates demonstrated that while a proportion of resting CD4+ T cells are susceptible to infection, activated CD4+ T cells produce higher levels of virus, likely facilitating viral dissemination and seroconversion [148]. Further, the presence of pre-existing sexually transmittable infections (STIs) and associated mucosal inflammation has been linked to increased sexual HIV-1 transmission [112, 149-151]. Conversely, observational studies of HEU individuls (commercial sex workers or men who have sex with men) have demonstrated that low levels of CD4 T cell activation and quiescent CD4 T cell phenotypes were associated with reduced HIV susceptibility [121, 122, 152]. Taken together, these results suggest that increased T cell activation, as observed in our study of BCG vaccinated, HIV-exposed infants, may also lead to increased HIV-1 transmission in the context of repeated exposures, potentially through the generation of more target cells, or through more efficient viral dissemination following initial infection. Interestingly, because we observed

increased CD4+ T cell activation of a very specific group of CD4+ T cells (CCR5-expressing - prime HIV targets), this may also increase the individual cellular susceptibiltiy to infection.

We were surprised to observe that monocyte activation was similar between the early and delayed BCG groups at 8 weeks of age, a time-point at which we saw increased T cell activation in the early group compared to the delayed. Because monocytes are some of the first responders to *Mycobacteria*, and their activation has the potential to activate CD4+ T cells, we hypothesized that we would see increased monocyte activation in BCG-vaccinated infants compared to unvaccinated (delayed) controls. Further, a recently published study of adults showed that BCG vaccination results in an altered monocyte phenotype as early as two weeks post vaccination, and lasts for as long as three months [153]. In this study, which used cells cultured and restimulated *ex-vivo*, BCG vaccination increased both the percentage of CD14+ monocytes as well as the mean fluorescence intensity (MFI) of TLR4 on monocytes. In this same study, non-specific proinflammatory cytokine production from PBMC was increased two weeks after BCG vaccination and remained elevated for at least three months compared to pre-vaccination levels [153].

We did not observe a difference in monocyte phenotypic subset distributions at 8 weeks post BCG vaccination in our study, nor did we detect increase in mRNA of proinflammatory mediators compared to the unvaccinated group at just 2 weeks post vaccination. Several differences between our study and the one above may account for the observed differences. While an increase in non-specific proinflammatory cytokine production by monocytes following BCG vaccination was noted by Kleinnijenhuis et al, this cytokine production was observed only following stimulation, while our study evaluated the basal output of these cytokines from PBMC and in plasma (not in response to stimulation). Further, and perhaps more importantly, while this study characterized responses to BCG in a population of healthy adults, our study evaluated monocyte responses of HIV-exposed infants. Several differences between the mature adult and immature infant immune system may lead to disparate responses to

immunogens, which may be important to consider [100]. And, as all the infants in our study were HIV-exposed, and this group has already been shown to have altered BCG responses compared to healthy infants [141], this may have further compromised monocyte responses to BCG vaccination in our study. Together, these differences may be responsible to the disparate outcomes between the two studies.

Available data supports the concepts that NK cells do respond to *Mycobacteria* and are important in the control of mycobacterial infection, however the NK response to BCG vaccination is less clear. It has been demonstrated that cord blood mononuclear cells (CBMC) stimulated with BCG *ex vivo* produced a cytokine response dominated by NK cell-derived IFNγ, while a minimal cytokine response was elicited from lymphocytes [154]. However, we did not determine BCG-induced NK cell activation in our study, as measured by the percentage of NK cells expressing Tim3 or HLA-DR as readouts of activation. While HLA-DR is routinely used as an activation marker on NK cells [155], it has only recently been demonstrated that Tim3 becomes upregulated on activated NK cells, which has been linked to substantially higher production of IFNγ [156]. All infants exhibited very high levels of Tim3 expression on almost all NK cell subsets examined (figure 3-5a), suggesting that perhaps infant NK cells are already primed for interferon production. Performing intracellular cytokine staining for IFNγ production as a functional readout might have ultimately been a better indicator of NK cell activation. However, as our goal was to measure BCG vaccine-induced, basal IFNγ production by PBMC in the absence of restimulation, IFNγ levels would likely have been minimal to undetectable.

Overall, despite the observed increase in BCG-induced CD4+ T cell activation, we did not observe differences in innate cell activation nor in plasma proinflammatory cytokine production, which leaves the mechanism for T cell activation following intradermal BCG vaccination of infants unknown. Because BCG in this study was administered intradermally, it is possible that Langerhans cells (skin-resident dendritic cells) may be the primary responders in this setting. Indeed, it has been demonstrated that Langerhans cells mature following a TLR 2-

mediated response to bacterial lipopeptides, and acquire the ability to activate CD4+ T cells [157]. Therefore, activation of skin-resident Langerhans cells may be a more physiologically relevant component of the immune response following intradermal BCG vaccination than is the activation circulating monocytes and NK cells.

Ultimately, these findings may have important clinical implications, as BCG-induced T cell activation ultimately has the potential to increase infant susceptibility to HIV infection. This becomes particularly important in the context of repeated exposure to HIV, such as through breastfeeding. This study is not the first to document the potential for unintended consequences of vaccination in the context of HIV exposure, as vaccine-induced increase in HIV-susceptibility of a subset of STEP trial participants was noted [158]. A better understanding of the complex interplay between vaccination, immune activation, and HIV susceptibility is needed. While delaying BCG vaccination is only safe when there are no known household or maternal contacts with active tuberculosis infection, it is perhaps one factor that should be considered as part of a pMTCT program when feasible.

CHAPTER 4:

USING THE SOOTY MANGABEY MODEL OF NON-PATHOGENIC SIV INFECTION TO ELUCIDATE MECHANISMS OF HIV PATHOGENESIS AND SUSCEPTIBILITY TO MYCOBACTERIAL INFECTIONS

OVERVIEW

HIV-related progression to AIDS is generally accompanied by immune system dysfunction and susceptibility to opportunistic infections (OIs). In contrast, nonpathogenic SIV infections of sooty mangabeys are characterized by maintained immune function despite high levels of replicating virus. This chapter evaluates immune responses to the opportunistic pathogen BCG in both progressive HIV+ humans and non-progressive SIV+ mangabeys to identify immune correlates of resistance to OIs in the SIV-mangabey model system. To do this, we performed transcriptomic analysis of BCG-stimulated PBMC from HIVneg and HIV+ donors as well as SIVneg and SIV+ mangabeys. In order to evaluate the contribution of monocytes in mediating the response to BCG, we also evaluated cytokine expression of BCG stimulated cells within whole blood. Our data reveal that HIV+ patients have significantly altered NK cell gene expression profiles and deficient monocyte IL-12 production in response to BCG stimulation compared to HIV-negative donors. In contrast, SIV+ mangabeys maintain similar gene expression profiles and increase the percentage of monocytes producing proinflammatory cytokines TNFα and IL-12 following BCG stimulation compared to uninfected controls. These data yield new insight into progressive HIV infections that result in susceptibility to OIs and demonstrate the utility of using hosts of non-progressive SIV infections to determine correlates of immune health despite chronic, high viremia.

INTRODUCTION

Coinfection with HIV and *M. tuberculosis* still accounts for a significant proportion of HIV-related deaths that occur annually [6]. As discussed in detail Chapter 1, HIV and *M. tuberculosis* (Mtb) synergize on several levels, which all contribute to the high incidence of HIV and Mtb coinfection associated with AIDS. Indeed, HIV increases not only the likelihood that latent tuberculosis will reactivate within a patient, but also the chance that a person will be infected with Mtb from initial exposure.

HIV-1 induces disease progression and AIDS through a combination of CD4+ T cell depletion and chronic immune activation/inflammation. Together, these mechanisms of pathogenesis damage the innate and adaptive host immune responses, leaving the host vulnerable to infection by opportunist pathogens. In contrast to pathogenic HIV infections, immune cell subsets generally retain function during the nonpathogenic infection of SIV natural host African monkey species, such as sooty mangabeys (discussed in-depth in chapter 1). The maintenance of immune function in SIV-infected natural host species, like mangabeys, coincides with a low level of immune activation, which begins at the end of the acute phase and persists throughout the chronic phase of the SIV infection [28].

Monocytes are circulating macrophage and dendritic cell precursors that play a key role in the innate immune response. Although human monocytes are rarely found to be directly infected with HIV-1 [85], several of their functions, including phagocytosis, intracellular killing, and cytokine production become altered during host infection with HIV-1 [86, 87, 89, 91-93, 159]. One key aspect of monocyte function is the ability to contribute to the elimination of opportunistic or pathogenic bacteria such as *Mycobacteria* spp.

Mycobacteria can be divided into two broad subgroups: non-pathogenic (rapid-growing) or pathogenic/potentially pathogenic (generally slow-growing). For example, *M. smegmatis* is a common, rapidly growing environmental saprophyte that typically remains non-pathogenic to even immunocompromised persons. In contrast, *M. bovis* Bacillus Calmette-Guérin (BCG) is a

slow-growing *Mycobacterium* used as a live-attenuated vaccine against *Mycobacterium tuberculosis* (*Mtb*). While BCG rarely causes disease in healthy persons, it has been shown that HIV-infected infants are at an increased risk of disseminated BCG disease following BCG vaccination [160], thereby defining BCG as an opportunistic pathogen. In contrast, *Mtb* (also slow-growing) can readily infect even healthy people, and while the majority of these persons do not progress to disease, HIV+ persons are at an increased risk for both *Mtb* acquisition as well as *Mtb* reactivation from a latent state to active disease irrespective of CD4+ T cell count [70, 161]. Importantly, *Mycobacteria* infect cells of the monocyte/macrophage lineage, of which HIV has the ability to dramatically impact the function.

One important monocyte functional response to *Mycobacteria* includes the production of cytokines, two of the most important being TNFα and IL12. The pivotal role for TNFα in the formation and maintenance of *Mycobacteria* immune responses has been demonstrated through the increased *Mtb* susceptibility within both TNFα knockout mice and patients given TNFα-blocking regimens for the control of autoimmune diseases [162-164]. IL-12 plays a central role in skewing the differentiation of naïve CD4+ T cells toward a Th1 response that is necessary for responding to a Mycobacterial infection. Th1 responses are associated with the production IFNg by T and NK cells and are critical in monocyte and macrophage activation (reviewed in [165]). Furthermore, IL-12 receptor deficiency is associated with increased susceptibility to several types of *Mycobacteria* [166-168], including the opportunist *M. avium,* which is cleared following IL-12 replacement therapy in a mouse model of infection [169].

Here, we evaluate responses of purified PBMC or whole blood to *Mycobacteria spp.* that vary in pathogenesis to test the hypothesis that progressive HIV infection alters innate immune cell responses from HIV+ patients while non-progressive infection of SIV+ mangabeys does not. Through a combination of transcriptome analysis and intracellular cytokine staining, we found that the overall Mycobacterial response between humans and mangabeys was similar, indicating that the two species respond via comparable pathways. Transcriptome analysis

determined that HIV+ subjects exhibit a unique gene expression profile compared to healthy subjects following 4h BCG stimulation, while sooty mangabey gene expression profiles remain similar despite SIV infection. Intracellular cytokine staining revealed that whole blood monocytes from HIV+ subjects do not upregulate IL-12 protein production to the same levels as HIVneg donors, while SIV+ mangabeys maintain and even increase production in response to BCG. These studies demonstrate the utility of using model hosts of non-pathogenic SIV infection in comparative analyses to determine correlates of HIV pathogenesis and identify key differences in gene regulation between HIVneg and HIV+ subjects. In addition, these findings implicate the potential to use IL12 and other therapeutics to increase NK cell activity to combat Mycobacterial infections in HIV infected patients.

METHODS

Human Subjects

All protocols involving human subjects were approved by the Western Institutional Review Boards (IRB protocol #20092089) and written informed consent was obtained from all study participants. Blood from chronically-infected, ART-naive donors was obtained from the University of Washington Center for AIDS Research Clinic. The mean viral load for this cohort was 4,062 copies/mL and viral loads ranged from 50-64,000 copies/mL of plasma. The median CD4+ T cell count was 584 and CD4+ T cell counts ranged from 385-833 cells/uL of blood. Healthy human blood was collected via the Seattle BioMed blood draw program.

Sooty Mangabeys

All animal experimentation was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and under the approval of the Institutional Animal Care and Use Committees (IACUC) of the Yerkes National Primate Research Center in Atlanta, Ga (YNPRC). Mangabeys were either uninfected controls or naturally infected at the YNPRC.

Mycobacteria Culture and Preparation

M. smegmatis (MC155[2]), *M. bovis* BCG (Russia), or *M. tuberculosis* (H37Rv) was grown in 7H9+GAT media at 37°C with constant rolling for at least two doublings. Upon reaching log growth phase (OD_{600} 0.3-0.7), the bacteria were washed twice in 1x PBS. For PBMC stimulation, bacteria were resuspended in complete RPMI and added to the PBMC at a ratio of 1:1 in a final volume of 200ul. For whole blood experiments, the culture was resuspended to a final OD_{600} of 1.0 in PBS for each experiment, allowing for the same volume to be added to the blood for every experiment.

PBMC Stimulation for Nanostring

Whole PBMC extracted from a Ficoll gradient were resuspended in complete RPMI (RPMI1640+10%FBC+1%PSL) and enumerated via hemocyometer. One million cells were plated in triplicate in 96-well flat-bottom plate and rested 1h at 37°C+5% CO_2. $1x10^6$ CFU BCG (in complete RPMI) was then added in 100ul, bringing all final stimulation volumes to 200ul. Cells were then incubated 4 or 18 hours at 37°C+5% CO_2. Following incubation, cells were pelleted at 1800RPM, 100ul supernatant was collected and stored for future analysis, and PBMC from the triplicate reactions were pooled into Eppendorf tubes, pelleted at 2000 RPM, and the supernatant was discarded. 100ul lysis buffer (RLT; Qiagen) + Beta-mercaptoethanol was used to wash/lyse remaining (adherent) cells in the plates, and this was used to resuspend the corresponding pooled lymphocytes in the Eppendorf tubes. All supernatants and disrupted cell pellets were stored a -80°C until RNA extraction.

Measurement of Gene Expression Profile

mRNA isolation from stimulated PBMC was performed using the Qiagen RNeasy kit according to the manufacturer's protocol. Following isolation, mRNA was quantified and quality checked using a Nanodrop by measuring the $A_{260/280}$ and $A_{260/230}$ ratios prior to Nanostring analysis. Probes specific for 244 target genes related to cytokine and chemokine signaling, TLR signaling, antigen processing, in addition to NK cell-specific markers were designed (Figure 1), manufactured by Nanostring Technologies, and analyzed using the Nanostring nCounter analysis system as previously described [170]. Briefly, mRNA from 3 million PBMC were incubated overnight at 65°C. The samples were then loaded onto the Nanostring prep station to remove unbound probes and to align probes for reading using the Nanostring digital analyzer. Each sample was spiked with 6 internal positive controls and 8 internal negative controls. The probeset contained 3 housekeeping genes, which cover a wide range of expression levels, and these were used to control for total RNA input into the Nanostring analysis.

Statistical Analyses

Raw Nanostring gene expression data were normalized using the internal controls. The arithmetic mean of the internal the negative control conditions plus 2 standard deviations (which totaled 35 copies) was used as a cutoff for gene expression. Genes with $\leq$75% of the samples lower than 35 copies were removed from the data set. Gene expression was then normalized to the housekeeping control genes GAPDH, ALAS1, and HPRT. Differences in baseline gene expression between HIVneg and HIV+ donors as well as SIVneg and SIV+ mangabeys were identified as having a p-value of <0.05 and a false discovery rate (FDR) of <10% was used to control for multiple comparisons through Benjamini–Hochberg methodology. Differentially expressed genes (DEGs) following BCG stimulation for each group of subjects were identified as genes having a fold change >2 with a $p<0.05$ through a paired t-test (unstimulated vs. stimulated) and an FDR <10% (the cutoff for SIV+ sooty mangabeys included an adjusted $p<0.1$, FDR <10% as no DEGs were apparent with the more stringent cutoff). For the identified DEGs, KEGG pathway enrichment analysis from WebGestalt (Web-based Gene SeT AnaLysis Toolkit) was used to identify significantly enriched pathways [171]. Through this method, the hypergeometric test was used to calculate the statistic for enrichment of each pathway within the human genome (as the reference gene set), and the p-value was adjusted by Benjamini–Hochberg (BH) methodology. Suggested KEGG pathways with adjusted $p<0.05$ and a minimum of 7 DEGs were considered to be enriched.

Whole Blood Stimulation for Intracellular Cytokine Staining

Whole blood (100ul) from HIV+/- human donors or SIV+/- mangabeys was dispensed into 5mL round-bottom polypropylene FACS tubes. *M. smegmatis, M. bovis* BCG, or *M. tuberculosis* cells were added at a final concentration of 10^4 (MOI 1) or 10^5 (MOI 10) were used for to detect proinflammatory cytokine production. The number of bacteria for MOI determination was based on the average number of human leukocytes in 100ul/whole blood. The stimulations

were placed at 37°C + 5%CO_2 for 1h before BFA (0.2ug/mL) was added. After 6h total incubation, the stimulations were placed at 4°C O/N. Each experiment (including stimulation with *M. smegmatis M. bovis* BCG, or *M. tuberculosis*) included a positive (5ug/mL *E. coli* lipopolysaccharide, LPS) and a negative (unstimulated) control.

Intracellular Cytokine Staining

Stimulations were washed once in 1x PBS+2%FBS before extracellular antibodies were added at the following volumes: CD3 APC-Cy7 (clone SP34-2) or CD3 FITC (clone SP34): 5ul; CD14 PE-Cy7 (clone M5E2): 1ul; Live dead Aqua (1ul, Invitrogen). Samples were stained for a total of 30m on ice and washed in 1x PBS+2%FBS. Samples were vigorously resuspended in residual wash buffer before 750ul FACS juice (25% BD Bioscience FACS lyse + 0.05% Tween20 in dH_2O) was added for 10 minutes with occasional vortexing. The samples were then washed twice in 1x PBS+2%FBS before intracellular antibodies were added at the following volumes TNF-α (clone MAb11): 5ul; IL-12 PE (clone C8.6, Miltenyi Biotec): 5ul. The samples were then incubated for 30m on ice before a final wash in 1x PBS. Cells were resuspended in 100ul paraformaldehyde prior to collection on a 16-color BD LSRII using FACSDiva. All antibodies listed are from BD Bioscience unless otherwise stated.

Data analysis

All flow cytometry data were analyzed using FlowJo version 8.8.6. Statistical analyses were performed using R or GraphPad Prism version 5.

RESULTS

Cluster Analysis of Subjects Following BCG Stimulation and Identification of Differentially Expressed Genes

Nanostring analysis of select gene targets (figure 4-1) was performed on purified mRNA from PBMC following 4h BCG stimulation from 4 ART-naïve HIV+ patients, 4 HIVneg controls, 4 SIV+ mangabeys and 4 SIVneg controls.

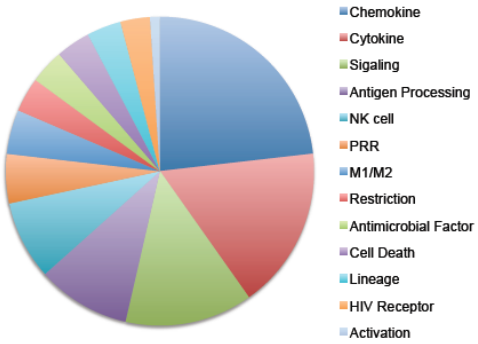

Figure 4-1. Categories of Gene Targets Used for Nanostring Analysis Before and After BCG Stimulation of HIVneg, HIV+, SIVneg, and SIV+ Subjects. Gene categories (and number in that category) included chemokines (45), cytokines (33), signaling (26), antigen processing (19), NK cell-specific (16), Pattern recognition receptors (PRR; 10), M1/M2 macrophage distribution (9), HIV restriction (7), antimicrobial defense (7), cell death (7), lineage-specific (7), HIV receptors (6), activation (2).

Genes for which ≥75% the samples for a given group (HIVneg, HIV+, SIVneg, SIV+) expressed higher levels than negative controls were used further for expression analysis (cutoff: mean +/- 2SD). A semi-supervised hierarchical clustering via principal component analysis (PCA) revealed that in all subjects (both humans and mangabeys), the effects of BCG stimulation are captured by the highest (human) or second highest (sooty mangabey) principal component for differential gene expression (Figure 4-1 a and b). Interestingly, while HIV status also contributes significantly to the pattern of gene clustering in humans, SIV status does not affect clustering to a similar extent (Figures 4-1 a and b).

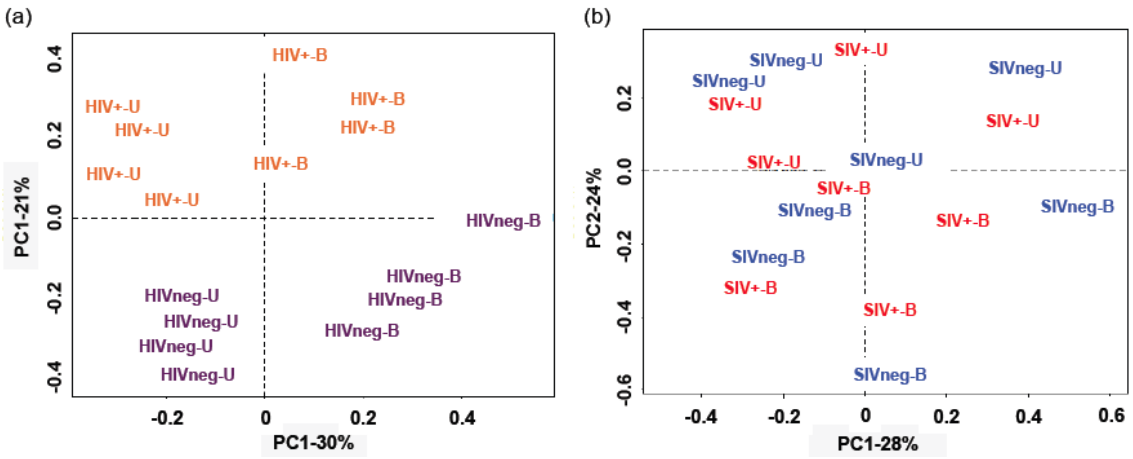

Figure 4-2. Principal Component Analysis of Expressed Gene Targets In Unstimulated and BCG-stimulated Samples. Genes with at least 75% of samples expressing a higher copy number than the negative cutoff (mean of negative controls +/-2SD) in humans (a) or sooty mangabeys (b) were used in a principal component analysis to determine patterns of gene clustering.

PCA demonstrated that even prior to BCG stimulation, HIVneg and HIV+ donor baseline gene expression formed distinct clusters (Figure 4-1a). We therefore measured gene expression in the unstimulated control groups from HIVneg and HIV+ donors as well as SIVneg and SIV+ mangabeys to gain insight into these baseline differences. Overall, 30 genes were differentially expressed between HIVneg and HIV+ donors at baseline, while 0 genes were differentially expressed between SIVneg and SIV+ mangabeys. The 30 different genes between HIVneg and HIV+ donors were submitted for KEGG analysis and found to be enriched in two main pathways: antigen presentation, including MHCI and MHCII processing pathways as well as cell-mediated cytotoxicity (Figure 4-3). While the genes in these two pathways were all expressed to higher levels in HIV+ donors compared to HIVneg (Figure 4-32), 5 genes including DEFA3, DEFB103A, LCN2, LTF, and SLC22A17 were expressed to higher levels in HIVneg donors.

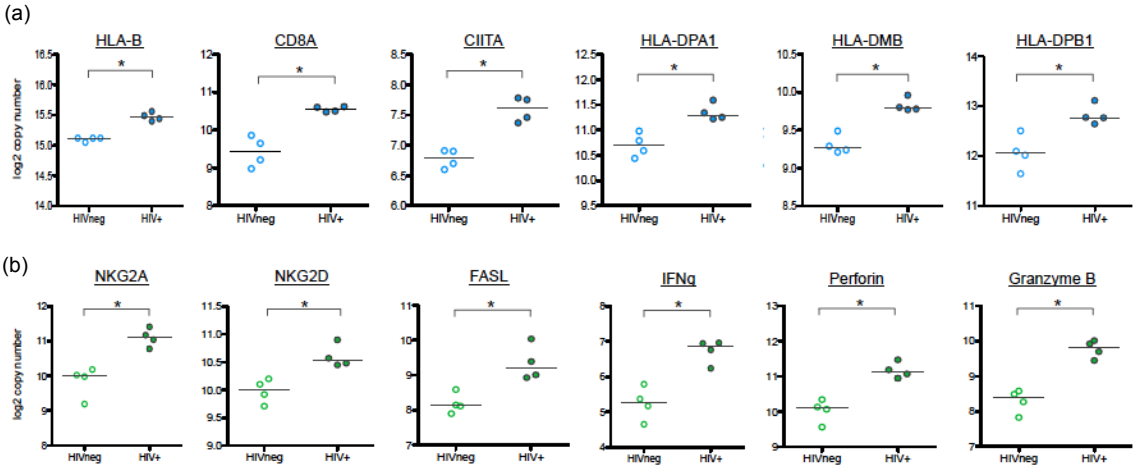

Figure 4-3. Differentially Expressed Genes in Unstimulated Samples From HIVneg and HIV+ Donors. Genes found to be differentially expressed were enriched in the (a) antigen processing and (b) cell-mediated cytotoxicity KEGG pathways.

Differentially Expressed Genes Following BCG Stimulation

Following BCG stimulation, differences in gene expression for each of the four subject groups were measured via pairwise comparison to unstimulated control samples. Differentially-expressed genes (DEGs) with $p<0.05$ and a false discovery rate of <10% were identified for each of the 4 groups. The number of DEGs for each group can be found in Table 4-1.

	HIVneg	HIV+	SIVneg	SIV+ (p<0.1)
Up	35	32	36	32
Down	10	7	6	5
Total	45	39	42	37

Table 4-1. Number of Differentially Expressed Genes Following BCG stimulation Compared to Unstimulated Controls. DEGs were defined as having an adjusted $p<0.05$ and a false discovery rate of <10%. Up: upregulated, down: downregulated

DEGs derived from the pairwise analysis were then used to determine enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (pathways that include at least 9 DEGs were considered to be "enriched"). As shown in Tables 4-2 and 4-3, similar pathways from both humans and mangabeys, were found to be significantly enriched, indicating that similar pathways are implicated in responding to BCG in both species and regardless of disease status. These included canonical immune response signaling pathways (TLR, NLR, chemokine), cytokine-cytokine receptor interaction pathways, pathways of inflammation (rheumatoid arthritis) and response to parasitic disease (malaria, Chagas disease, and African trypanosomiasis; not shown). Out of 10 enriched pathways in HIVneg subjects. 9 pathways from HIV+ subjects overlapped, including the canonical immune gene regulation and signaling pathways (Table 4-2).

		# Genes	
Gene Symbol	**Pathways Involved**	**HIVneg**	**HIV+**
IFNG, TNF, CCL7, CCL20, CCL2[♮], CXCL3, CXCL6, CCR2, CXCL2, IL1A[♮], IL23A, IL1B, IL12B, CXCL5, CXCL9[♮], CCL24, TNFRSF9[♮], IL6, IL8, CCL4[♮], CXCL1, IL2RA[♮], CCL3[♮], IFNGR1[♮], CXCR1, CCL18, CSFR1R[♮], CCL23*, IL18*, CCL8*	Cytokine-Cytokine Receptor Interactions	27	21
CXCL5, IFNG, TNF, TLR2[♮], CCL20, CCL2[♮], IL6, IL8, CXCL6, CXCL1, CCL3[♮], IL1A[♮], IL23A, IL1B, ICAM1, IL18*	Rheumatoid arthritis	15	12
CCL7, CCL20, CCL2[♮], CXCL3, CXCL6, CCR2, CXCL2, CXCL9[♮], CXCL5, CCL24, NFKB1, CCL4[♮], IL8, CCL1[♮], CCL3[♮], CCL18, CXCR1, CCL23*, CCL8*, CXCL1*	Chemokine Signaling	17	18
TNF, CXCL9[♮], TLR2[♮], IL6, IL8, CCL4[♮], NFKB1, CCL3[♮], IL1B, TLR6, TLR1, IL12B	TLR Signaling Pathway	12	10
TNF, NLRP3, CCL2[♮], IL6, IL8, NFKB, CXCL1, CXCL2, IL1B, IL18*	NLR Signaling Pathway	9	10

Table 4-2. Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Analysis of Differentially Expressed Human Targets. Human gene targets that were found to be differentially expressed upon BCG stimulation (fold change >2; pairwaise t-test adjusted $p<0.05$) were used to determine pathway enrichement. [♮]upregulated only in HIVneg donors, * upregulated only in HIV+ donors.

All of the 10 enriched pathways in SIVneg sooty mangabeys were also enriched in SIV+ sooty mangabeys (Table 4-3). These pathways were identical to pathways enriched in human PBMC and included canonical signaling, inflammation pathways as well as pathways known to be enriched during parasitic infection (not shown).

		# Genes	
Gene Symbol	**Pathways Involved**	**SIVneg**	**SIV+**
IFNG, TNF, CCL20, CCL2, CXCL3, CCR2, CXCL2, IL22[♭], IL1A, IL1B, IL23A, IL12B, CCL24, IL6, CCL14, IL8, CCL4, CXCL1, CCL22, IL2RA, CCL3, CCL1, CSF1R, CXCL6[#]	Cytokine-Cytokine Receptor Interactions	23	21
IFNG, TNF, TLR2, CCL20, CCL2[♭], IL6, IL8, CXCL1, CCL3, IL1A, IL1B, IL23A, ICAM1[♭], CXCL6[#]	Rheumatoid arthritis	13	12
CCL24, CCL20, CCL2[♭], CXCL3, CCL14, IL8, CCL4, NFKB1, CCR2, CXCL1, CCL22, CXCL2, CCL3, CCL1, CXCL6[#]	Chemokine Signaling	14	14
TNF, TLR2, IL6, IL8, CCL4, NFKB1, CCL3, CD14, IL1B, IL12B, TLR8	TLR Signaling Pathway	11	11
TNF, TNFAIP3, CCL2[♭], IL6, IL8, NFKB1, CXCL1, CXCL2, IL1B, CASP1[♭]	NLR Signaling Pathway	10	8

Table 4-3. Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Analysis of Differentially Expressed Sooty Mangabey Targets. Sooty mangabey gene targets determined to be differentially expressed upon BCG stimulation (fold change >2; pairwise t-test adjusted $p<0.05$) were used to determine pathway enrichement. [♭] upregulated only in SIVneg mangabeys, [#] upregulated only in SIV+ mangabeys.

Despite differences in the number of genes implicated in the KEGG pathways between HIVneg and HIV+ as well as SIVneg and SIV+ mangabeys, the change in gene expression following BCG stimulation was overall found to be similar between the groups. For example, genes involved TLR-mediated recognition of *Mycobacteria*, including TLR2 were found to be significantly upregulated across all the groups, suggesting that the stimulation was effective for all subjects tested. Interestingly, while humans expressed significantly lower levels of TLR6 expression upon BCG stimulation, sooty mangabeys did not (Figure 4-2 a, second panel from left and b, second panel from left), suggesting a species-specific difference in response to BCG. Depite this difference, however, sooty mangabeys still demonstrated significant upregulation of genes downstream of TLR recognition, such as NFkB and IL-6.

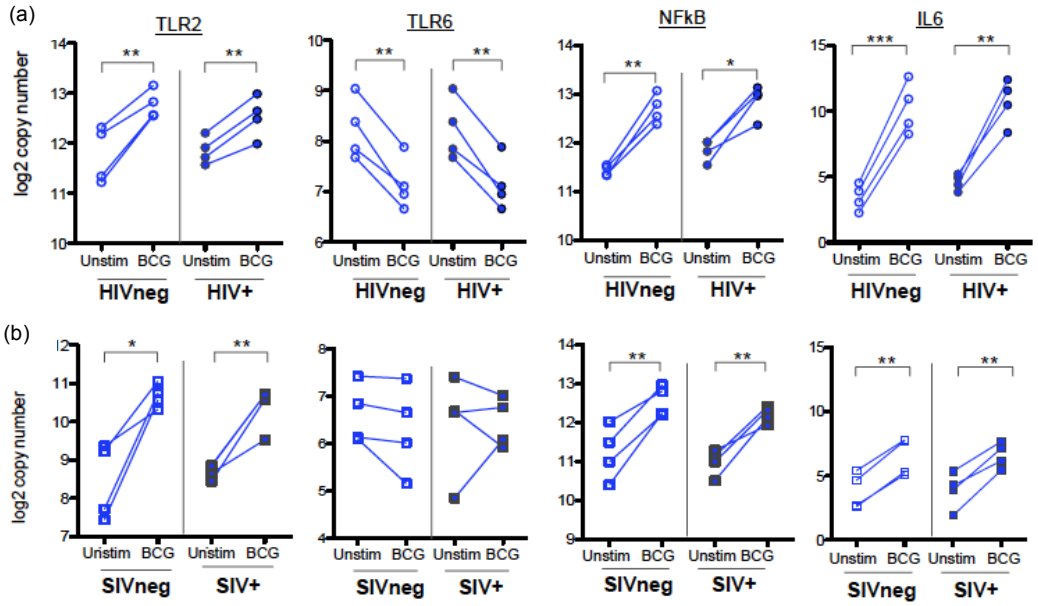

Figure 4-4. TLR Pathway Regulation Following Stimulation With BCG in Humans and Sooty Mangabeys. Nanostring analysis of gene expression in whole PBMC revealed differential regulation of several TLR pthway-related genes in both humans (a) and sooty mangabeys (b) following BCG stimulation. *p<0.05, **p<0.01, p<0.005 paired t-test.

Despite the large percentage of genes found to be similar among all groups, genes specific for NK cell activation, including activating KIRs (the probe for this gene set was specific for the following KIRs, which display a degree of homology too high to be eparately identified: KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR3DS1), NKG2D, NKp46, and the effector moelcule perforin became significantly down-regulated upon BCG stimulation in healthy donors, while their expression levels remain unchanged in HIV+ donors (figure 4-5).

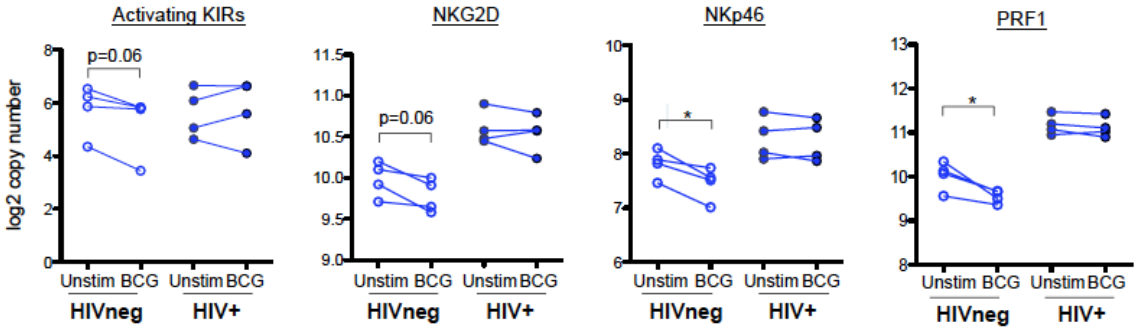

Figure 4-5. NK Cell Response to BCG in HIVneg and HIV+ Subjects. Nanostring analysis of NK Cell gene expression following BCG stimulation of whole PBMC from HIVneg and HIV+ donors. (*p<0.05, paired t-test).

Proinflammatory Cytokine Response Associates with Pathogenic Potential of Mycobacteria in Humans and Sooty Mangabeys

We also performed an analysis of proinflammatory cytokine production of monocytes from chronically HIV- and SIV-infected subjects following stimulation with fully pathogenic *M. tuberculosis,* opportunistic BCG, and non-pathogenic *M. smegmatis* to analyze the responses of each species to *Mycobacteria* of varying pathogenic potential. We found a remarkably consistent trend of suppressed proinflammatory cytokine production from both humans and mangabeys, regardless of infection status (HIV-negative and HIV+ or SIV-negative and SIV+) following 6h stimulations with increasingly pathogenic *Mycobacteria* at both MOIs tested (Figure 4-6 d-f). Despite this overall trend, however, the proportion of TNFα+ monocytes from human subjects was similar in response to *M. tuberculosis* compared to BCG, while mangabeys exhibited a 10-fold decrease (MOI1; medians=1.8 for BCG and 0.19 for Mtb; p=0.006) or 17-fold (MOI 10; medians=9 for BCG and 0.53 for Mtb; p=0.004) following stimulation with *M tuberculosis* compared to BCG (Figures 4-6 c and d).

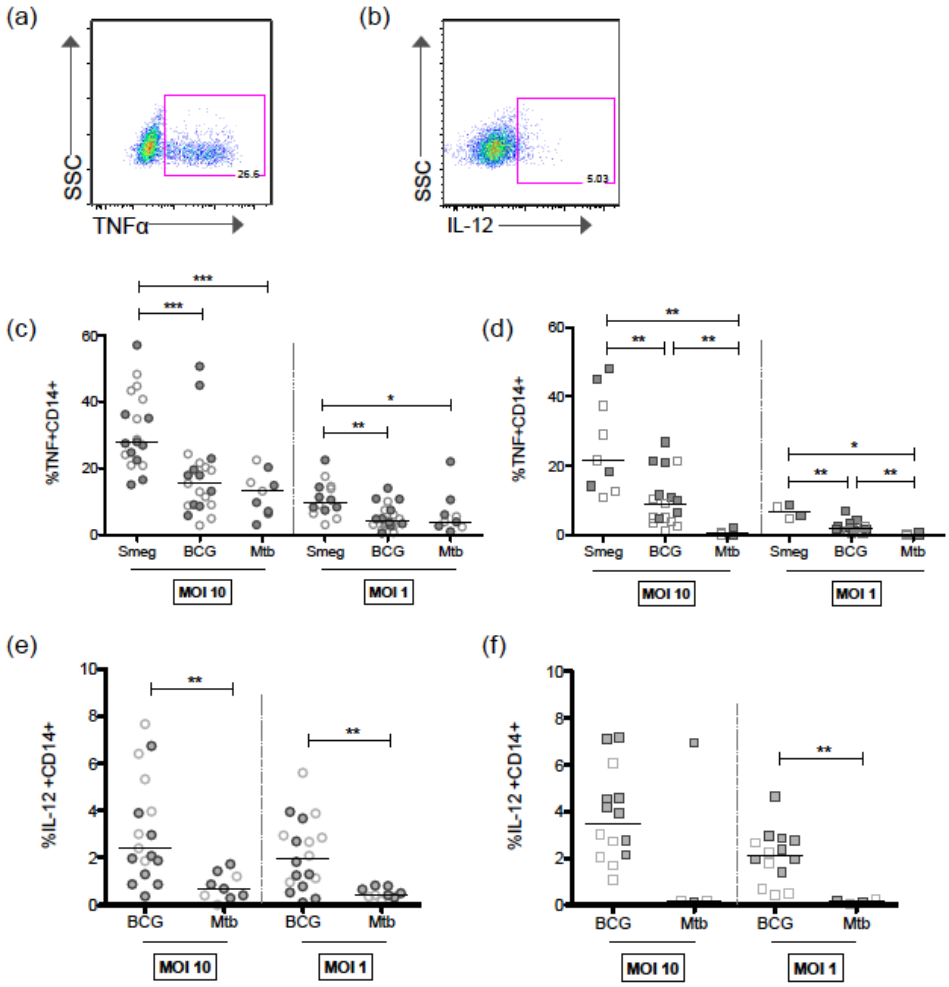

Figure 4-6. Percentage of Inflammatory Cytokine-secreting Monocytes Decreases in Response to Increasingly Pathogenic *Mycobacteria* in Humans and Sooty Mangabeys. Representative flow plots of the percentage of TNFα+ (a), or IL-12+ (b), monocytes following BCG stimulation. TNFα (c, d) or IL-12 (e, f) production from humans (c, e) or sooty mangabeys (d, f) at MOI 10 or MOI 1 following stimulation with *M. smegmatis, M. bovis* BCG, or *M. tuberculosis*. Lines represent the median for each group (*p<0.05, **p<0.01 Mann-Whitney).

Proinflammatory Cytokine Response to Mycobacteria Stimulation in HIV/SIV Infected humans and mangabeys

Chronic HIV infection can result in impaired monocyte proinflammatory cytokine production [90-92]. Our assessment of nonpathogenic *M.smeg* stimulation in ART-naïve HIV+ donors determined that monocyte TNFα production was similar to uninfected patients at MOI 10, (Fig. 4-7a), MOI 1 (data not shown) and the unstimulated controls. SIV+ mangabeys also exhibited a similar proportion of TNFα producing monocytes compared to uninfected mangabeys in response to *M. smegmatis* at MOI 10 (Figure 4-7a).

BCG has potential to become pathogenic [73, 160, 172] and was evaluated as an opportunistic pathogen with regard to monocyte proinflammatory cytokine production. We observed a similar percentage of TNFα-producing monocytes in response to *M. bovis* BCG between HIV-negative and HIV+ individuals at MOI 10 (Figure 4-7a) and MOI 1 (not shown). In contrast, SIV+ mangabeys exhibited an increased frequency of TNFα-positive monocytes at MOI 10 (median=11.2%) compared to SIV-negative mangabeys (median=4.9; p=0.03) and MOI 1 (SIV+=2.1; SIV-negative=1.1; p=0.02; not shown). Therefore, SIV infection of mangabeys was associated with nearly double the percentage of TNFα-producing monocytes in response to BCG, indicating a distinct TNFα response in the context of a nonpathogenic SIV infection.

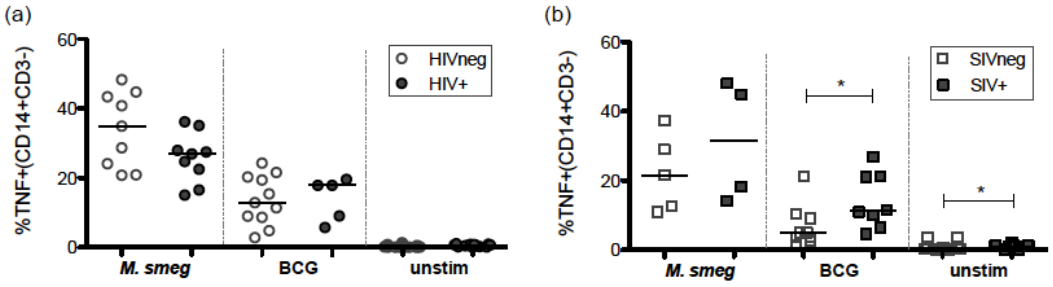

Figure 4-7. Percentage TNF-α -producing blood monocytes after 6h stimulation with *M. smegmatis* or *M. bovis* BCG. IL-12 production by monocytes in uninfected or ART-naive, HIV+ humans (a) or uninfected and SIV+ sooty mangabeys (b). Black lines represent the median of each group (*p<0.05; Mann-Whitney).

While monocyte IL-12 production was similar both basally and in response to stimulation with LPS among HIV-negative and HIV+ subjects, we found that HIV+ subjects had decreased proportion of IL-12 producing monocytes (median=1.9%) compared to uninfected subjects (median=4%) at MOI10 (p=0.017; Figure 4-8a). However, this decrease was not seen at MOI1. In contrast to the decreased proportion of IL-12-producing monocytes in HIV+ subjects, SIV+ mangabeys displayed a clear trend toward an increased proportion of IL-12+ monocytes in response to BCG at MOI 10, although this did not reach statistical significance (p=0.059; Figure 4-8b). SIV+ mangabeys also displayed a trend toward an increased percentage of IL-12-producing monocytes basally (median=2.5%) compared to SIV-negative mangabeys (median=0.72%; p=0.08) but similar proportions following stimulation with LPS, suggesting that the observed increase may be specific to *Mycobacteria* stimulation.

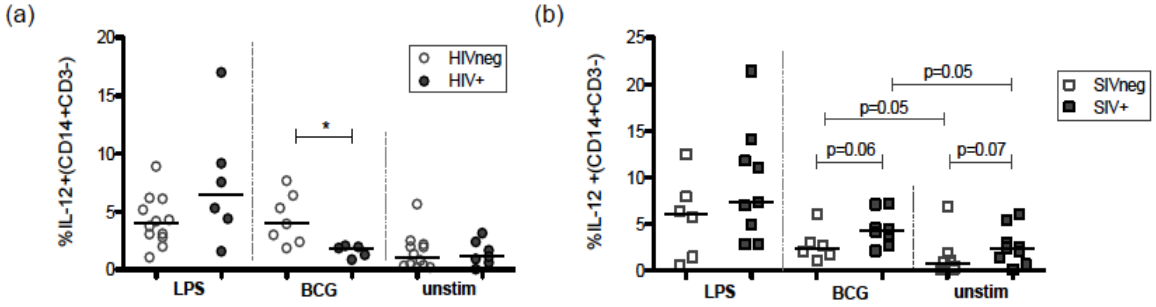

Figure 4-8. Percentage IL-12 -producing blood monocytes after 6h stimulation with *M. bovis* BCG or LPS. IL-12 production by monocytes in uninfected or HIV+ humans (a) or uninfected and SIV+ sooty mangabeys (b). Black lines represent the median of each group (*p<0.05; Mann-Whitney).

DISCUSSION

In contrast to pathogenic HIV infections, African primate hosts of non-pathogenic SIV infections undergo a disease course that does not typically result in AIDS or the acquisition of opportunistic infections despite persistent viremia [19, 173-177]. Here we performed a comparative analysis of the immune responses during these differentially pathogenic infections to identify correlates of immunologic signatures that are associated with non-pathogenic SIV infections. Overall, our data demonstrate that 1) both humans and mangabeys mount a similar proinflammatory response to *Mycobacteria* defined by differential gene expression and pathway enrichment analysis 2) HIV-infected donors exhibit distinct gene expression patterns following BCG stimulation that discriminates them from uninfected donors, and these differences are characterized primarily by differential expression of genes associated with NK cell activation, 3) SIV+ mangabeys have similar gene expression profiles to their uninfected counterparts following BCG stimulation, and 4) monocytes from whole blood of HIV+ donors exhibit a similar or reduced proportion of proinflammatory cytokine-secreting monocytes (TNFα and IL12) following whole blood stimulation with BCG compared to uninfected donors, while sooty mangabeys exhibit an increased percentage of proinflammatory cytokine-secreting monocytes compared to uninfected mangabeys.

The analyses undertaken focused on assessing within-species differences (i.e. HIV+ compared to uninfected humans or SIV+ compared to uninfected mangabeys), so that assay-specific variation between human and mangabey samples would not impact our findings. However, it was first important to ensure that mangabeys and humans exhibited an overall similar response to *Mycobacteria*, as this would strengthen our confidence that we can identify correlates of immunopathogenesis in these two systems. Our transcriptome and enriched pathway analysis indicated that overall, mangabeys mount a comparable response to *Mycobacteria* compared to humans. First, BCG stimulation emerged as the first (humans) or second (mangabeys) principal component of gene clustering in a semi-supervised cluster

analysis (Figure 4-1). Second, humans and mangabeys show a similar proportion of DEGs (Table 4-1) and similar enriched pathway modulation following stimulation with BCG (Tables 4-2 and 4-3). While the selected genes from which the transcriptomics data is largely enriched for immune response genes, it clearly demonstrates that a robust proinflammatory immune response is mounted by hosts of both species, regardless of HIV or SIV status. Further, as shown in figure 4-6, monocyte proinflammatory cytokine production negatively correlates with the level of *Mycobacteria* pathogenesis in both species. Together, these data suggest that sooty mangabeys qualitatively and quantitatively respond to *Mycobacteria* to a similar extent as humans. This data is consistent with two documented cases of mycobacteriosis in SIV-infected natural hosts demonstrate that very rarely, these non-progressing hosts (an African Green Monkey and a sooty mangabey) may suffer from disseminated Mycobacterial disease, further supporting the notion that these non-progressors may also serve as hosts for *Mycobacteria* [178].

Principal component analysis demonstrates that HIV infection is a key driver of gene clustering of mRNA from whole PBMC (in both stimulated and unstimulated conditions), while SIV infection is not. This observation suggests more similar gene regulation between SIVneg and SIV+ mangabeys (in both stimulated and unstimulated controls) than between HIVneg and HIV+ subjects. While our Nanostring analysis is limited in the number of samples for each condition, which lowers the power to be able to detect smaller changes in gene regulation between the groups, using stringent cutoff methods (adjusted $p<0.05$; FDR <10%) to define DEGs likely enriched our data with true DEGs. The overlap of DEGs between all groups, including even SIV+ sooty mangabeys, in which a relaxed p-value cutoff was used to determine DEG status, further supports the notion that our analysis identified true DEGs.

In order to better understand differences between HIVneg and HIV+ donors encompassed by PCA, we performed an analysis of baseline gene expression (unstimulated control samples) between HIVneg and HIV+ donors. Through this analysis, we observed higher

expression of several genes that were found to be enriched within antigen processing or cell-mediated toxicity pathways in HIV+ donors (Figure 4-3). Together, these data suggest the presence of a higher level of antigen processing and cellular activity consistent with increased immune activation in HIV+ donors. Interestingly, despite a similar overall proinflammatory response to BCG by both species regardless of disease status (Figure 4-4), HIV+ donors had a specific maintenance of gene expression associated with NK cell activation compared to HIVneg donors, which displayed a down-modulation of genes related to NK cell activation. This result was initially surprising due to the probable importance of NK cells in mediating responsiveness to BCG. However, a recently published paper demonstrated a physiological response of NK cells from healthy donors to BCG characterized by apoptosis of activated, IFNg-producing NK cells [179]. Our data showing a down-modulation of gene expression of activated NK cells is consistent with this finding, and suggests that this activation-induced apoptosis of NK cells in response to BCG is not occurring in HIV+ donors (where there was no change in mRNA expression). Further studies are warranted to examine the functional consequences of the lack of response by NK cells to BCG in our study and are described in chapter 5 of this dissertation.

Due to the relative difficulty of delineating monocyte functional responses in whole PBMC, we analyzed whole blood monocyte responses following stimulation with environmental *M. smegmatis,* opportunist BCG, or fully pathogenic *M. tuberculosis* to evaluate specifically the role of monocytes in mediating the inflammatory response to *Mycobacteria.* Using whole blood for these *ex-vivo* analyses provides monocytes with the most physiologically relevant environment for responding to the bacteria. Through this system, we observed a significant increase in whole blood monocyte proinflammatory cytokine production following stimulation with BCG with decreasing pathogenicity of the *Mycobacteria* (Figure 4-6 a and b). Increased proinflammatory cytokine responses by non-pathogenic *Mycobacteria* has been previously documented in tissue macrophages, and is thought to be due in part to the phospho-myo-inositol modification of the cell wall component lipoarabinomannan compared to the mannosyl

modification of lipoarabinomannan found in pathogenic *Mycobacteria* [180]. However, our results for the first time to our knowledge, demonstrate this suppressed proinflammatory cytokine production in whole blood monocytes as soon as 6h after stimulation.

Despite a slight increase in the level of TNFα+ monocytes in uninfected mangabeys in unstimulated control (Figure 4-7b), we found that SIV+ mangabeys not only compensate for, but overcome for this difference, and exhibit a two-fold increase in TNFα producing monocytes in response to *M. bovis* BCG at MOI10, but not to non-pathogenic *M. smegmatis* or LPS. This may suggest an enhanced ability of SIV+ mangabeys to respond to potentially opportunistic *Mycobacteria* compared to their uninfected counterparts. HIV+ donors maintained similar TNFα production following stimulation with BCG compared to their uninfected counterparts. In contrast, HIV+ donors displayed a limited ability to upregulate IL-12 in response to BCG, leading to a significantly lower IL-12 production in response to BCG at MOI10 compared to uninfected donors. Because IL-12 is critical in guiding the immune response to an intracellular pathogen like *Mycobacteria*, largely through stimulating the production of IFNg from NK cells resulting in macrophage activation (reviewed in [165]), the observed difference in IL-12 response to an opportunistic *Mycobacteria* as early as 6h after exposure may represent one correlate of increased susceptibility to opportunistic infections in HIV+ patients.

Our data suggest that the ability to mount a robust, monocyte-mediated IL-12 response is protective against opportunistic *Mycobacteria* in SIV+ mangabeys. Further, because IL-12 is important for NK cell activation, this may provide the help necessary for NK cells from HIV+ donors to become activated to produce cytokines prior to undergoing activation-induced cell death, as has been described for healthy donors [179]. To this end, administering IL-12 to *Mycobacteria*-infected HIV+ patients may potentially help to “kick-start” the immune response needed to clear the pathogen. However, as IL-12 can activate CD4+ T cells, providing a larger reservoir for HIV, this type of therapy would be best reserved for virally suppressed patients (through successful ART treatment). Because Mtb infections are a significant contributing factor

to HIV-related deaths even following ART [181], therapies aimed at restoring immune function during ART are warranted. Indeed, cytokine therapy (IL-7) in combination with ART has been implicated in enhanced T cell recovery in HIV+ patients [182] and provides a good example of the potential utility of cytokine therapy. However, further research including the proper timing and dosage of IL-12, as well as the efficacy and safety are needed before patient treatment can be considered.

Overall, our data suggest the potential of dysfunctional synergy in innate immune cell (monocyte and NK cell) response to *Mycobacteria.* Thus, attempting to augment innate cell functionality in the face of invading pathogens exhibits potential in further improving immune cell health in HIV+ patients. The work described here has the potential to complement ongoing vaccine research through the discovery of novel immune therapeutics aimed at enhancing innate immunity and, ultimately, by lowering the risk of opportunistic infection incidence in HIV+ patients.

CHAPTER 5:

MONOCYTE AND NK CELL FUNCTIONAL INTERACTIONS IN THE CONTEXT OF CHRONIC HIV-1 INFECTION

OVERVIEW

This chapter describes an ongoing study aiming to design a novel, flow cytometry-based assay to assess the functional interactions of two important innate immune cells, NK cells and monocytes, in response to *Mycobacteria* during chronic HIV-1 infection. The rationale for this study is based in part on data generated in chapter 4 showing that whole blood monocytes from HIV+ donors do not upregulate IL-12, an important cytokine for NK cell activation, in response to opportunist BCG, while monocytes from healthy donors do. For these studies, we first assessed each cell type individually in order to better understand their baseline functional characteristics in our Seattle-based cohort. Preliminary data shows that while the phenotype of monocytes from HIV+ patients is skewed toward an increase in CD14+CD16+ inflammatory monocytes compared to healthy patients, monocyte phagocytic capacity of both opportunist BCG and pathogenic *Mtb* remains intact. Interestingly, monocyte phagocytosis is highly correlated with HIV+ patient viral load, suggesting that HIV may impact monocyte phagocytosis. Killing of target K562 cells by NK cells from HIV+ patients was also impaired compared to that of healthy donors, suggesting that chronic HIV infection impacts both monocyte and NK cell functionality. Because our Seattle-based HIV+ cohort is relatively healthy (based on CD4 count), we predict that the effects of HIV pathogenesis are even more pronounced in patients with low CD4 counts. Ultimately, these data yield useful preliminary data on which to build an assay to measure the functional interactions of innate immune cells during chronic HIV infection.

INTRODUCTION

Innate immunity is critical not only to initiating a timely defense against pathogens but also to influencing the development of a quality adaptive immune response. Chronic HIV infection and its associated pathologies, however, can impact the functional capacity of numerous innate cells, including those that become directly infected, such as dendritic cells, as well that those that are relatively resistant to direct viral infection, like monocytes, NK cells, and neutrophils. This impairment of innate immunity during HIV-1 infection is likely a key factor in increased susceptibility to pathogens, and the subsequent inability to contain or clear infection, ultimately leading to the opportunistic infections that define AIDS.

Data presented earlier in this dissertation (chapter 4) revealed several differentially regulated genes following BCG stimulation of PBMC from HIV+ subjects compared to controls. Interestingly, several of these genes are involved in inflammatory pathways characteristic of monocyte activation as well as NK cell-specific genes. Additionally, upregulation of the production of IL-12, a cytokine important for activating NK cells, by whole blood monocytes was impaired in HIV+ subjects compared to healthy donors. These data, together with previously published reports, support a role for monocytes and NK cells in response to and the control of *Mycobacteria,* as well as a functional alteration of these cellular responses during chronic HIV infection.

Blood monocytes are typically divided into two groups based on the surface expression of CD14 and CD16: CD14++CD16- and CD14+CD16+. While both groups are exquisite producers of potent, proinflammatory cytokines, like TNFα, IL-12 and IL-6, CD14+CD16+ monocytes are less capable of producing anti-inflammatory cytokines, like IL10, and are therefore denoted as “proinflammatory” [183]. Increased proportions of CD14+CD16+ monocytes have been described in several autoimmune diseases, such as lupus and rheumatoid arthritis, as well as in HIV-associated disease [184-187]. In addition to and perhaps

as a consequence of phenotypic alterations, monocyte effector functions critical for their response to pathogens including *Mycobacteria*, such as cytokine production, phagocytosis, and antigen presentation become dysregulated during HIV pathogenesis. These dysfunctions which likely have a negative consequence regarding ability of monocytes to respond to pathogens as well as to interact with other cells [188-192].

NK cells, like monocytes, function as a part of the innate immune system. NK cells in blood can be identified through the lack of lineage markers (CD3, CD14, and CD19) as well as expression of CD56 and/or CD16. NK cells are exquisite producers of IFNγ, a cytokine responsible for activating monocytes and granulocytes to carry out killing of intracellular microbes via reactive intermediates [193]. Unlike their CD8+ T cell counterparts, NK cells are able to secrete IFNγ prior to the establishment of immune memory, and therefore are likely the major source of IFNγ immediately following pathogen infection. While currently little is known about NK cell responses to *Mycobacteria*, they have been shown to respond to *Mycobacteria* through TLR 2 stimulation [103], and to localize to the site of *Mycobacteria* infection *in vivo* [109, 110]. Together, these data suggest that NK cells are an important driver of the initial immune response to *Mycobacterial* invasion.

Available data suggests a role for monocyte and NK cell interactions in controlling Mycobacteria infections. For example, NK cell-derived IL-22 contributes to monocyte phagolysosome fusion, ultimately inhibiting intracellular mycobacterial growth [194]. Further, NK cells can lyse *Mycobacteria*-infected monocytes, primarily through interactions between activating receptors NKp46 and NKG2D expressed on NK cells and the stress-induced ligand for NKG2D, ULBP1, on infected monocytes [195]. A pictorial representation of known monocyte and NK cellular interactions can be found in chapter one of this dissertation (Figure 1-5). While these studies provide evidence that monocytes and NK cells can work together in clearing

Mycobacteria infections, little is known regarding how these anti-Mycobacterial interactions are altered during pathogenic HIV-1 infection.

Because monocytes and NK cells each play a role in mediating the very early events following *Mycobacteria* infection, we hypothesized that a resulting "dysfunctional synergy" of these cell types occurs during chronic HIV-1 infection. Further, we speculated that this synergy contributes to the increased incidence and severe pathogenic effects associated with HIV/TB coinfection. To test this hypothesis, we designed a flow cytometric assay to directly measure the ability of NK cells to kill autologous *Mycobacteria*-infected monocytes. The results described herein provide the foundation to perform a comprehensive analysis of the effect of HIV-1 on monocyte and NK cell crosstalk.

MATERIALS AND METHODS

Detailed information regarding human subjects protocol, PBMC isolation, and *Mycobacteria* culture can be found in Chapter 4.

Monocyte Isolation

Monocytes were separated from whole PBMC via magnetic bead isolation according to the manufacturer's protocol (Miltenyi). Briefly, PBMC were resuspended in bead buffer (PBS + 2% BSA + 0.5mM EDTA) at a concentration of 10^7cells/80ul. Anti-human-CD14-labeled microbeads were added at a concentration of 20ul/10^7 cells for 15 minutes. Following the incubation, cells were washed with 8 mL bead buffer and pelleted at 1500 RPM for 10 minutes. Cells were then resuspended in 500ul bead buffer, and added to a preconditioned (with 3 x 3mL with bead buffer) Miltenyi LS column in a magnetic separator. Columns with bound bead-cell complexes were then washed 3x with 3mL bead buffer. The unlabeled (CD14-) flow-through fraction was collected for later NK cell isolation (see below). Columns were then removed from the separators and placed in a 15mL conical (Falcon) for collection. Magnetically-labeled CD14+ cells were mechanically detached from the columns into 5mL bead buffer through use of a plunger. Cells were then pelleted at 1500 RPM, counted, and resuspended to a final concentration of 1x10^6 cells/mL in cold RPMI1640. Cells were plated in either 5mL polystyrene FACS tubes (350ul) or 96-well (100ul) tissue culture plates for 2h at 37°C prior to *Mycobacteria* infection, to allow the cells to rest and attach. Monocyte purity was assessed via flow cytometry and monocytes were consistently determined to be greater than 93% pure (as measured by CD14 expression),

NK Cell Isolation

Flow-through (CD14- cells) from the monocyte isolation was enriched for NK cells using the StemCell NK cell enrichment kit, according to the manufacturer's instructions. Briefly, cells were resuspended in bead buffer to a final concentration of $5x10^7$ cells/mL in a polystyrene FACS tube (12x75mm; Falcoln) before addition of the EasySep™ Human NK Cell Enrichment Cocktail (50 µL/mL cells) at room temperature for 10 minutes. EasySep Magnetic Particles were then added (100 µL/mL cell) to the reaction, which was then mixed well and incubated at room temperature for 5 minutes. Following the incubation, the cell suspension was brought up to a total volume of 2.5 mL in bead buffer. The cells were then mixed by gently pipetting up and down and then placed into an EasySep magnet for 2.5 minutes. The EasySep magnet (and tube containing cell suspension) was inverted, and the non-labeled fraction (NK cells) were poured off into a new 5 mL polystyrene FACS tube. Cells were then pelleted at 1500 RPM for 5m, resuspended in 500ul complete RPMI, and incubated at 37degC until use in killing assay. NK cell purity was assessed via flow cytometry and was found to be approximately 85-90% purity as measured by the expression of CD16 and/or CD56.

Monocyte and NK Cell Phenotyping

Monocyte and NK cell baseline phenotypes were assessed via flow cytometry immediately following isolation. For this, cells were washed 1x in 2%FBS in PBS and stained with a cocktail of antibodies for 30m on ice. Dead cells were identified using 50ul LIVE/DEAD Fixable Aqua Dead Cell Stain (diluted 1:500 in PBS; Invitrogen). Monocytes were co-stained with 1ul anti-CD14 PE-Cy7 (clone M5E2) and 1ul anti-CD16 FITC (clone 3G8). NK cells were co-stained with 0.5ul anti-CD56 PE-Cy7 (clone NCAM16.2) and 1ul and anti-CD16 FITC (clone 3G8). All antibodies were obtained from BD Biosceinces unless otherwise noted.

Monocyte Phagocytosis Assay

Following isolation, monocytes were incubated for 2h prior to infection with a fluorescent (mCherry) reporter strain of either BCG (strain: Russia) or *M. tuberculosis* (strain: H37Rvs) in RPMI+20% FBS in a volume equal to that used for initial plating (yielding RPMI+10%FBS final concentration). *Mycobacteria* were enumerated based on optical density at a wavelength of 600nm and added at an MOI (multiplicity of infection) of 10 (10 bacteria per monocyte) for 6h. Following the infection period, FACS tubes were gently shaken, media was decanted, and the adherent monocytes were washed a total of 3x with fresh complete RPMI (+10% FBS+1%Penicillin/Streptomycin/L-Glutamine). Infected monocytes were incubated for 18h longer, for a total of 24h prior to dissociation from the FACS tubes using ice cold EDTA (5mM) and vigorous pipetting up and down. Monocytes were then pelleted at 1500RPM for 10 minutes, the supernatant was decanted, and cells were resuspended and washed once more in PBS+2mM ice cold EDTA. Monocytes were then stained with the cocktail of markers described above for 30m on ice. Cells were then washed 1x and fixed for 2h in 2% paraformaldehyde prior to analysis using a BD LSRII. Monocyte phagocytosis was ultimately measured by the percentage of cells that stained positive for mCherry BCG (PE channel).

K562 Cell Culture and Label With Claret Dye

K562 cells were obtained as a gift from the laboratory of Dr. Helen Horton. Cells were maintained in complete RPMI and passaged every other day until use. In order to discriminate K562 cells from NK cells during flow cytometric analysis, K562 cells were pre-labeled with CellVue Claret Far Red Fluorescent Cell Linker Kit for cell membrane labeling (Sigma) according to the manufacturer's directions. Briefly, K562 cells were pelleted, resuspended in serum-free RPMI, and enumerated via hemocytometer. Cells were again pelleted and resuspended in 1mL Diluent C buffer (Sigma kit). This cell suspension was subsequently added to a 15mL conical tube containing another 1mL of diluent C and claret dye. Following a 4

minute incubation, an equal volume of serum was added to the reaction for quenching. Cells were then washed three times in complete RPMI prior to being added to cultured NK cells.

NK Cell Killing Assay

Following O/N culture, NK cells were enumerated and resuspended to a concentration of $5x10^6$cells/mL, and 100ul ($5x10^5$ cells) were added to a 96-well round bottom tissue culture treated plate. Freshly washed and claret-labeled K562 cells in complete RPMI were added to the NK cells at an E:T (NK:K562) of 8:1, 4:1, and 1:1 for 4h in a final volume of 200ul. When the number of NK cells permitted, cocultures were performed in triplicate. NK and K562 cells were cultured together for 4h at 37°C+5%CO_2. K562 cells cultured alone were used as a negative control to assess background/spontaneous cell death. Upon harvest, cells were pipetted up and down 4-5 times and transferred to FACS tubes for multicolor flow cytometric analysis. K562 viability was assessed by the percentage of cells that had incorporated the LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen). NK cell killing was calculated via the following calculation:

Mean percentage of dead K562 cells (K562+NK cells) – Mean percentage of dead K562 cells (K562 only control)

NK Cell and Mtb Infected Monocyte Coculture and Killing Assay

In order to measure the ability of NK cells to kill Mtb- or BCG-infected autologous monocytes, both monocytes and NK cells were isolated via magnetic bead isolation as described above. Monocytes that had rested for 2h in 5mL polystyrene FACS tubes were infected with mCherry-BCG at an MOI 10 for 6h. Following the infection period, monocytes were washed three times in complete RPMI and NK cells (that had been rested approximately 8h and enumerated prior to addition to the monocytes) were added at an E:T of 4:1 in a final volume of 200ul. Cocultures were allowed to proceed for 18h. Following the incubation, NK cells were pipetted off the

cultures, and adherent monocytes were detached from the FACS tubes as described above. Monocytes were assessed for infection status and viability via LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen)

RESULTS

Cohort Description

This study used healthy specimen collected from the Seattle BioMed Blood Draw Program or from chronically HIV-infected donors collected in collaboration with the University of Washington Center for AIDS Research Madison Clinic. HIV+ patients were antiretroviral naïve or have ceased antiretroviral treatment for at least 2 years to be enrolled in the study and did not have a history of presenting with TB symptoms. Additionally, all subjects enrolled into the study were PPD negative.

Monocyte Phenotype in Healthy and HIV-infected Individuals

Following monocyte isolation, monocytes were assessed for purity and baseline phenotype (Fig 5-1a). Monocyte purity was greater than 93% and was not different between the groups. HIV-infected subjects had a significantly lower proportion of CD14++ monocytes (71% vs 91%; p=0.004; Fig 5-1b), and a concomittant increase in CD14+CD16+ inflammatory monocytes (16.5% vs. 6%; p=0.004; Fig 5-1b). A small proportion of the isolated monocytes from both healthy and HIV+ subjects were CD14-CD16+, and likely represent monocytes that

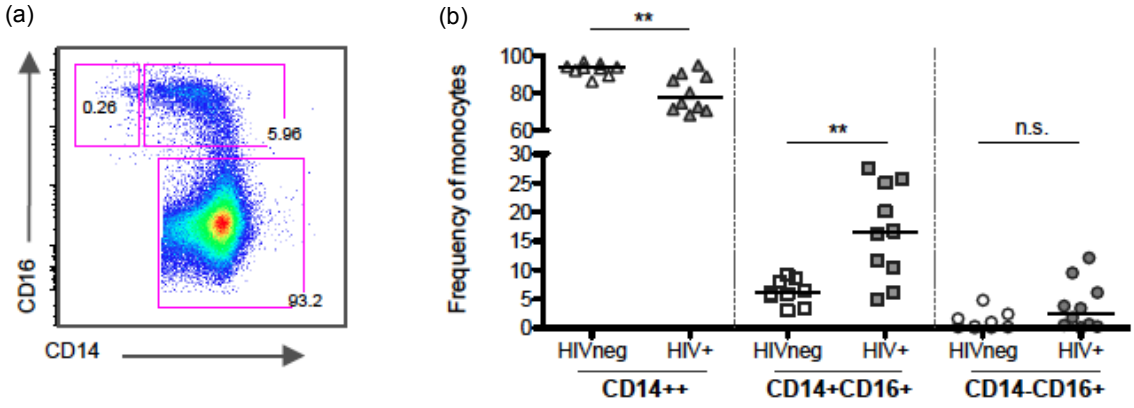

Figure 5-1. Monocyte Phenotypes at Baseline Between Healthy and HIV+ Subjects. Following magnetic bead isolation, monocytes were assessed for purity and phenotypic subset distribution via flow cytometry. (a) Representative flow plot showing the distribution of CD14+ and CD14+CD16+ monocytes. The frequency of monocytes was calculated as what proportion each phenotype contributes to the sum of the three gates in A, which was normalized to 100% (b). Lines represent the medians for a given subset. Statistic analysis was performed via a Mann-Whitney test (**p<0.005).

have down-modulated CD14 or perhaps had their antibody-recognizable epitope on the CD14 protein masked by magnetic beads. However, this small population was not different between healthy and HIV+ groups.

Monocyte Phagocytosis of Fluorescently-Labeled Mycobacteria

To evaluate monocyte phagocytosis prior to measuring combinatorial effects of monocyte and NK cell dysfunction, the uptake of fluorescently-labeled opportunistic BCG or pathogenic Mtb was measured over a 6 hour time period. In order to ensure that detection of fluorescence in the PE channel was indicative of the presence of phagocytosed *Mycobacteria*, monocyte phagocytosis was also assessed in the presence of the actin inhibitor, cytochalasin D, and a negative, uninfected control was run for each subject (Figure 5-2a). Monocytes from healthy and HIV+ subjects exhibited comparable phagocytosis of both opportunist BCG and pathogenic Mtb following 6h assay (Figure 5-2b and 5-2c). This may be in part due to the large variation in the capacity to phagocytose Mtb (range 9%-81%). A modest inverse correlation of decreased phagocytosis with increasing HIV+ subject VL was detected ($r=-0.61$, $p=0.12$; Figure 5-d), but no correlation was detected with subject CD4 count ($r=0.1$; $p=0.5$; Figure 5-e). No correlations were found between monocyte phagocytosis of BCG and HIV+ clinical disease parameters (not shown).

While initial experiments assessing phagocytosis of either BCG or Mtb suggested that a lower proportion of monocytes phagocytosed Mtb compared to BCG (from both groups), follow-up experiments using matched donors under demonstrated that monocytes from healthy and HIV+ subjects both have similar capacity for phagocytosing both BCG and Mtb (Figure 5-2e). A deeper analysis of monocyte phenotype showed that CD14+CD16+ monocytes and CD14-CD16- monocytes (likely indicaitve of monocytes that had downmodulated or shed CD14 upon activation) were comparatively better at phagocytosing Mtb for both healthy and HIV+ individuals compared to CD14+CD16+ monocytes (Figure 5-2f). A similar trend was found in

response to BCG (not shown). Together, these data suggest that while HIV may impact the ability of monocytes to phagocytose Mtb, monocyte subsets from healthy and HIV+ donors from this cohort display equal capacity to phagocytose BCG and Mtb.

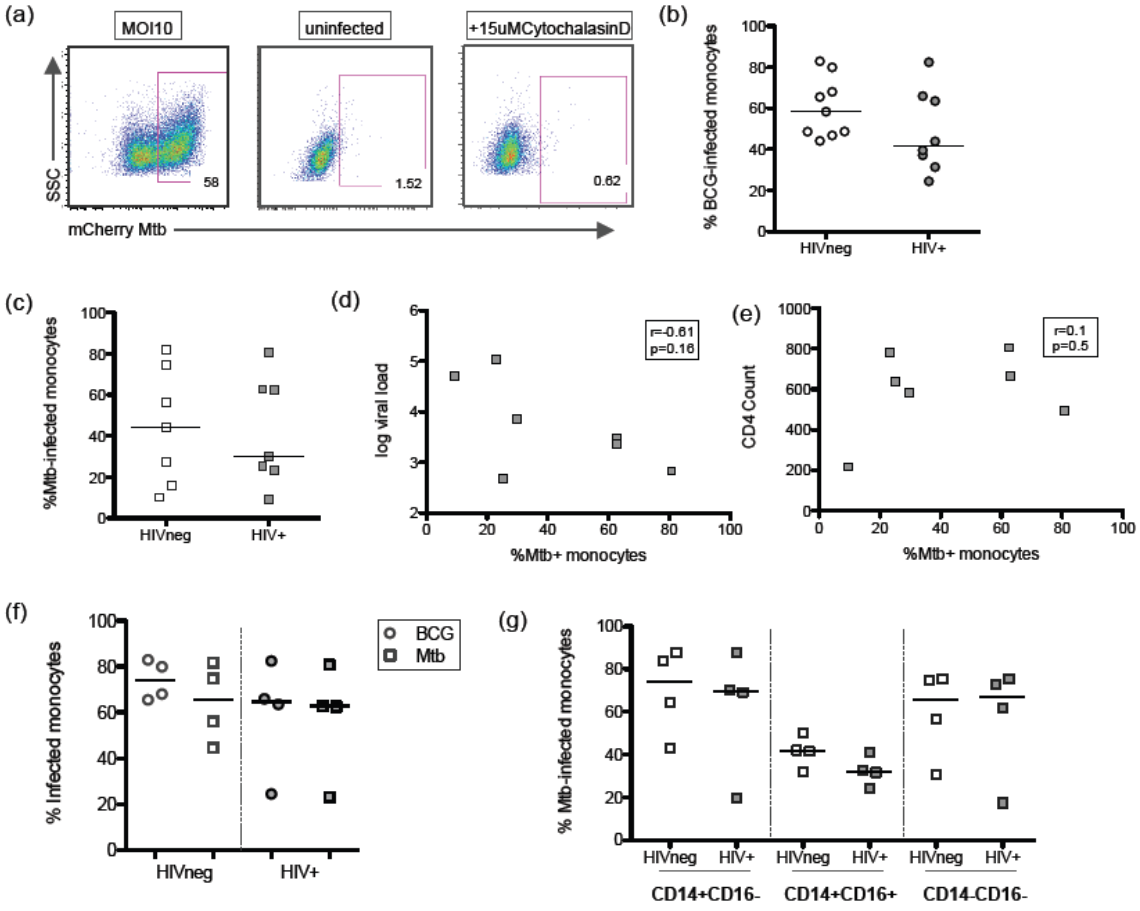

Figure 5-2 Monocyte Phagocytosis of Fluorescent *Mycobacteria* in Healthy and HIV+ Subjects. (a.) Representative flow plots of monocytes following 6h phagocytosis of mCherry-labeled BCG or Mtb, an uninfected control, or in the presecne of 15uM actin inhibtor cytochalasin D. Cells were first gated on singlets and live cells before detecting the percentage of cells positive for mCherry-*Mycobacteria*. Monocyte phagocytosis of (b) BCG and (c) Mtb in healthy and HIV+ subjects patients was assessed as well as the association of HIV VL (d) or subject CD4 count (e) with phagocytosis. A subset of patients were assessed for both BCG and Mtb phagocytosis in the same experiment (f) where phagocytosis by each monocyte subset was also measured (g).

Monocyte Viability Following Phagocytosis of mCherry BCG and Mtb

Monocyte viability was measured following phagocytosis of mCherry BCG and Mtb in order to establish the contribution of infection with *Mycobacteria* to overall monocyte death. Following 24h total culture (6h infection+18h equilibration), significantly fewer live monocytes were detected in the Mtb condition (48.3% healthy; 61% HIV+) compared to either BCG (81.6%

healthy; 87% HIV+; p=0.03) or the unstimulated control (85-2% healthy; 88% HIV+; p=0.03) in both groups (Figure 5-3).

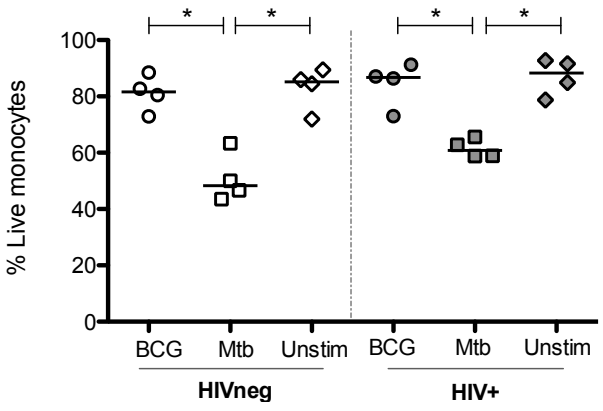

Figure 5-3. Percentage of Live Monocytes Following 18h Culture with BCG and Mtb. Following a 6h phagocytosis assay and 18 hour incubation period, monocyte death was assessed via incorporation of live/dead aqua. The percentage of singlets that excluded the dye (indicative of viable events) are displayed. Statistics were generated via a Mann-Whitney (*p<0.05).

NK Cell Phenotypic Distribution in Healthy and HIV-infected Individuals

Following NK cell enrichment, NK cells were assessed for purity and baseline phenotype (Fig 5-4a). NK cell purity was typically greater than 85% and was not different between the groups. NK cells from HIV-infected subjects had a significantly higher proportion of CD16+CD56- NK cells (1.7% vs 7.3%; p=0.01; Fig 5-4b) and similar proportions of CD16+CD56+, CD56++ and CD16-CD56+ NK cell phenotypes.

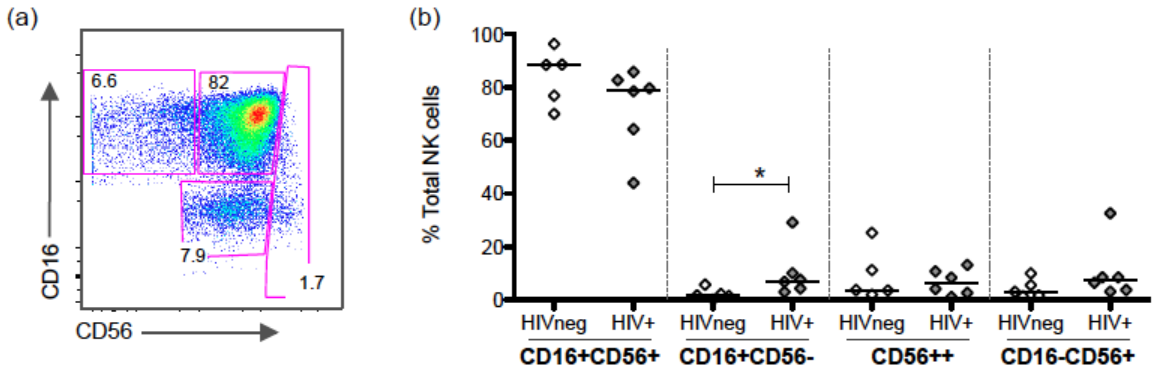

Figure 5-4. NK Cell Phenotypes at Baseline Between Healthy and HIV+ Subjects. Following magnetic bead enrichment, NK cells were assessed for purity and phenotypic subset distribution via flow cytometry. (a) Representative flow plot showing the distribution of CD16+CD56+, CD16+CD56-, CD56++ and CD16-CD56+ NK cells. The frequency of NK cells was calculated and the proportion each phenotype is displayed (b). Lines represent the medians for a given subset. Statistics were generated via a Mann-Whitney test (*p<0.05).

NK Cell Killing of Target K562 Cells

To assess the killing capacity of NK cells from HIV+ patients, purified NK cells were cultured with MHCI-deficient K562 cells for 4 hours at effector (NK cells) to target (K562 cells) ratios of 1:1, 4:1, and 8:1. NK cells from HIV+ patients displayed a reduced capacity to kill K562 target cells compared to controls at an effector:target ratio of 4:1 (but not 1:1) following 4h coculture (Figure 5-5b). NK cell killing was not correlated with HIV+ subject CD4 T cell count or VL (data not shown).

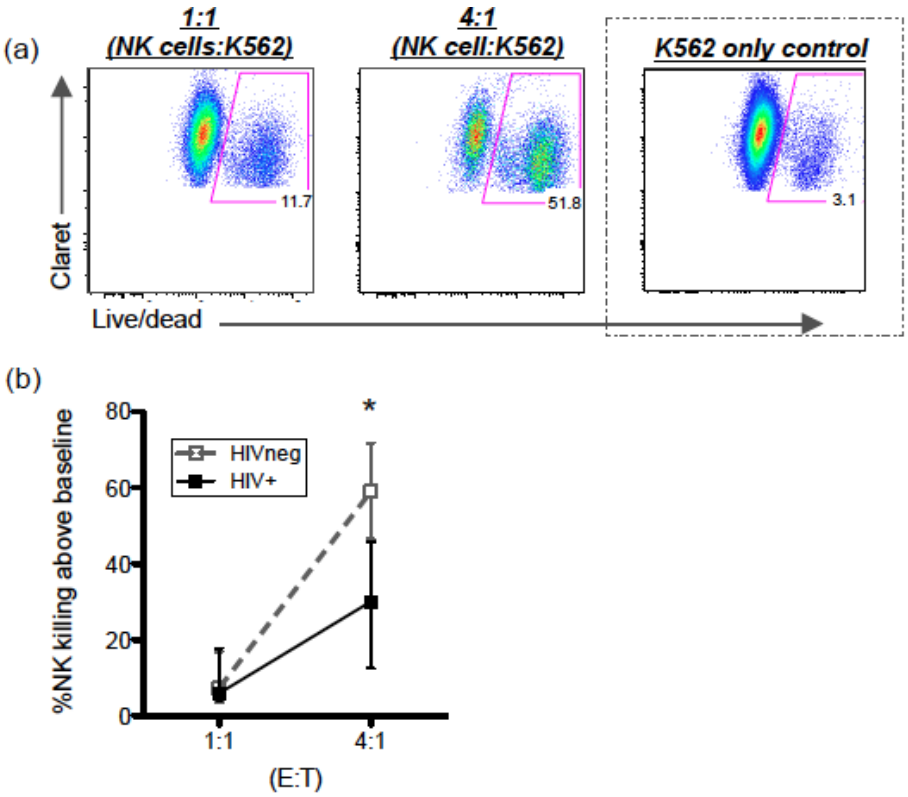

Figure 5-5. NK Cell Killing Capacity in Healthy and HIV+ Patients. Purified NK cells were assessed for their capacity to kill MHCi-deficient K562 cells in a 4h killing/coculture experimenr. K562 death was assessed via incorporation of a live/dead stain and measured via multicolor flow cytometry (a). Percent NK cell killing above baseline was calculated as the mean percentage of death in cultures with NK cells – spontaneous K562 cell dead (no NK cells) at an effector:target 1:1 and 4:1(b).

DISCUSSION

Innate immune cell dysfunction during HIV pathogenesis was first observed very early in the temporal unfolding of the HIV pandemic [196]. Since then, numerous studies have confirmed initial reports of innate immune dysfunction and have further classified this dysfunction. In this novel study, we have begun to classify multiple cell types and their interactions within the same individuals to investigate the potential for synergistic dysfunction that very likely occurs following HIV-mediated impairment of several cell types. Here we have characterized critical effector functions of monocytes and NK cells in an effort to better understand the role of impaired cellular crosstalk in HIV pathogenesis and, ultimately, susceptibility to opportunistic pathogens.

Through an assessment of monocyte phagocytosis, we found that monocytes in our Seattle-based HIV+ cohort exhibit equivalent phagocytosis of an opportunistic pathogen, BCG, as well as the fully pathogenic Mtb. This contrasts previously published reports, which have demonstrated that in addition to several other functional parameters, monocyte phagocytosis becomes dysregulated during HIV pathogenesis. These studies have investigated monocyte phagocytosis of apoptotic neutrophils [197], and a wide array of pathogens, including *T. gondii* [86], P. falciparum-infected erythrocytes [198], *C. albicans* [87], as well the opportunist *M. avium* [190]. Because the majority of our patients had a CD4+ T cell count in the healthy range, it is quite possible that our cohort does not represent the majority of untreated, progressive HIV infections. This is consistent with the findings that decreased monocyte functionality correlates with CD4+ T cell count [199] and that monocyte functionality improves when patients are administered antiretroviral therapy [200, 201]. Although we did not detect any significant association between patient CD4 count and monocyte phagocytic capacity, this may be due in part to the relatively maintained CD4 counts of the donors in our HIV+ cohort. We did see that the one patient with a CD4 count near only 200 cells/ul exhibited the lowest phagocytosis of the group (Figure 5-2e), in accordance with previously published data. Further, we found a modest

correlation between monocyte phagocytosis and donor viral load, suggesting that disease status may impact monocyte function. Finally, although the proportion of infected monocytes was similar between groups, the number of *Mycobacteria* taken up by monocytes may differ by disease status. Employing microscopy might help to elucidate these differences.

Monocytes exhibited significantly higher death following infection with Mtb than BCG or uninfected control cells. Indeed, only 50-60% of monocytes remained viable following 24h culture at an infection of MOI 10. These data are consistent with another report showing that human macrophages infected with Mtb at MOI10 undergo necrosis including extensive DNA fragmentation, loss of mitochondrial membrane potential, and loss of plasma membrane integrity, and these effecte were not seen at MOI 1 [202]. Further, this was only seen with the ESAT-6 protein-expressing Mtb, which likely is the reason we did not see this extensive death in the BCG conditions (as BCG does not secrete ESAT-6). While an MOI 10 was chosen for these experiments based on high infectivity, future experiments that ultimately aim to quantify monocyte death should employ a lower MOI, which would likely result in less Mtb-induced death and allow a better discernment of NK cell-induced death.

Interestingly, NK cells from HIV+ donors in our cohort were found to have significantly reduced killing capacity of K562 cells compared to healthy donors following 4h coculture. This was only significant at 4:1 and not 1:1. These data are consistent with previous observations showing that NK cells from HIV+ donors have reduced functional responses to K562 cells [99]. Interestingly, in a previous study of a cohort of Brazilian HIV+ patients, only patients with CD4 counts <200 cells/ul exhibited dysfunctional NK killing at high E:T ratios (40:1) in a chromium release assay [203]. Our data, showing impaired NK cell killing of target cells in a comparatively healthy population of HIV+ subjects and at a much lower E:T may be indicative of increased sensitivity in the detection of killing through use of multicolor flow cytometry. Impaired NK cell function during HIV infection has been demonstrated in multiple studies, and may be attributed to the expansion of a functionally ‘anergic’ or ‘immature’ CD16+CD56- NK subset [100, 101,

204]. Indeed, a phenotypic analysis of enriched NK cells revealed a characteristic expansion of this subset in HIV+ individuals in our cohort as well, and this may provide mechanistic insight into our functional findings.

To our knowledge, our data is the first to examine functional alterations in both monocyte and NK cells in the same HIV+ patients. Further, these data offer insight into cellular alterations in a cohort of viremic subject with relatively high CD4 T cell counts. Additionally, these data yield a foundation on which to build future studies to better evaluate 1) NK cell killing of autologous *Mycobacteria*-infected monocytes in HIV-infected donors 2.) the impact of patient clinical parameters and antiretroviral therapy on monocyte and NK cell function and cross talk, which will be discussed in detail in Chapter 6 of this dissertation. Ultimately, these studies will provide useful insight regarding mechanisms by which HIV-induced alterations in innate immunity leads to tuberculosis or disseminated Mycobacterial diseases.

CHAPTER 6:

DISCUSSION AND FUTURE DIRECTIONS

OVERVIEW

This chapter summarizes the results presented in the preceding chapters and discusses their scientific implications regarding 1.) The role of cellular activation in mother-to-child HIV-1 transmission (Chapters 2 and 5), and 2.) The assessment of innate immune cell dysfunction during HIV-1 pathogenesis that contributes to the acquisition of AIDS-defining opportunistic infections (Chapters 3 and 4). Each of these studies is connected to the central tenets of this dissertation, which include delineating the contributions of innate immunity to HIV-1 transmission and to disease pathogenesis, with an emphasis on susceptibility to opportunistic infections. The data are presented in the context of what is currently known regarding cellular immunity and immune activation in HIV-1 transmission or in HIV and *Mycobacteria* pathogenesis. Finally, potential future directions for each topic are proposed, including a thorough analysis of potential sources of cellular activation for the HIV-1-exposed infant that may enhance transmission as well as the use of animal models to better delineate the contributions of specific cell types to HIV-1 pathogenesis in the context of a complex immunological milieu.

Despite being recognized as an important factor in HIV-1 progression extremely early on in the HIV-1 pandemic [196, 205-207], innate immunity has been comparatively understudied as an important factor in HIV-1 transmission and disease pathogenesis. A relatively large pool of resources have been devoted to better understanding adaptive immune responses. This is partially because CD4+T lymphocytes are the major target of HIV-1 replication and destruction, but perhaps more importantly because these T and B-lymphocytes generate immune memory, a concept that is absolutely key to vaccine design. For a vaccine against HIV-1 to be efficacious, the intricacies of adaptive immunity that ultimately yield a correlate of protection as well as long-lasting memory responses need to be defined. However, innate immune responses are important for not only immediate response to HIV-1 and/or opportunistic pathogens, but it also guides the quality and quantity of the adaptive immune response [208, 209]. Therefore, a better understanding the importance of immune activation and HIV-1 pathogenesis remains an important path to be explored not only for the discovery of immune therapeutics as a complement to vaccine discovery, but perhaps also to effective vaccination design in and of itself.

Innate immunity is undoubtedly critical in the response to pathogens, but innate cells like monocytes and NK cells have the capacity to be highly proinflammatory, which may not always be beneficial for the host. In the case of HIV-1 transmission, a high level of innate immune cell activation has been shown to be protective against transmission [137, 210]. In contrast, T cell activation, which is directly linked to innate cell activation, has been correlated to increased susceptibility to HIV-1 transmission [122, 123]. In addition to the potential to increase transmission, immune activation of several cell types is highly correlated to HIV disease progression, and this activation may serve as a better predictor of HIV progression than even viral load or CD4 T cell depletion [25, 211, 212]. Finally, the importance of eliciting a proper immune response while limiting the detrimental effects of inflammation-induced tissue destruction is not unique to HIV-1. Several observations of *Mycobacteria* pathogenesis also

implicate inappropriately controlled inflammation in driving tissue destruction [213-215]. Thus, an inherent immunological complexity exists in promoting the protective effects of inflammation and immune activation that are important for protecting a host from disease while minimizing detrimental factors that promote disease acquisition and/or pathogenesis.

POTENTIAL CONTRIBUTIONS OF INNATE IMMUNITY TO T-CELL ACTIVATION AND MATURATION STATUS IN HIV-1 TRANSMISSION

Cellular immune activation correlates with HIV-1 disease progression [216-219], but its role in HIV-1 transmission is less well-defined. For example, proinflammatory cytokines result in the recruitment of activated target dendritic cells and CCR5-expressing CD4+ T cells [220-222] creating an environment that favors HIV-1 infection, replication and dissemination. Conversely, immune activation may lead to production of anti-HIV proteins such as interferons and/or beta-defensins [223], which inhibit HIV-1 infection and replication. Thus, immune activation serves as a "double-edged sword" in the context of HIV-1 transmission, and maintaining a balance in activation of cells with antiviral capacity while minimizing target cell availability may be critical for preventing HIV-1 infection.

Previous data support a model in which T cell activation increases susceptibility to HIV-1 acquisition, while decreased CD4+ T cell activity, termed "quiescence," may be protective during repeated HIV-1 exposure [113, 121, 122]. In contrast, an activated innate response, particularly by NK cells, may provide protection against HIV-1 [137, 210]. Our findings in chapter 2 are consistent with this observation, and demonstrate that activation of non-target NK cells and CD8+ T cells is protective during repeated exposure to HIV, while CD4+ T cell maturation is positively associated with MTCT of HIV-1. Because our case-control study design controlled for maternal HIV load, increased cellular activation of NK cells present in cord blood of infants prior to infection cannot be explained by HIV-1 load alone. Additionally, as cases and controls were drawn from the same cohort, other immunologic and environmental risk factors

are also likely to be similar between the groups. One hypothesis that may account for increased activation of innate immunity is the degree of maternal-infant HLA concordance, a previously described risk factor for MTCT of HIV-1 [224], which we were not sufficiently powered to address in our study. Hence, the source of differential T cell maturation and T cell/NK cell activation in infants described in our study remains unknown. It is possible that maternal factors play a key role in influencing the infant immune milieu and, ultimately, susceptibility to HIV-1. Examples of maternal factors and lifestyle that might affect the immune status and of and subsequent HIV-1 transmission to the fetus include maternal coinfection with other pathogens [140], diet [225, 226] or vaginal microbiota [227, 228], which all have potential to induce immunological changes and therefore alter HIV-1 susceptibility in the fetus.

One driver of T cell activation and maturation in HIV-1-exposed infants includes the intentional manipulation of the immune environment through vaccine administration. While vaccines have undoubtedly provided millions of people with life-saving immune education against several pathogens, they have the potential to induce a state of inflammation that, upon certain circumstances, could be of greater detriment to the vaccinee than the risk of going unvaccinated. Although vaccination status was not considered as a factor that may induce differential immune system activation/maturation in chapter 2, the fact that cases and controls were drawn from the same cohort suggests that vaccination rate between cases and controls was likely similar. Chapter 3 of this dissertation addresses this point, and demonstrates one particular scenario in which BCG vaccination leads to an increased proportion of activated HIV-1 target CCR5+CD4+ T cells. Interestingly, we did not find a causal role for innate immune activation in influencing this T cell activation, but examining other time points post vaccination or other markers of monocyte/NK cell activation (such as cytokine secretion) might yield more insight into the innate immune status (discussed in detail in Chapter 3). Our study is not the first to document potential for adverse vaccine-related outcomes in the context of HIV-1 exposure. In 2008, the STEP trial for HIV-1 vaccines was terminated prematurely due to an observed

increase in HIV-1 acquisition among male, Ad5 seropositive vaccinees. As Ad5-generated immunity likely involves lymphocytes homing to the mucosa, this is suggestive of the existence of a complex interplay of mucosal immune activation and HIV-1 acquisition.

Because successful implementation of pMTCT programs in our study site, Khayelitsha, limited the number of HIV-1 transmissions in our cohort, we were unable to measure the consequences of this increased CD4+ T cell activation directly on HIV-1 acquisition. However, several studies have previously documented a positive association between CD4+ T cell activation and HIV-1 acquisition in adult populations [122, 123]. Together, these studies of MTCT of HIV-1 yield novel insight into protective and risk-enhancing immune correlates of infant HIV-1 acquisition.

Future Studies to Examine Mother-to-Child HIV-1 Transmission

A recent study showed that the gut mucosa is the preferential site for memory CD4(+) T cell residence in infants [229]. As data from Chapter 2 of this dissertation shows that effector memory CD4+ T cells are associated with an increased risk of HIV-1 acquisition in infants (independent of maternal VL status), memory T cells particularly located in the infant mucosa provide a large pool of susceptible cells for ingested HIV-1 at birth and during breastfeeding. Interestingly, in addition to BCG, another live-attenuated vaccine administered to infants at birth in developing countries includes the oral polio vaccine (OPV), which preferentially replicates in the intestinal mucosa. Because HIV-1 has been shows to specifically target mucosal immune cells, their activation by a live-attenuated vaccine (LAV), like OPV may enhance MTCT during breastfeeding. To better understand the effects of LAV administration in HIV-1-exposed infants, a carefully-controlled study using animal models of HIV-1 transmission, in which cellular location, activation, and generalized immune activation can be correlated with transmission would greatly contribute to the knowledge surrounding mucosal immunology as well as the immunogenicity of childhood vaccines in the context of HIV-1 exposure. If our hypothesis is supported by these

studies, and demonstrate that live-attenuated vaccines result in increased HIV-1 target cells and subsequent HIV-1 acquisition, infants with risk of HIV-1 acquisition should be considered for exemption from vaccination as a part of their overall clinical treatment plan. However, it is important to stress that because the BCG and OPV vaccines are efficacious in preventing childhood and disseminated tuberculosis and polio, respectively, only those HIV-1-exposed infants with no known tuberculosis contacts (maternal and/or household) or risk of polio acquisition should be exempted from vaccination.

Additional studies that can be undertaken to forward the studies presented here with the goal of identifying maternal and infant factors that contribute to differential infant HIV-1 susceptibility are warranted. It is currently known that maternal viral load and CD4+ T cell counts can independently predict transmission [230, 231]. Further, the presence of inflammation, including mastitis, breast abscesses, or infant oral thrush can all increase the likelihood of a successful transmission event [45, 231, 232]. Although known risk factors for transmission were likely similar between groups in chapter 2 of this dissertation, determining interventions for HIV-1-exposed infants might decrease overall HIV-1 transmission, and may be particularly useful for infants with no other known risk factors who would otherwise acquire HIV.

Maternal nutritional status is one predictor of adverse clinical outcomes HIV-1-exposed infants [233]. Studies in both humans and the pigtail macaque model demonstrate that probiotics may help to restore normal immune function of the gastrointestinal system following HIV-1-related damage through increased activity of antigen presenting cells, enhanced T cell numbers and functionality, and through decreasing proinflammatory cytokine levels [234, 235]. Lactobacilli, for example, not only plays a role in suppressing TNF-α transcription, but also in regulating the formation of adherence junction proteins, which are critical in maintaining gut epithelial integrity and function [236, 237]. Because existing studies suggest that some bacteria present in the maternal gut could reach the mammary gland during late pregnancy and lactation (reviewed in [238]), administration of probiotics to pregnant women during ART initiation may

have important benefits to both maternal and fetal gastrointestinal tract health. Ultimately, this may help prevent transmission of HIV-1 to infants through lowering maternal immune activation (which results in part from HIV-1-mediated GI tract destruction [50]) and its associated increase in viral load, which is the single largest predictor of MTCT of HIV-1 [125] or through the direct maintenance of infant GI tract integrity.

However, a more thorough understanding of the relationship between the maternal and fetal interface in the context of probiotic exchange should be elucidated before these studies can be carried out. For example, are there probiotics that more effectively pass between mother and infant compared to others? Does this exchange happen solely through breastmilk or are there other factors that may lead to gut mucosal microbiome similarities between mother and infant? Additionally, a better understanding of the specific types and relative proportions of probiotic bacteria is needed in order to formulate the most appropriate and protective probiotic regimens. While pMTCT programs have been successful thus far, it is possible that improve GI tract health, mediated through probiotic administration, may help prevent break-through cases of infection or act as a "plan B" in the case of treatment interruption. Overall, administering probiotics with ART treatment to pregnant HIV+ mothers offers a feasible and relatively economical intervention for improving maternal and child health.

The results from these proposed studies may provide insight into factors that alter infant immunity and increase susceptibility to HIV-1 acquisition in the context of repeated exposure, particularly through breast milk. Ultimately, these studies have the potential to yield tangible targets for intervention and the reduction of MTCT.

Innate Immune Cell Response and Crosstalk in Mediating Protection from Opportunistic Pathogens During HIV Infection

Previous studies have demonstrated a role for monocytes and NK cells in mediating defense against various opportunistic pathogens, including *Mycobacteria* (discussed in Chapter 5 and below). Our systems analysis data in chapter 4 add to these studies by implicating differential regulation of several genes involved in canonical immune-response and signaling pathways during chronic HIV-1 infection following PBMC stimulation with BCG. These results have implications for studies aiming to better characterize the dysregulation of immunological response to *Mycobacteria* during HIV, as described in Chapter 4, or even following BCG vaccination in HIV-1-exposed infants (Chapter 3). Finally, results from both chapters 4 and 5 can be used to better guide studies aiming to characterize innate immune cell crosstalk during HIV-1 pathogenesis, such as the studies described in Chapter 5.

Monocytes and NK cells, which are the primary focus of the studies described in this dissertation, are particularly interesting in that they display altered phenotypic and functional characteristics that contribute to HIV-1 pathogenesis despite a lack of direct infection by the virus (discussed in detail in chapters 4 and 5). Although the cause of the dysfunction has not been fully characterized, it may be a consequence of an overall disrupted immune milieu caused by the depletion of CD4+ T cells, through direct exposure to virus and/or stimulation by viral products, or chronic immune activation and resultant cellular exhaustion. While a small number of studies have a implicated direct infection of these cells, which may contribute to their dysfunction [239, 240], the majority of studies support the notion that they remain uninfected (although monocytes become more permissive to HIV-1 as they differentiate into macrophages; [241]). Dendritic cells (DCs) are another cell subset critical for responding to both HIV-1 and Mtb and for interacting with other immune cells to link innate and adaptive immunity. Therefore, while these cells were not a focus of these studies, future studies aimed at better understanding the role of dendritic cells in mediating HIV-1 infection and HIV/Mtb pathogenesis are warranted.

Studies to address NK cell function during HIV have shown that HIV-1 protein VPR triggers NK cell-mediated lysis of HIV-infected T cells by upregulating ULBP2, a ligand of the NKG2D receptor [242]. ULBP1, another NKG2D ligand, is upmodulated on monocytes or regulatory T cells upon activation with *Mycobacteria* or LPS [243] and promotes NK cell-mediated killing of Mtb-infected monocytes and T cells [195]. Following exposure to Mtb-stimulated monocytes, NK cells are able to lyse *M. tuberculosis*-expanded regulatory T cells through a ULBP1-NKG2D interaction [244]. If monocyte and NK cell dysfunctional interactions reduced the ability of NK cells to kill Tregs, this might ultimately dampen the quality of the immune response to Mtb, as Tregs have been shown to slow the immune response to Mtb, thereby negatively affecting the course of Mtb disease pathogenesis [245]. These data provide an *in vivo* example for the importance and the application of studies focused on immune cell crosstalk.

Although tissue-resident macrophages likely play a major role in the *in vivo* responses to Mtb and opportunistic pathogens, monocytes were chosen as the primary cell type for investigation in these studies for several reasons. First, they are the precursors to mature macrophages, and have similar functional profiles dominated by inflammation and phagocytosis, and these functions are known to become dysregulated by HIV-1 infection in both cell types. Second, in contrast to tissue macrophages, they are more readily available for collection from patients and in sufficient numbers for performing replicate experiments. Finally, monocytes themselves can become important in the context of disseminated disease or in the context of new migration and differentiation into tissue macrophages. Despite these important similarities, key differences between monocytes and macrophages exist and are important to consider. The most important difference is likely that while monocytes remain relatively refractory to HIV-1 infection, macrophages can become productively infected with virus, which presents unique opportunities for molecular and cellular interactions between Mtb and HIV-1 within macrophages, but likely not within monocytes. However, the vast majority of macrophages are present in

tissues, for which it is not feasible to sample during HIV-1 infection of humans. Therefore, using non-human primate models of pathogenic SIV infection, in which sampling of diverse tissues is not only feasible, but routinely performed, is critical to carry out studies aimed at characterizing tissue macrophage function during pathogenic lentiviral infection.

Future Directions to Study HIV/Mtb Pathogenesis

The results in chapters 4 and 5 provide a solid foundation on which to propose studies aimed at understanding innate immune cells as part of a complex milieu of interactions in the context of HIV-1 infection in place of as an isolated entity. Due to the complexity of experiments aimed at examining cellular crosstalk, it may be necessary to delineate functional profiles of individual cells prior to or in combination with such a complex assay in order to better understand and interpret the results of that experiment. Chapter 5 demonstrated maintained monocyte phagocytosis of BCG or Mtb, but dysfunctional NK cell killing of MHC-deficient K562 cells. These results indicate that in the context of *Mycobacteria* infections, altered NK killing of *Mycobacteria*-infected monocytes is not due to exposure of the NK cells to fewer infected monocytes, which may have resulted in an obscured interpretation the interaction. Further, our data from chapter 4 suggest that altered monocyte cytokine profiles may also account for decreased killing activity by NK cells, which become activated in the presence of IL-12. Thus, a more comprehensive analysis of monocyte function in these experiments, including a thorough analysis of cytokines produced during the infection period (instead of only proinflammatory cytokines), would yield more insight into monocyte functionality in the context of interactions with NK cells.

Our data is consistent with a recent, published study, which found that NK cell-derived IFN-γ produced in response to heat-killed *E. coli* and pathogenic *S. typhimurium* is diminished in untreated HIV-1 infection and that this dysfunction persists despite viral suppression [246]. Interestingly, while monocytes were necessary for NK cells to respond to the bacteria, results

from this same study suggest that the HIV-associated defect was intrinsic to NK cells, because the addition of healthy monocytes did not restore IFN-γ production [246]. While this data is intriguing, the authors did not examine the ability of monocytes to subsequently kill the bacteria in the environment of decreased NK-cell-derived IFN-γ. Further delineating whether altered killing occurs solely because of a deficiency in NK-cell-derived IFN-γ or as a result of other inherent dysfunction resulting from HIV-1 in either monocytes or NK cells would provide useful insight into monocyte and NK cell interactions.

NK cell-mediated lysis of bystander T cells that have undergone a VPR-associated upregulation of ULBP2 may contribute to bystander T cell killing during HIV-1 infection [242]. While bystander killing of monocytes has not yet been observed, we hypothesize that this might be one mechanism of HIV-mediated pathogenesis that results in susceptibility to intracellular pathogens, like *Mycobacteria* due to similar mechanisms of stressed-cell recognition by NK cells. Employing flow cytometry to better discriminate between specific NK cell killing (of infected monocytes) versus non-specific NK cell killing (of non-infected monocytes) may uncover novel mechanisms of HIV/TB synergism in disease pathogenesis. For example, host infection with either HIV-1 or *Mycobacteria* resultant inflammation and pathogenesis could result in upregulation of ULBP1, known to be expressed on monocytes, and to facilitate NK cell killing of monocytes, which might contribute to the depletion of healthy monocytes and macrophages observed during SIV infection [211]. Further, using fluorescence-activated cell sorting to sort *Mycobacteria*-infected monocytes and macrophages from uninfected (from the same cultures) prior to transcriptome analysis could yield insight into regulation of cell surface and stress molecules as well as canonical inflammation and signaling pathways, to further discriminate the effects of direct *Mycobacteria* infection on cellular transcription from potential bystander effects of non-infected cells.

The vast majority of studies demonstrating the importance of *in-vivo* experiments to fully understand functional parameters of immune cells in their natural milieu has utilized the mouse

model of Mtb infection. While this model has contributed greatly to our understanding of Mtb pathogenesis, its ability to recapitulate HIV-1 and/or human tuberculosis disease is limited due to its lack of ability to model either HIV-1 infection or hallmark caseous granuloma formation. Because NK cells are found in granulomas of tuberculosis patients [110], one major place that NK cells and Mtb-infected monocytes and macrophages likely interact is within a granuloma. Using the non-human primate model may therefore be informative in guiding research to examine granuloma and other tissue-related cellular interactions. Indeed, the ability to frequently sample diverse tissues, control the dose and timing of infection, and to coinfect non-human primates with SIV to induce a model of HIV/Mtb coinfection makes this an appealing model. Although non-human primate models are significantly less genetically tractable compared to mice, experimental depletions of specific cell types, like NK cells, in order to determine their specific role in the contribution to pathogenesis vs. maintaining immune health, is feasible [247].

Finally, a better understanding of the role of monocytes in tuberculosis-associated immune reconstitution inflammatory syndrome (TB-IRIS) provides a very relevant clinical context for studying the effects of monocyte-induced inflammation and resulting pathogenesis. TB-IRIS is a clinical condition in which immunocompromised patients who receive antiretroviral therapy to treat HIV-1 infection develop a robust immune response to previously-acquired opportunistic pathogens. This ultimately results in a severe disease characterized largely by immunopathogenesis, for which there is no known treatment other than the administration of antibiotics or antivirals to treat the coinfection. A recent study has implicated monocytes and common immunological/inflammatory pathways associated with monocytes in the development of TB-IRIS by demonstrating that monocyte gene expression was perturbed in patients who subsequently developed TB-IRIS even prior to initiating ART [248]. This study has identified useful biomarkers that present the potential of predicting the likelihood of an IRIS event, but also

provides several tangible pathways, like pattern recognition receptors and complement, that could serve as targets for therapy to better harness monocyte function upon ART initiation.

FINAL THOUGHTS

At the end of 2012, there were over 35 million people infected with HIV-1. Even those that are fortunate enough to have access to life-saving antiretroviral therapy will experience persistent immune activation that could drive immune cell dysfunction [155]. Further, a portion of those people will develop IRIS after the initiation of ART, which is largely mediated by innate cells like monocytes and NK cells. Thus, even in the event of successful HIV-1 vaccine development, a better understanding of inflammation and its resolution during HIV-1 is urgently needed. Using non-human primate models that progress to AIDS, like Rhesus macaques, will be critical to developing a better understand the double-edged-sword of innate immune cell-mediated inflammation and to develop therapeutics to restore proper immune functionality. Ultimately, a better understanding of the specific factors, including magnitude, timing, and location, of immune responses that protect against disease pathogenesis and the acquisition of OIs without contributing to inflammation-induced tissue destruction, will be critical to creating and implementing effective therapies to harness innate immune function.

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