ASSESSMENT OF CD4+ T CELL DEPLETION AND MONOCYTE FUNCTION DURING NONPATHOGENIC SIV INFECTION OF SOOTY MANGABEYS

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by

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DEDICATION

This work is dedicated to my husband, Jeremy Hudgeons, whose unwavering support of my endeavors, both academic and personal, has made possible its completion. I am eternally grateful for his love, trust, and willingness to relocate.

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SIV-infected sooty mangabeys maintain low levels of chronic immune activation and do not progress to AIDS, making them an important model for elucidating the mechanisms contributing to AIDS pathogenesis. The studies presented here utilize the mangabey model of nonpathogenic SIV infection to assess the impact of CD4+ T cell depletion on immune activation in SIV-infected mangabeys and to assess the contribution of monocytes to nonpathogenic SIV infection.

The Sodora laboratory previously identified a cohort of SIV-infected sooty mangabeys that experienced a virally-mediated severe decline in

CD4+ T cells yet did not develop opportunistic infections or AIDS. Here, we assessed the immune competence of three mangabeys following viral passage from one CD4-low mangabey that resulted in a dramatic decline in CD4+ T cells within 21 days-post-infection. Despite the rapid depletion of CD4+ T cells, all mangabeys maintained low levels of chronic immune activation and mounted adaptive immune responses to SIV and influenza vaccination. To investigate the contribution of monocytes to the low levels of immune activation, we assessed the effector function of monocytes in SIV-infected CD4-low and CD4-healthy sooty mangabeys. We found that, compared to SIV-negative mangabeys, monocytes from SIV-infected mangabeys produced significantly less TNF-α upon stimulation with lipopolysaccharide (LPS). In contrast, hosts of a pathogenic infection, including SIV-infected macaques and HIV-infected humans, displayed no change in monocyte TNF-α responses relative to uninfected controls. In mangabey PBMC cultures, stimulation with LPS led to increases in CD8+ T cell activation that could be inhibited in a dose-dependent manner by TNF-α –blocking antibodies. Taken together, these results suggest that TNF-α production from monocytes can contribute to increases in immune activation and that SIV-infected sooty mangabeys regulate the monocyte response to LPS as one means to avoid chronic immune activation during SIV infection.

These studies expand the current knowledge of the mechanisms by which SIV-infected natural hosts avoid progression to AIDS and underscore the importance of controlling immune activation during lentiviral infection, which may inform the next generation of therapies and vaccines for HIV patients.

TABLE OF CONTENTS

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LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
AGM	African green monkey
ART	Anti-retroviral therapies
BAL	Bronchoalveolar lavage
cDNA	Complementary DNA
CTL	Cytotoxic T lymphocyte
DPI	Days post infection
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HIV	Human immunodeficiency virus
IFN-α	Interferon alpha
IFN-β	Interferon beta
IFN-γ	Interferon gamma
IL	Interleukin
LN	Lymph node
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
mDC	Myeloid dendritic cell

NK cell	Natural killer cell
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PHA	phytohaemagglutinin
PTM	Pig-tailed macaque
RB	Rectal biopsy
RM	Rhesus macaque
SIV	Simian immunodeficiency virus
SM	Sooty mangabey
ssRNA	Single-stranded RNA
TNF-α	Tumor necrosis factor alpha
T-reg	T-regulatory cell

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

Exactly how human immunodeficiency virus (HIV) infection induces the clinical sequela characteristic of AIDS is a complex question that to date has not been fully answered. Despite more than 25 years of active research on HIV, the correlates of protection from the virus are not known and an effective vaccine is far from reach. To date, an estimated 30 million people have perished as a result of HIV infection and another 33 million are currently infected, with the heaviest disease burden occurring in sub-Saharan Africa (1). The recent failure of a major HIV vaccine trial in September 2007 emphasized the need for basic research into the pathogenesis of lentiviral disease to identify the correlates of protection and enable a more rational basis for the design of vaccines and therapeutics.

The Life Cycle of HIV

A member of the family Retroviridae, genus lentivirus, HIV is an enveloped virus with a diploid single-stranded RNA genome encoding 9 proteins (2, 3): Gag, Pol, Env, Tat, Rev, Vif, Vpr, Vpu and Nef (Fig. 1-1). The Gag protein is comprised of the capsid, matrix, and nucleocapsid proteins which form the core and encapsulate the viral genome in mature various. The viral core also houses the viral enzymes polymerase, protease and

integrase (encoded by Pol) as well as the accessory proteins (Vif, Vpr, Vpu, Tat, Nef). The surface of HIV virions are adorned with the glycoproteins gp120 and gp41 which together form the gp160 protein encoded by the *env* gene (4). The

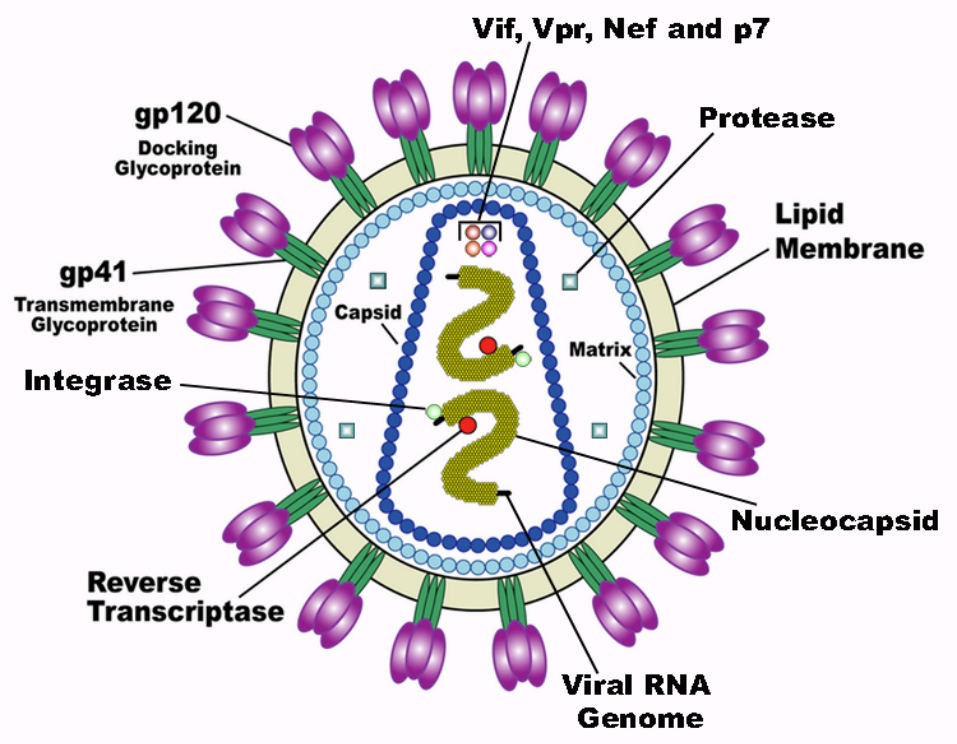

Figure 1-1. The Structure of HIV-1.

HIV-1 is an enveloped virion containing a diploid, single-stranded RNA genome packaged by the structural matrix, capsid, and nucleocapsid (together comprising the Gag protein). Also present inside the virion are the accessory proteins Vif, Vpr, and Nef, and the viral enzymes Reverse transcriptase and Integrase. The glycoproteins gp120 and gp41 (together comprising gp160) are present on the surface of the virion.

Env glycoproteins facilitate HIV entry into target cells via interactions between gp120 and the CD4 molecule on the surface of cells, including CD4+ T cells, macrophages, and some dendritic cells. Upon initial binding to CD4, gp120 undergoes a conformational change allowing it to bind to additional cell surface molecules, termed co-receptors, which are also required for entry. The most widely utilized co-receptors are CCR5 and CXCR4, found predominantly on T cells (5, 6) (reviewed in (7, 8)). CCR5 is typically expressed on effector T cells located at mucosal sites and is usually associated with HIV transmission. In contrast, CXCR4 is expressed by naïve and central memory T cells in the peripheral blood or at lymphoid sites and is typically utilized as a co-receptor for HIV only later in infection when sequence evolution of the *env* gene enables protein-protein binding with CXCR4 (CXCR4 tropism is observed in roughly 50 % of infected individuals and is usually associated with faster disease progression). After binding to both CD4 and a co-receptor, a second conformational change allows the gp41 molecule to initiate fusion between the lipid bilayers of HIV and the cell membrane. Following membrane fusion and entry, the viral core translocates into the cell cytosol where the viral genome and enzymes become uncoated. At this point, reverse transcription of the viral RNA begins, driven by the error prone viral reverse transcriptase enzyme and culminating in the production of double-stranded complementary DNA. The DNA is next transported into the cell nucleus where the viral integrase facilitates the integration of the viral DNA into genomic DNA, at which point the virus is termed a provirus. Proviral DNA is then transcribed by host cell machinery, including multiple transcription factors such as NF-κB (which are found at higher concentrations in activated cells, thus explaining the preferential infection of activated cells). Upon export from the nucleus, spliced proviral mRNA

is translated to generate viral proteins. Later in infection these viral proteins facilitate the nuclear export of unspliced viral RNAs, which are packaged along with viral proteins into new progeny virions, which bud from the host cell and proceed to infect new cells (Fig. 1-2, reviewed in (3, 9, 10)). This process allows HIV to infect and kill multiple immune cells, the majority of which are CD4+ T cells, or “helper T cells”, resulting in a major insult to the host immune system (11, 12).

The complex nature of HIV presents a particular challenge to researchers aimed at developing anti-viral therapies and vaccines. The error-prone nature of HIV reverse transcriptase results in a high rate of mutation of the viral genome leading to a heterogeneous population of virions within an infected individual, termed the viral quasispecies. Indeed, it has been estimated that in a single infected cell every possible mutation at every single base pair occurs once a day (13). This method of replication results in a high rate of mutation and variability not only within a single host but also on a population level, as evidenced by the worldwide distribution of multiple clades and subspecies of HIV (14-16). One of the regions of HIV that is most susceptible to variability is the Env protein, which is important for infection of host cells as described above, and is one of the major viral proteins targeted by the host immune system (17). In fact, the host immune response only exacerbates the mutation of HIV, as it drives immune escape in the viral quasispecies. Thus the highly variable nature of HIV, combined with the virally-mediated depletion of immune cells, makes an effective immune response capable of protecting against or clearing the virus nearly impossible.

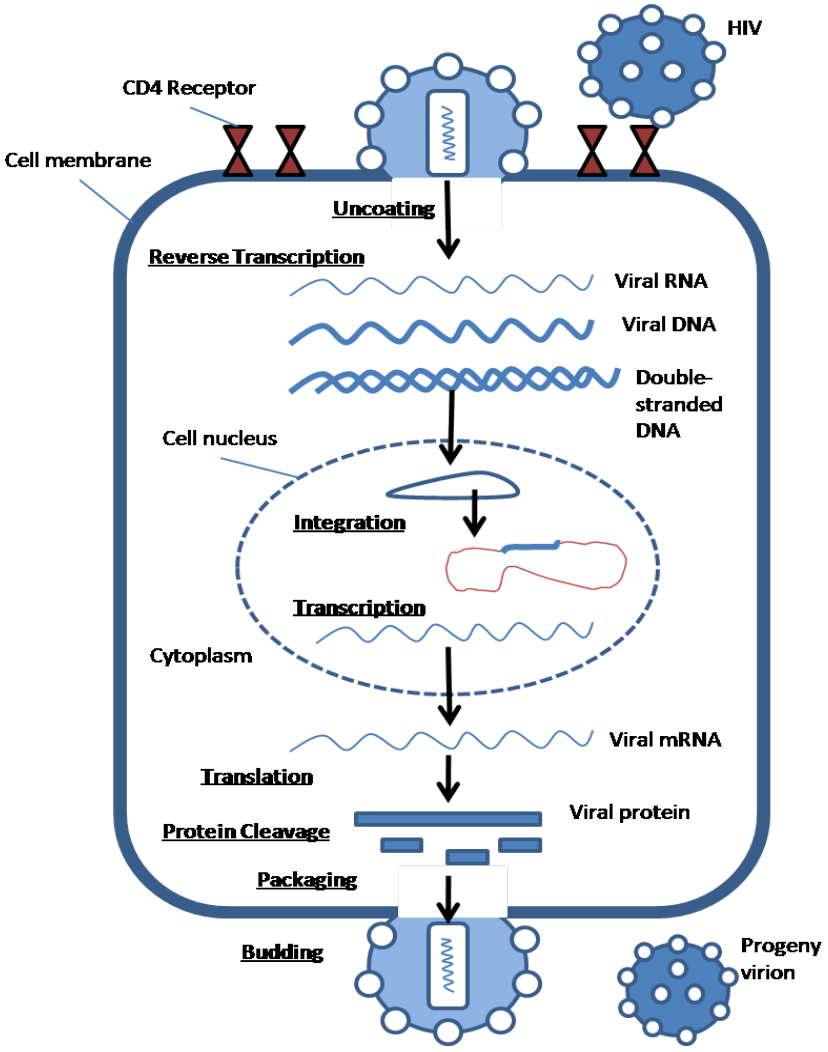

Figure 1-2 The life cycle of HIV-1.

HIV virions bind to CD4 on the surface of target cells, facilitating membrane fusion and entry. The virion is uncoated, revealing the viral RNA genome and allowing reverse transcription to commence. Double-stranded viral DNA enters the cell nucleus, where it is integrated into the host genome by the viral Integrase. Next, the host cell transcription machinery produces viral mRNA, which is exported to the cytoplasm for translation into the viral polyprotein and subsequent cleavage by the viral protease. Viral RNA and proteins are packaged and progeny virions bud from the surface of the host cell membrane.

Immune responses to HIV

While an effective immune response capable of suppressing HIV replication is not observed in the majority of infected individuals, the immune system does respond to HIV in multiple ways. However, these responses generally seem to do more harm than good, as they contribute to viral escape and overall disease pathogenesis. Studies of regular plasma donors who contract HIV have illuminated the earliest events following infection, which include increases in acute phase proteins such as serum amyloid A, and multiple cytokines coinciding with an increase in plasma viremia (18). Among the cytokines which increase in the plasma are anti-viral type-I interferons (IFNs), interleukin (IL)-15, IL-22, and the pro-inflammatory cytokines IFN-γ and tumor necrosis factor (TNF)-α, likely produced by innate immune cells such as plasmacytoid dendritic cells (pDCs), myeloid dendritic cells (mDCs), monocytes/macrophages, and natural killer (NK) cells. (11, 18, 19). Collectively, this early immune response to HIV is referred to as the "cytokine storm" and, due to the magnitude of the response, is thought to contribute to the immunopathogenesis of HIV disease rather than clearance of the virus, similar to what occurs in avian influenza virus infections (20).

The first adaptive immune responses to HIV occur in the form of antiviral $CD8^+$ T cells that are detectable during peak viral replication (10-20 days post infection) (21). As the CD8+ T cell response approaches its peak approximately 1-2 weeks later, the plasma viral load declines to reach a steady set point, suggesting that anti-viral cytotoxic T lymphocytes (CTL) help control initial virus replication (22). However, during this time viral escape mutants begin to arise in great number (23, 24). CD4+ T cells also mount an early response to HIV despite the fact that the virus

infects and depletes these cells. Again, however, this response acts as a double-edged sword, as the benefits of CD4+ T cell help for both cellular and humoral adaptive immune responses are tempered by the fact that HIV preferentially infects HIV-specific CD4+ T cells (25). Thus, the very cells designed to protect against the virus become fuel for its expansion. Finally, the antibody response to HIV infection first occurs at approximately 13-25 days post infection in the form of non-neutralizing antibodies, a majority of which are specific for gp41 and gp120. Mathematical modeling has determined that these early antibodies do not result in viral escape mutants, suggesting that they are ineffective at quelling viral replication (26). Antibodies capable of neutralizing autologous virus do not appear until 12 weeks post infection. However, these more potent antibodies contribute significantly to HIV immune evasion, as neutralization-resistant viral variants become rapidly abundant (27). The rapid immune escape of HIV from anti-viral CTL and antibodies begets a compensatory increase in immune cells specific for new viral variants, beginning a distressing cycle between the host immune system and the virus which persists throughout infection. Ultimately, this cycle contributes to immune dysfunction and exhaustion and underlies a major challenge of developing effective vaccines for HIV.

Following acute infection, HIV-infected individuals who are untreated generally maintain a state of clinical latency for months to up to ten years. The term “clinical latency” denotes the absence of outward symptoms in spite of a vigorous ongoing internal immune struggle. Eventually, the immune system reaches a point of no return, generally coinciding with a depletion of peripheral CD4+ T cells to below 200 cells/μl blood, and the individual presents with signs of illness and is diagnosed

with AIDS. The first symptoms of AIDS usually include moderate and unexplained weight loss, recurring respiratory tract infections (such as sinusitis, bronchitis), prostatitis, and skin rashes. At this stage, HIV-positive individuals are more susceptible to opportunistic infections, including oral thrush, tuberculosis, and pneumonia. During AIDS, patients also develop other protozoal, bacterial, viral and fungal infections as well as neurological disorders and malignancies. Indeed, prior to the advent of anti-retroviral therapies (discussed below), HIV infection meant certain death.

HIV-infected non-progressors

Though the vast majority of HIV-infected patients are incapable of controlling virus replication on their own, a small group of individuals (5 to 15 %) remain clinically and immunologically stable for years without the need for anti-viral therapy (28-31). These long-term non-progressors exhibit low to moderate viral loads and relatively stable CD4+ T cell counts. A sub-group of the long-term non-progressors, termed elite controllers, is able to suppress viral loads to undetectable levels and remain healthy without therapy. Genetic analyses have identified a correlation between HIV disease progression and certain alleles of the human major histocompatibility complex (MHC), also known as the human leukocyte antigen (HLA). Both rapid and slow progressors have been routinely found to have specific HLA alleles that correlate with differing rates of disease progression. Specifically, the most notable correlations include HLA-B27 and B57 alleles, which correspond to delayed progression to AIDS, and HLA-B35 and the HLA-B7 supertypes, which

correspond to more rapid progression to AIDS(32, 33). Furthermore, there are several studies that report similar associations between disease progression in SIV-infected non-human primates in correlation with specific Mamu alleles (34). In addition, associations have been identified between delayed HIV disease progression and the expression of the NK cell receptors KIR3DS1 and KIR3DL1 in combination with particular HLA class I alleles (35, 36). These associations provide strong evidence that CD8+ T cells and NK cells can play an important role in antiviral immunity. However, it is important to note that the vast majority of HIV-infected individuals maintain high viral loads and progress to AIDS. Moreover, follow up has revealed that most non-progressors eventually develop AIDS (37-40). These studies demonstrate that maintaining control of HIV replication, even for several years, is not sufficient to avoid developing AIDS and opportunistic infections.

Anti-retroviral therapies and vaccine candidates for HIV

One of the most important advances in HIV research was the development of anti-retroviral therapies (ART), which are associated with decreased mortality and morbidity in HIV-infected individuals(41-43). Azidothymidine (AZT) was the first drug approved for the treatment of HIV/AIDS in 1987 (44). However, due to the high mutation rate of HIV, it did not take long until AZT-resistant viruses were identified. It became clear that successful therapy would require a combination of drugs, known as highly active antiretroviral therapy (HAART). Three or four drugs from different classes are usually taken during HAART, which first became available in 1996. Patients with advanced HIV disease who began a

HAART regimen experienced dramatic decreases in morbidity and mortality (45). The combination of drugs slows the emergence of drug-resistant mutations by inhibiting several different stages of the viral life cycle at the same time. More than twenty ART have been developed which decrease viral load by acting directly or indirectly against the virus. The antiretrovirals used in HAART can be classified by the mechanism of action of the drug:

- Nucleoside and nucleotide reverse transcriptase inhibitors (nRTI) inhibit viral reverse transcription by incorporation into the newly synthesized viral DNA, thus preventing further elongation.
- Non-nucleoside reverse transcriptase inhibitors (NNRTI) inhibit the viral reverse transcriptase by binding directly to the enzyme and impairing its function.
- Protease inhibitors (PIs) target viral assembly by inhibiting the activity of protease, an enzyme used by HIV to cleave nascent proteins for final assembly of new virions.
- Integrase inhibitors inhibit the enzyme integrase, which is responsible for integration of viral DNA into the DNA of the infected cell.
- Entry (or fusion) inhibitors interfere with binding, fusion and entry of HIV-1 to the host cell by blocking one of several targets.

While the advent of ART dramatically extended the life expectancy of HIV-infected patients, these drugs are expensive and require complex daily regimens, making adherence challenging. This is particularly true in developing countries, where more than 90% of all HIV infections exist yet life-saving anti-retrovirals are not available in numbers sufficient to treat those in need (46, 47). Also, because of the extensive variability of HIV, escape mutations against ART drugs are common. Thus patients need to

be closely monitored and drug regimens must be changed often(48). Furthermore, the majority of these antiretroviral drugs have toxic side effects, making daily adherence difficult for patients(49). These factors make the effectiveness of ART difficult to achieve. Thus an alternative therapy or vaccine is crucial in order to better treat and/or prevent HIV infection worldwide.

The long sought after goal of a vaccine for HIV remains the holy grail of HIV research. After more than two decades of research, no vaccine to date has been soundly efficacious in humans. The goal of an effective vaccine is to stimulate an immune response against a specific antigen (i.e. HIV) and promote the development of a memory response. Most successful vaccines elicit primarily B cell responses in the form of antibodies, and secondarily, T cell responses in the form of antigen-specific memory T cells (50, 51). B cells become activated and differentiate into plasma cells that produce neutralizing antibodies (nAb) which can quickly neutralize pathogens upon a secondary exposure to antigen, while CD8+ T cells (often referred to as “cytotoxic T cells,” or CTLs) can directly kill virus-infected cells. Effective memory CD8+ T cell responses can quickly clear viral infections upon secondary exposures (52). CD4+ T cells, or “helper T cells” help stimulate B cells and CD8+ T cells by producing factors that support growth and differentiation of these cells (52). Thus, historically, vaccinations that induce responses from these cell types have been efficacious in protecting against infection (i.e. measles, mumps, rubella, small pox, rabies, flu, etc.) (53). In the case of HIV infection, typical vaccination strategies have failed to elicit complete control in human trials. In the past 25 years, multiple constructs aimed at eliciting B and T cell responses have been tested for safety and efficacy,

including whole inactivated virus, envelope subunit protein, Gag, Pol, and Nef proteins, and expression vectors ranging from canarypox to adenovirus (Reviewed in (54, 55). None have succeeded. Among the many challenges to developing an effective AIDS vaccine is the fact that the major target for HIV infection is CD4+ T cells, specifically HIV-specific CD4+ T cells (25, 56, 57). Thus a vaccine that induces proliferation of these cells would likely be more harmful than helpful, by generating excessive targets for viral infection. Additionally, the extreme variability of HIV, including multiple clades and subspecies, presents a challenge to developing a single vaccine capable of protecting against the variety of viral strains worldwide. Studies have also demonstrated that superinfection occurs frequently, suggesting that immune responses to one strain of HIV (vaccine-elicited or not) does not guarantee protection from another (54). As a testament to these challenges, in September 2007, results from a large, highly publicized trial of a CTL-based HIV vaccine sponsored by Merck showed that the vaccine candidate did not reduce the incidence of new infections and did not have any effect on viral loads in those participants who contracted HIV(55, 57). The failure of the Merck vaccine, once referred to by leaders in the HIV field as the best hope going forward, was a major blow to HIV vaccinology and prompted a “back to the drawing board” mentality aimed at redoubling basic research efforts to understand the complex interaction between HIV and the immune system and identify the correlates of immune protection in lentiviral disease.

More recently, however, there is cause for hope on the horizon for HIV vaccines. In September 2009, precisely two years after the Merck announcement, promising results were released from a large phase III

vaccine trial in Thailand co-sponsored by the U.S. National Institutes of Health and Walter Reed Army Medical Center. The Thai vaccine, named RV144, utilizes a prime-boost strategy consisting of two earlier vaccine candidates—the ALVAC canarypox expression construct encoding HIV Gag, Pol, and Env (prime; aimed at eliciting CTL) and the AIDSVAX gp120 protein subunit (boost; aimed at eliciting antibodies). Despite the fact that neither ALCAC nor AIDSVAX proved efficacious when administered separately (58, 59), the combination of the two in RV144 resulted in a 31% reduction of new HIV infections in vaccinated volunteers (compared to placebo) (60). These results are modest, but RV144 is the first HIV vaccine candidate in history to yield any degree of efficacy. The immunologic basis for protection by RV144 is currently under investigation but is speculated to result from vaccine-elicited antibodies (61).

Non-human Primate Models for AIDS and the Origin of HIV

The field of HIV research would not be where it is today without the non-human primate simian immunodeficiency virus (SIV) model. Multiple species of non-human primates of Asian and African origin are utilized to study both pathogenic and non-pathogenic infections with SIV (Table 1). In the case of pathogenic models, which primarily utilize the Asian rhesus and pig-tailed macaques, infection with SIV results in a disease course very similar to HIV-1. Acute SIV infection is characterized by the loss of CD4+ T cells in mucosal tissues and high peak viral load (62). SIV replication typically peaks in the plasma between 10-17 days post-infection, before reaching set-point levels, which are comparable to HIV infection in humans (63). Since SIV also replicates predominantly in

CD4+ T cells, there is a massive depletion of CD4+ T cells from the mucosal associated lymphoid tissues (MALT) during both acute and chronic infections, as observed in humans(64-66). Chronic infection is characterized by high levels of immune activation (discussed below), chronic high levels of virus replication, and a progressive loss of CD4+ T cells in the periphery, after which these animals eventually develop opportunistic infections and succumb to AIDS (67-70). These pathogenic models afford the opportunity to study multiple aspects of lentiviral infection which would not be possible in HIV-infected patients. For example, experiments with non-human primates allow researchers to study the earliest events of HIV transmission and infection. They also permit access to multiple tissue samples that would be difficult to obtain from human subjects. Finally, the pathogenic SIV macaque model is an important testing ground for new therapies and vaccines prior to administration to humans.

The pathogenic SIV models arose from the cross-species transmission of SIVsmm from captive sooty mangabeys to captive rhesus macaques (71). Likewise, a zoonotic transmission from sooty mangabeys to humans gave rise to the HIV-2 epidemic, which is geographically confined to West Africa (71). The HIV-1 pandemic arose following a cross-species transmission of SIVcpz from wild chimpanzees (*Pan troglodytes*) to humans, most likely as a result of the regular butchering of chimpanzee meat (72). Genetic analysis suggest that three independent cross-species transmission events occurred, giving rise the HIV-1 groups M, N, and O, with the earliest event occurring in 1908 near modern-day Kinshasa, Democratic Republic of the Congo (73). Currently, Group M (main) comprises the largest group of HIV-1 and accounts for the majority

of infections worldwide; group O (outlier) contains strains that are highly divergent and are localized to equatorial Africa (74); and group N (non-M, non O) contains only a small number of strains localized to Cameroon (75). Utilizing knowledge of the origins of HIV is a valuable tool for better understanding HIV pathogenesis in humans by allowing extensive *in vivo* studies to be performed in the different models for HIV infection.

Table 1-1. Commonly studied non-human primate models of AIDS

	Species	Origin	Virus
Nonpathogenic/ Natural Hosts of SIV	Sooty Mangabey (*Cercocebus atys*)	Western Africa	SIVsmm
	African Green Monkey (*Cercopithecus aethiops*)	Central Africa Caribbean	SIVagm
	Mandrill (*Papio sphinx*)	Western Africa	SIVmnd
Pathogenic/ Non-natural Hosts of SIV	Rhesus Macaque (*Macaca mulatta*)	Asia: China and India	SIVmac SIVsmm SHIV
	Pig-tailed Macaque (*Macaca nemestrina*)	Southeast Asia	SIVmac SHIV

Natural, non-pathogenic SIV infection of sooty mangabeys

Over thirty non-human primate species in Sub-Saharan Africa are naturally infected with SIV, and collectively these animals are termed the natural hosts of SIV (72, 76, 77). SIV infection of the natural hosts is sexually transmitted and lifelong, yet unlike pathogenic SIV/HIV infection, there is generally no pathogenic consequence of lentiviral replication in these species. Interestingly, these natural host species are able to avoid progression to AIDS while replicating SIV at levels comparable to those found in HIV+ patients (78). Studies aimed at understanding natural SIV infections have also allowed important insight into pathogenic SIV/HIV infections. Among the most studied natural hosts species are sooty mangabeys, found in coastal forests of West Africa, and African Green monkeys (AGMs; *Cercopithecus aethiops*), which inhabit sub-Saharan Africa and the Caribbean (Caribbean AGMs were brought over from Africa as part of the slave trade and have established thriving populations) (Table 1) (71). The virologic and immunologic parameters of SIV infection in sooty mangabeys and AGMs are quite similar, though the focus of this chapter will be sooty mangabeys. SIVsmm infection of sooty mangabeys occurs at sexual maturity (approximately 4-5 years of age) and is common both in the wild and in captivity (79-81). SIV-infected sooty mangabeys generally maintain normal CD4+ T cell counts and rarely progress to AIDS (only one reported case to date), despite chronic high levels of virus replication (80, 82). Understanding how these animals remain healthy despite viral loads consistent with progression to AIDS in other primates has yet to be completely clarified; however, important insights into natural infection of SIV have been made.

One method for understanding how SIV-infected natural hosts remain free of AIDS is to identify the features of nonpathogenic infection which are both similar and distinct from pathogenic infection. SIVsmm infection of sooty mangabeys shares some similarities with pathogenic SIV/HIV infections. SIV infection of sooty mangabeys results in levels of virus replication that are similar to plasma levels of virus observed in pathogenic SIV infection of rhesus macaques and HIV infection in humans (Fig. 1-3) (79). The high viral load in these animals indicates that sooty mangabeys may have an ineffective SIV-specific CTL response, and it indeed has been observed that sooty mangabeys have very low levels of SIV-specific CD8+ T cell responses (83). It has also been found that more than 90% of SIV replication in sooty mangabeys occurs in short-lived cells, indicating that CD4+ T cells are the major site for viral replication, similar to pathogenic models (84). While most SIV-infected sooty mangabeys maintain healthy levels of peripheral CD4+ T cells, there is an acute and severe mucosal CD4+ T cell depletion that persists in both models of infection (84, 85). Importantly, a subset of SIV-infected sooty mangabeys experiences peripheral CD4+ T cell depletion to levels comparable to HIV-infected patients with AIDS (<200 cells/μl blood) (86, 87). Yet even these CD4-low sooty mangabeys remain clinically healthy and free of opportunistic infections. Despite these characteristics of SIVsmm infection that are similar to pathogenic infections, sooty mangabeys stay healthy and rarely progress to AIDS (79, 80). This is most likely due to the differences observed between pathogenic and natural infections. Despite their high SIV viremia, depletion of CD4+ T cells is rarely observed in the peripheral blood and lymph nodes of sooty mangabeys (88). Sooty mangabeys have a general lack of cell cycle dysregulation and T cell apoptosis, and have normal T cell regeneration (88-90). One of the most

striking characteristics of natural infection, though, is the lack of immune activation, which is one of the major correlates of disease progression in pathogenic models (64, 87). In SIV infection of sooty mangabeys, there is an inverse correlation between CD4+ T cell counts and the amount of activated T cells (78). However, many aspects of natural SIV infection of sooty mangabeys are still not well understood, including the determinants of viremia and the role of CD4+ T cells and their effect on viremia. Specifically, in naturally SIV-infected sooty mangabeys, do CD4+ T cells play their canonical helper role, thus contributing to protection from AIDS, or are CD4+ T cells driving viremia by providing the main site for viral replication? If CD4+ T cells are a determinant of viremia, how do these cells maintain homeostasis in the face of virus replication? A more definitive understanding of the determinants of viremia during natural SIV infection of sooty mangabeys may clarify how these animals remain disease-free despite highly replicating SIV. Further understanding the mechanisms underlying maintenance of CD4+ T cell homeostasis and lack of disease progression during natural infection of SIV may give us insight into how we can more effectively treat and prevent HIV disease progression in humans.

One very useful model for studying the contributions of CD4+ T cells, immune activation, and high viremia to HIV/SIV pathogenesis is the CD4-low SIV-infected sooty mangabeys. These animals can be found within the naturally SIV-infected sooty mangabey colony at the Yerkes National Primate Research Center (87) as well as within a cohort of mangabeys infected via plasma transfer that subsequently developed a multitropic (CCR5/CXCR4/CCR8) infection (86). The depletion of CD4+ T cells in the peripheral blood, lymph nodes and GALT in these mangabeys

did not result in any evidence of immune activation and no clinical signs of simian AIDS, suggesting that the preservation of total CD4+ T cells is not required for maintaining low levels of immune activation in the SIV-infected natural host monkey species. The identification of SIV-infected natural hosts that remain free of simian AIDS even when CD4+ T cell levels are below levels normally considered to be 'AIDS defining' was difficult to reconcile with the central role of CD4+ T cells in the immune system. Recent findings have begun to unravel how the natural hosts are able to maintain low levels of immune activation and healthy immune systems even when the CD4+ T cell levels are depleted. First, there may be a shift in which cells are infected in natural hosts due to the significantly low levels of CCR5 coreceptor expression on some subsets of CD4+ T cells in natural SIV hosts (91). This low level of CCR5 expression may restrict viral replication to those CD4+ T cells that are in a more advanced stage of activation and/or differentiation (i.e. - effector cells). As such, restricting the expression of CCR5 to activated CD4+ T cells may preserve the homeostasis of the pool of "resting" naïve and memory CD4+ T cells while supporting high levels of virus production. In this way only the effector CD4 cells are likely to die as a consequence of activation-induced cell death (AICD) (74). It follows that the preserved pool of naïve and central memory T cells would be available to replenish the effector memory T cell repertoire, which would provide a continuous source of these important helper T cells capable of contributing to immune responses to both SIV and other pathogens, as well as a steady source of viral target cells (64, 92). Second, there may be cells in the natural host that can perform functions of CD4+ T cells but do not express the CD4 protein on their surface. Indeed, assessment of the AGMs identified a population of CD3+ cells that did not express CD4 but did express the

CD8-α subunit (93). In addition, sooty mangabeys have a population of CD3+ T cells that do not express CD4 or CD8 (double negative T cells) (86). The presence of these CD3+ cells that are refractory to infection, yet perform functions of CD4+ T cells, represent yet another adaptation for how the natural hosts have evolved to circumvent simian AIDS even after many years of SIV infection. Third, although the GALT CD4+ T cells are depleted during the acute phase of SIV infection of natural hosts, this CD4 depletion is qualitatively different from that observed during pathogenic HIV/SIV infection (94-96). In particular, the preferential preservation of Th17 T cells may be critical for maintaining mucosal health in SIV infected natural hosts (94-96). In summary, these findings regarding CD4 and CD4-like T cell populations suggest that natural hosts have methods for preserving their critical helper T cell populations, or at least maintaining helper function in the context of CD4+ T cell depletion.

Viral determinants of nonpathogenic SIV infections of natural hosts

Recent findings suggest that African monkeys have been naturally infected with SIV for at least 32,000 years (97). This lengthy relationship begs the question of what impact the primate immune systems have had on the viruses with which they co-evolved. Little is known about the genetic evolution of SIV; however there is evidence to suggest that the lengthy relationship between African monkeys and SIV has not resulted in a dramatic attenuation of the virus. Namely, passage of SIVsm from sooty mangabeys, in which infection has a nonpathogenic outcome, to rhesus macaques results in a pathogenic disease course (71). More likely, African monkeys have experienced an adaptation of the interaction

between the immune system and the virus. In particular, the ability of the host to minimize aberrant immune activation is a key feature of nonpathogenic SIV infection (78, 98). Regardless, there remains significant diversity between primate lentiviruses, which have been categorized into four groups: SIVagm, SIVsmm/mac/HIV-2, SIVmnd, and HIV-1. Assessment of the *pol* gene has revealed only 55-60% amino acid identity between each group (71). However, there is currently no evidence to suggest that specific sequences within each viral group are associated with particular disease outcomes (ie- nonpathogenic vs. pathogenic). The precise viral contributions to nonpathogenic disease outcomes in SIV-infected natural hosts remain to be determined.

Immune activation and HIV/SIV pathogenesis

A striking distinction of SIV infection in the natural hosts is the absence of aberrant immune activation during chronic infection despite viral loads rivaling those of pathogenic HIV and SIV infections (Fig. 1-3) (78, 79, 99, 100). Comparative studies of nonpathogenic and pathogenic infections have revealed multiple differences in the parameters of immune activation and have offered strong support for the role of immune activation in HIV/SIV disease pathogenesis. Early descriptions of immune activation in HIV-infected patients included high levels of plasma IFN-α, increased expression of the IL-2 receptor on lymphocytes, and increased expression of the activation markers CD38 and HLA-DR on CD8+ T cells, (101, 102). It was noted then that these abnormalities are found at elevated levels in HIV-infected patients and that patients at more advanced stages of disease have the highest levels (101). Indeed, Giorgi et al found that

disease progression was more closely correlated with the levels of CD8+ T cell activation than with viral load (103). Presently, excessive proliferation, activation, and/or dysfunction has been described in almost every immune cell type during chronic pathogenic infection, including T cells, B cells, NK cells, macrophages, and neutrophils (104-107). Additionally, high levels of pro-inflammatory cytokines and chemokines can be detected in the plasma during pathogenic HIV/SIV infections.

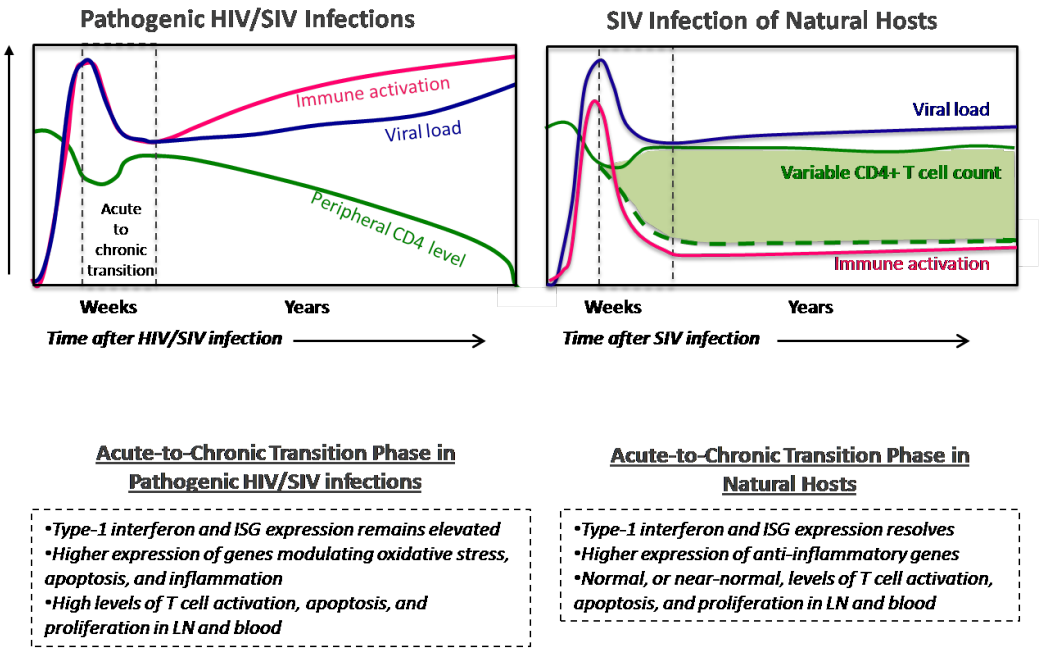

Figure 1-3. Comparison of pathogenic and non-pathogenic HIV/SIV disease courses.

Summary of the differences between pathogenic HIV/SIV infections and SIV-infected natural host monkey species, with specific emphasis on the acute-to-chronic transition phase of infection. Within the graphs the blue line represents typical viral load levels, the green line and shaded green area represent typical CD4+ T cell levels, the red line represents general systemic immune activation which can be measured in a number of ways including the activation state of the immune cells and levels of interferon stimulated genes (ISGs). Vertical dotted lines indicate the time of acute-to-chronic transition which generally occurs between approximately 21 and 40 days. Some specific differences in the acute-to-chronic phase between pathogenic and natural host infections are listed within the dashed boxes below the graphs.

The causes of this widespread immune cell activation are not fully elucidated but likely are multi-factorial. Potential contributing factors include the direct effects of the virus infection, both through virally-mediated killing of CD4-expressing cells and the adaptive immune response to HIV. Certainly, the initial dramatic depletion of CD4+ T cells during acute infection leads to a homeostatic burst of proliferation which could be both helpful, in that it provides effector T cells, and harmful, in that it contributes to an activated immune environment and provides more targets for infection (11). It has also been suggested that the virally-mediated killing of CD4+ T-regulatory T cells (Treg) circumvents the role of Tregs in resolving immune activation (98, 108, 109). The direct effects of the virus cannot account for the overall frequency of activated cells observed in HIV-infected patients, however, as the proportion of CD4+ cells that are infected is quite low (< 1%) (110) as are the numbers of HIV-specific T cells (11). Indirectly, the virus may contribute to immune activation through protein interactions with innate immune cells, which elicit cytokines such as IFN-α and TNF-α (111). It has also been shown that, unlike its ancestral primate lentiviruses, the Nef protein of HIV-1 has lost the ability to down-regulate T cell receptor (TCR) expression on the surface of infected cells, which may contribute to TCR-mediated activation of T cells (112). However, the virus is unlikely to be the singular culprit of HIV-associated immune activation, as initiation of anti-retroviral therapy (ART) reduces the viral burden but does not reduce immune activation in all patients (113, 114). Moreover, the natural hosts of SIV maintain persistently high levels of virus in the absence of chronic immune activation (88, 115), while the same virus results in widespread increases in immune activation in non-natural Asian macaques (71, 72). An additional contributor to chronic immune activation is the stimulation of

immune cells by non-viral TLR ligands originating from the intestine and translocating across a mucosal barrier severely compromised by virally-mediated CD4+ T cell depletion (98). Indeed, early during infection, approximately 20% of GALT CD4+ T cells are infected and another 60% of GALT CD4+ T cells become activated and die by apoptosis (11, 116). Thus, approximately 80% of GALT CD4+ T cells are depleted within the first three weeks of HIV infection, constituting a major blow to the mucosal immune system (117). Evidence for the role of microbial translocation in HIV-associated immune activation came in the form of measured increases in bacterial lipopolysaccharide (LPS) in the plasma of infected patients that were correlated with increases in immune cell activation and plasma IFN-α (118). These findings have been recapitulated by several groups and in the pathogenic SIV macaque model (119). Moreover, SIV-infected sooty mangabeys, which have low levels of chronic immune activation, have no increase in plasma LPS during chronic infection (118). Thus, microbial translocation, along with the direct and indirect effects of the virus, contributes to increases in aberrant immune activation during pathogenic infection.

Given the dramatic difference in chronic phase immune activation status between nonpathogenic and pathogenic infections, it is perhaps more striking that these diverse disease courses both initiate with increases in immune activation during the acute phase of infection (Fig. 1-3) (115). During acute SIV infection, T cell proliferation occurs rapidly within 1-5 weeks post infection in both rhesus macaques and sooty mangabeys, but only sooty mangabeys return to baseline during chronic infection (120-122). In both blood and LN, the expression of ISGs by CD4+ T cells is increased during acute infection of both rhesus macaques

and AGMs. However, AGMs show a decline in these genes by day 28 while rhesus macaques maintain elevated expression (123). While acute phase increases in immune activation are likely linked to the development of anti-viral adaptive immune responses, the degree of immune activation may not correspond to the magnitude of immune response in both types of host. Specifically, though rhesus macaques and sooty mangabeys display comparable levels of IFN-γ-producing cells during acute infection, rhesus macaques display markedly higher levels of CD8+ T cell proliferation (124). The mechanisms by which SIV-infected natural hosts resolve acute phase immune activation while HIV-infected patients and SIV-infected macaques go on to suffer chronic increases are unclear but likely include programmed death receptor-1 (PD-1), which is increased on sooty mangabey T cells at 14-30 days post infection coincident with a decline in T cell activation (122).

Recent studies assessing global changes in gene expression during both pathogenic and nonpathogenic SIV infection have provided a detailed view of the immunologic events occurring during the acute phase. These studies illustrate that both progressive and nonprogressive hosts experience dramatic changes in the expression of genes associated with initiating innate and adaptive immune responses. However, distinct from rhesus macaques, only AGMs and sooty mangabeys show modulations of genes involved in restricting inflammation and proliferation. During acute SIV infection of sooty mangabeys, Bosinger et al. detected widespread upregulation of genes involved in cellular immunity, such as granzyme A, immunoglobulin genes, and chemokines responsible for T cell trafficking (125). SIV-infected rhesus macaques also upregulated genes involved in innate and adaptive immune responses, however, the peak of gene

expression did not occur until day 14, compared to an earlier peak at day 10 in sooty mangabeys (125). Moreover, expression of this gene family resolved to baseline levels during chronic infection of sooty mangabeys, whereas expression remained elevated in chronically infected rhesus macaques. Lederer et al. observed that the majority of gene expression changes during SIV infection occurred in the lymph nodes (LN) of both AGMs and PTMs (126). At day 10, both PTMs and AGMs experienced upregulation of IFN-α signaling, but only AGMs resolved this response by day 45. Likewise, SIV-infected sooty mangabeys experienced early increases in type I interferon signaling, including RIG-I, IRF-3, and IRF-7 (125). When comparing acute and chronic time points among AGMs and PTMs in the LN, blood, and colon, the differences in gene expression were consistently higher at day 10 (126). Specifically, PTMs had higher expression of genes involved in oxidative stress, apoptosis, and inflammation, including neutrophil chemotaxis and degranulation, and chemokines such a CXCL9 and CXCL10. Interestingly, the increase in these gene transcripts at day 10 in PTMs correlated with higher levels of CD8+ T cell proliferation (96, 126). In contrast, AGMs had higher expression of genes involved in modulating the cell cycle, and restricting both inflammation and Th1 responses, including IL-10. Similar results were seen in sooty mangabeys, in which the genes for immunoregulatory proteins which contribute to controlling T cell proliferation and activation, such as CD274/PDL1 and IDO1/INDO, were upregulated at days 7 and 10 post infection (125).

Immune cell contributions to immune activation

One of the key topics of research with regard to HIV/SIV infection is to understand how the acute phase increase in immune activation is resolved in the natural hosts while hosts of pathogenic infection go on to maintain high levels of activation throughout infection that are closely correlated with disease progression. HIV/SIV infections have traditionally been divided into two segments: the initial acute phase and the chronic phase that follows. Acute infection in both pathogenic and natural SIV hosts display striking similarity in the majority of viral and immune parameters (86, 124, 127-131). Chronic infection, however, is markedly different in pathogenic and nonpathogenic infections. Specifically, during chronic infection the natural hosts of SIV generally maintain healthy levels of peripheral CD4+ T cells, low levels of aberrant immune activation, and no clinical signs of AIDS. The apparent departure between pathogenic and nonpathogenic disease outcomes evident during chronic infection despite generally similar acute phases has ignited interest in the immune and viral events which occur during the transition between these two phases, termed the acute-to-chronic transition phase. To better understand this transition phase, numerous studies have focused on defining the roles of individual immune cell subsets and their contributions to resolving or potentiating an activated immune environment during the acute-to-chronic transition phase. We hypothesize that how the host deals with this transition predicts the overall outcome of the disease. The following sections will summarize relevant data to describe the role of various immune cell subsets in HIV/SIV immune activation, and postulate mechanisms by which natural hosts may control or evade long-term, pathogenic activation.

CD4+ T cells

CD4+ T cells serve a key role in eliciting both humoral and cellular immune responses and are likely to play an important role in HIV/SIV-induced immunologic activation. Their key role in shaping the adaptive immune response makes them a potential source of immune activation, as the antiviral immune response is persistent and dynamic in the context of a chronic infection such as HIV/SIV. The ability of these cells to produce inflammatory cytokines and chemokines in response to antigen recognition adds to their potential to mediate immune activation. Of course, the potential contributions of this cell population to immune activation are balanced by the fact that CD4+ T cells are depleted by HIV/SIV infection. Therefore, understanding the role that CD4+ T cells might play in eliciting immune activation requires an understanding of how HIV/SIV infection impacts these cells during the progression to AIDS, as well as in the nonprogressive SIV infection of natural hosts.

The assessment of CD4 depletion following SIV infection in the natural host models provides insights to the similarities and differences between pathogenic and nonpathogenic infections. If the depletion of CD4+ T cells was sufficient to induce the chronic phase immune activation observed during pathogenic infections then one would predict that the natural hosts would maintain healthy CD4+ T cell levels in association with the low levels of immune activation. During the acute phase of SIV infection a decrease in CD4+ T cell levels can be observed associated with the early peak in viral replication in both sooty mangabeys and AGMs (132, 133). Still, these reduced CD4+ T cell levels are generally within the 'healthy' range (above 500 cells/μl of blood) for SIV-infected sooty mangabeys and are normally maintained throughout the disease course

(78, 130). In SIV-infected AGMs, CD4+ T cell levels generally rebound to near baseline levels during the transition from acute to chronic infection, providing further evidence for the immune recovery that occurs during this phase of the infection (131, 134). In contrast, the CD4+ T cells within the gut associated lymphoid tissue (GALT) of sooty mangabeys and AGMs undergo a rapid decrease following SIV infection, similar to that observed during pathogenic infections (131, 132). Indeed, the GALT CD4+ T cells remain low throughout the chronic phase of the infection in natural host infections. One might hypothesize that in the context of massive GALT CD4+ T cell depletion maintaining the peripheral blood and lymph node associated CD4+ T cells is sufficient for maintaining low levels of immune activation. However, a subset of SIV-infected sooty mangabeys maintains low levels of immune activation despite global depletion of CD4+ T cells in the blood, LN, GALT, and BAL (86, 87). Hence, preserving CD4+ T cell levels may not be a critical factor in resisting increases in immune activation.

The acute-to-chronic phase shift in immune activation may also be modulated by regulatory T cells (T-regs). Studies have found maintained or increased levels of T-regs during acute SIV infection of natural hosts (134, 135). Kornfeld et al. found increases in Foxp3 gene expression concurrent with increased plasma TGF-beta within 24 hours of SIV infection of AGMs (134). This early increase was followed by an increase in plasma IL-10 at day 6. Possibly, the presence of T-reg cells early during SIV infection contributes to establishing an anti-inflammatory environment during acute infection which suppresses the immune activation at later stages (134). However, there is no direct correlation between T-reg levels and the resolution of immune activation in natural hosts (136).

Specifically, both PTMs and AGMs experience increases in lamina propria T-regs at the same time as colonic immune activation increases (45 dpi), suggesting that these cells are not controlling immune activation in either pathogenic or nonpathogenic infection (96). Other studies suggest that during acute pathogenic infection the ratio of T-reg to other cell subsets is most important (96). In SIV-infected PTMs, a specific depletion of Th17 cells and a concurrent increase in FoxP3+ T-regulatory cells during the first 10 days of infection led to a perturbed ratio of these subsets. By day 45, PTMs with lower Th17:T-reg ratios had higher levels of T cell activation. In contrast, both Th17 and T-reg cells were preserved in SIV-infected AGMs and there was no correlation between the Th17:T-reg ratio and T cell activation. A similar preservation of Th17 cells was observed in the blood and GI tract of SIV-infected sooty mangabeys whereas HIV patients had significant depletions in this subset (95). These findings suggest a potential role for the CD4+ T-reg cells, although it is likely that both the timing of the T-reg activation as well as the cellular milieu that is present will influence the ability of T-regs to elicit a beneficial or detrimental effect with regard to acute-to-chronic phase immune suppression as well as the disease outcome.

CD8+ T cells

A role for CD8+ T cells in controlling HIV/SIV infection was initially suggested by experiments demonstrating that (1) autologous CD8+ T cells are able to suppress *ex vivo* HIV replication (137), (2) there is a temporal correlation between the development of antiviral CD8+ T cells and post-peak viral decline (22), and (3) depletion of CD8+ cells in the SIV macaque model resulted in rapid increases in viral replication (138). In

natural hosts, the contribution of CD8+ T cells is less clear, as CD8+ cell depletion in SIV-infected sooty mangabeys results in only minor increases in viral load which may result from the depletion-associated increase in CD4+ T cell activation rather than the lack of CD8+ cells (139). More recently, Schmitz et al. assessed the contribution of adaptive immune responses to a nonpathogenic outcome by administering antibodies to deplete both CD8+ and CD20+ cells during the first two weeks of $SIV_{agmVer90}$ infection in PTMs and AGMs (140). This dramatic depletion of the effector cells responsible for both arms of adaptive immunity had dramatically different outcomes in the two species. PTMs experienced a one log increase in peak viral loads and approximately a four-log increase in set point viral loads following lymphocyte depletion (140). These animals exhibited rapid disease progression, including CMV reactivation. In contrast, depletion of CD8+ and CD20+ cells in AGMs had no effect on peak viral load and only a small delay in post-peak decline compared to control animals and all AGMs remained clinically healthy (140). A similar delay in post-peak viral decline was observed in a separate study in which CD8+ cells were depleted during acute SIV infection of AGMs (141). Moreover, when only B cells were depleted in acutely SIV-infected AGMs, there was no effect on viral load, suggesting that CD8+ cells but not CD20+ cells exert minor control of viral replication during acute infection of natural hosts (142). Importantly, these studies demonstrate that delaying the initiation of CTL and antibody responses has no impact on the nonpathogenic outcome in SIV-infected natural hosts. These findings are supported by earlier studies demonstrating that the magnitude of SIV-specific adaptive immune responses is comparable between natural hosts and pathogenic HIV/SIV-infected hosts and that depletion CD8+ T cells in SIV-infected sooty mangabeys has a limited effect on viral load (83, 124,

139, 143, 144). Moreover, these studies point to the possibility that the natural hosts of SIV have evolved immune strategies to deal with SIV infection which act independently of CTL and antibody responses. These strategies may include the ability to limit chronic immune activation, the paucity of CCR5+ CD4+ target cells, and downregulation of CD4 on CD4+ T cells, all of which may prevent increases in viral replication (11, 88, 91).

We previously described the role of interleukin 7 (IL-7) in T cell homeostasis of SIV-infected rhesus macaques and sooty mangabeys (121, 130). We found that SIV infection resulted in an early decline of CD4+ T cells in both rhesus macaques and sooty mangabeys (between weeks 1-5). However, only sooty mangabeys experienced an immediate compensatory increase in plasma IL-7 (121). This IL-7 response was followed by an increase in T cell proliferation, as measured by Ki67 expression, and resulted in a stabilization of both CD4+ and CD8+ T cell levels that was maintained throughout the study period (37 weeks) (121). In contrast, despite similarly early declines in T cell levels, SIVmac239-infected rhesus macaques did not display increases in plasma IL-7 until 20 weeks post infection, a delay of approximately 15 weeks relative to sooty mangabeys (121, 130). By 20 weeks post infection and in the context of high viral replication (between 10^5-10^8 viral copies/mL plasma), the increases in IL-7 were unable to overcome the low levels of T cells in SIV-infected rhesus macaques, as there were no correlations between plasma IL-7 and T cell proliferation and T cell numbers continued to decline. Notably, plasma IL-7 levels remained elevated at later stages of infection in rhesus macaques and were correlated with disease progression (130). In SIV-infected sooty mangabeys, however, plasma IL-7 levels were positively correlated with T cell proliferation (121). The considerable delay

in IL-7 increases in SIV-infected rhesus macaques suggests that immune dysfunction develops early during pathogenic SIV infection. Moreover, the differential responses of rhesus macaques and sooty mangabeys to declines of peripheral T cells during the early stages of SIV infection illustrate that the immunologic events occurring during acute infection are critical for influencing disease outcome.

Other T cell subsets: Double Negative T cells, Natural Killer T cells and γδ T cells

Natural killer T (NKT) cells are T cells that express both CD3 and the NK receptor CD16 and recognize non-peptide antigens on non-classical MHC molecules, responding by secreting a vast array of cytokines. In SIV-infected rhesus macaques, an early increase in IL-17 production by NKT cells in the LN was recently found to be associated with subsequent progression to simian AIDS (145). In this study, IL-17 production peaked at 14d.p.i. and was correlated with increased IL-8 and TGF-beta production (145). Moreover, these cytokines were continuously produced during chronic infection of macaques that did not control viremia. Conversely, in SIV-infected AGMs, there was no increase in IL-17 production during acute infection (145). Possibly, regulating the production of pro-inflammatory cytokines such as IL-17 from NK T cells during acute infection allows for a swift resolution of other “permissible” acute phase inflammatory responses during nonpathogenic SIV infection.

A second interesting T cell subset is the gamma delta (γδ) T cells, which play key roles in innate immunity and the initiation of adaptive immune responses. In humans, γδ T cells comprise a relatively minor

subset (1 – 5% on average) of circulating T cells, but may represent as much as 50% of the T cells present within the mucosal associated lymphoid tissue (146). γδ T cells can produce both Th1 and Th2 cytokines, thereby influencing adaptive immune responses (147). Two main γδ T cell subsets exist as defined by the expression of one of two delta variable region, Vδ1 or Vδ2, in the TCR (148, 149). The Vδ1+ γδ T cells are found predominately at mucosal sites and can respond to non-classical MHC molecules expressed on stressed cells, while Vδ2+ γδ T cells are predominately in the peripheral circulation and respond to non-peptide phosphoantigens (148, 150). γδ T cells are influenced by HIV infection as evidenced by a phenotypic switch from predominately Vδ2 before infection, to predominately Vδ1 within the peripheral blood of HIV+ patients (151). Following an oral SIV infection, decreased γδ T cell levels were observed at the oral and esophageal mucosa, while increased γδ T cell levels were observed in the lymph nodes of rhesus macaques (152). In addition, the Vδ2 T cells of rhesus macaques did not exhibit a sound primary or recall response following Mycobacterium BCG infection or reinfection (153). γδ T cells have also been assessed in SIV-infected sooty mangabeys with regard to their ability to express cytokines following a mitogenic or phosphoantigen stimulation (152). The sooty mangabey γδ T cells maintain or increase their ability to produce IFN-γ or TNF-α following stimulation, whereas human γδ T cells exhibit a decreased cytokine response following HIV-infection (152). Based on these studies γδ T cells could potentially have a role in the acute to chronic transition and the down-modulation of the immune response in the SIV natural host monkeys.

Little is known about the activity of the subset of T cells which are CD4/CD8-double negative (DN) during lentiviral infections, though recent studies suggest they may contribute to nonpathogenic SIV infections in the natural hosts. AGMs naturally maintain low frequencies of CD4+ T cells and high frequencies of CD4-negative/CD8alpha-dim T cells regardless of SIV status (93, 154). Beaumier et al. found that the distribution of these cells arises as a result of down modulation of the CD4 molecule concurrent with differentiation into memory cells in the blood (93). Notably, these CD4-negative/CD8alpha-dim T cells retain helper function, including IL-2 and IL-17 production, are MHC class II-restricted, and—due to the lack of CD4 expression—are resistant to SIV infection (93). Similar to AGMs, the levels of DN T cells in sooty mangabeys are unaffected by SIV infection, even in a cohort of sooty mangabeys with dramatic CD4+ T cell depletion (86). In contrast to natural hosts, SIV-infected rhesus macaques experience high rates of DN T cell apoptosis as early as one week post infection, even in controlled SIVsmE041 infection (124). In natural hosts, the presence of a pool of functional helper T cells which are impervious to SIV infection likely contributes the nonpathogenic outcome. More detailed analyses of this understudied subset of cells will likely yield further insight into the mechanisms of nonpathogenesis in natural hosts and may identify new targets for therapeutic intervention which could extend the healthy lifespan of HIV-infected patients.

Plasmacytoid dendritic cells

Plasmacytoid dendritic cells (pDCs) are likely among the first responders to lentiviral infection. Their unique signature of pattern recognition receptors, including TLR7, 8, and 9, as well as their capacity to produce more type I interferon on a per cell basis than any other immune cell demonstrates that they are built to respond quickly to viral infections. Indeed, pDCs have been shown to play a role in the earliest stages of HIV/SIV infections. During the first two weeks of pathogenic SIV infection, the levels of circulating pDCs are dynamic- early increases are followed by transient decreases, with the nadir occurring at two weeks post infection (155). Concurrent with the decline of pDCs in the blood, pDC numbers increase in lymph nodes and plasma IFN-α levels reach their peak, suggesting that pDCs traffic to the LN, where they encounter high levels of virus (155). In AGMs, the first weeks of SIV infection result in a transient decrease in the absolute numbers of blood pDCs with a return to baseline by 42 d.p.i. (156). Just as in pathogenic infection, plasma IFN-α levels increase early in SIV-infected AGMs (156). Others have found that while pDC kinetics are similar in pathogenic and nonpathogenic SIV infection, the magnitude of IFN-α-producing cells is lower in AGMs (157). Interestingly, in SIV-infected rhesus macaques, there is no correlation between the numbers of SIV+ cells and IFN-α+ cells in the LN, suggesting that the virus is not the only driving force behind pDC IFN-α-production during pathogenic infection (157). Controlling aberrant IFN-α production by pDCs during acute infection may be critical for avoiding subsequent increases in global immune activation during nonpathogenic infection. sooty mangabeys may do so by maintaining a reduced sensitivity to TLR7/9 stimuli (158). However, pDCs from AGMs display considerable sensing of viral TLR7/8 and TLR9 ligands, producing levels of type I IFN

comparable to rhesus macaques (157). While the mechanisms have yet to be elucidated, the ability of SIV-infected natural hosts to mount an early yet controlled IFN-α response likely sets the stage for an appropriate acute phase immune activation event which can be resolved during the transition to chronic infection.

Natural Killer Cells

Recent studies have identified a key difference in the function of natural killer (NK) cells between sooty mangabeys and rhesus macaques. Specifically, in uninfected sooty mangabeys, the major subset of NK cells (CD16+ CD56neg) exhibit higher *ex vivo* cytolytic activity relative to uninfected rhesus macaques (106). Additionally, CD16neg CD56+ NK cells from uninfected sooty mangabeys were found to have higher IFN-γ and IL-2 responses to mitogenic stimulation than uninfected rhesus macaques (159). The higher baseline NK cell activity in sooty mangabeys may contribute to a rapid, robust response to SIV, as sooty mangabeys display an increase in the frequency and function of these cells during acute infection (106). Moreover, sooty mangabey NK cells were found to have lower expression of the inhibiting receptor NKG2A than rhesus macaques, suggesting that the profile of activating and inhibiting receptors may contribute to the enhanced NK cell performance in sooty mangabeys (106). Taken together, these findings suggest a role for NK cells in the acute phase of nonpathogenic infection. It is tempting to speculate that NK cells contribute to controlling early viral replication by eliminating infected cells that display reduced MHC class I expression. This idea is supported by experiments in which CD8-depletion (which targets both CD8+ T cells and NK cells) results in a delayed post-peak decline of viral

replication in natural hosts (discussed above). It is likely that bringing early viral replication down to a lower set point is critical for the resolution of acute phase immune activation.

Monocytes/Macrophages

Macrophages and their blood precursors monocytes are innate immune cells that specialize in clearing microbial infections by ingesting and killing fungi and bacteria, promoting inflammatory responses, and recruiting and influencing adaptive immune cells (Fig 1-4). They express a number of pattern recognition receptors that enable them to detect bacterial antigens, including the mannose receptor, dectin-1, the nucleotide oligomerization domain (NOD) receptors, and the toll like receptors (TLR) 1, 2, 4, 5, 6, and 8. Macrophages originate in the bone marrow from a common hematopoietic stem cell and differentiate into a committed myeloid cell (160). Upon release into the blood, they are termed monocytes. Historically considered only a precursor to the larger tissue resident macrophages, monocytes are effector cells in their own right capable of detecting and responding to microbial antigens through phagocytosis, generation of reactive oxygen and nitrogen species, and the production of pro-inflammatory cytokines such as IL-1, IL-6, IL-8, and TNF-α. Exposure to microbial antigens or to inflammatory cytokines results in differentiation of monocytes into myeloid dendritic cells or macrophages and their concomitant migration into tissue sites (160). The exact fate of monocyte differentiation depends on the specific microbial patterns recognized and the surrounding cytokine milieu (161).

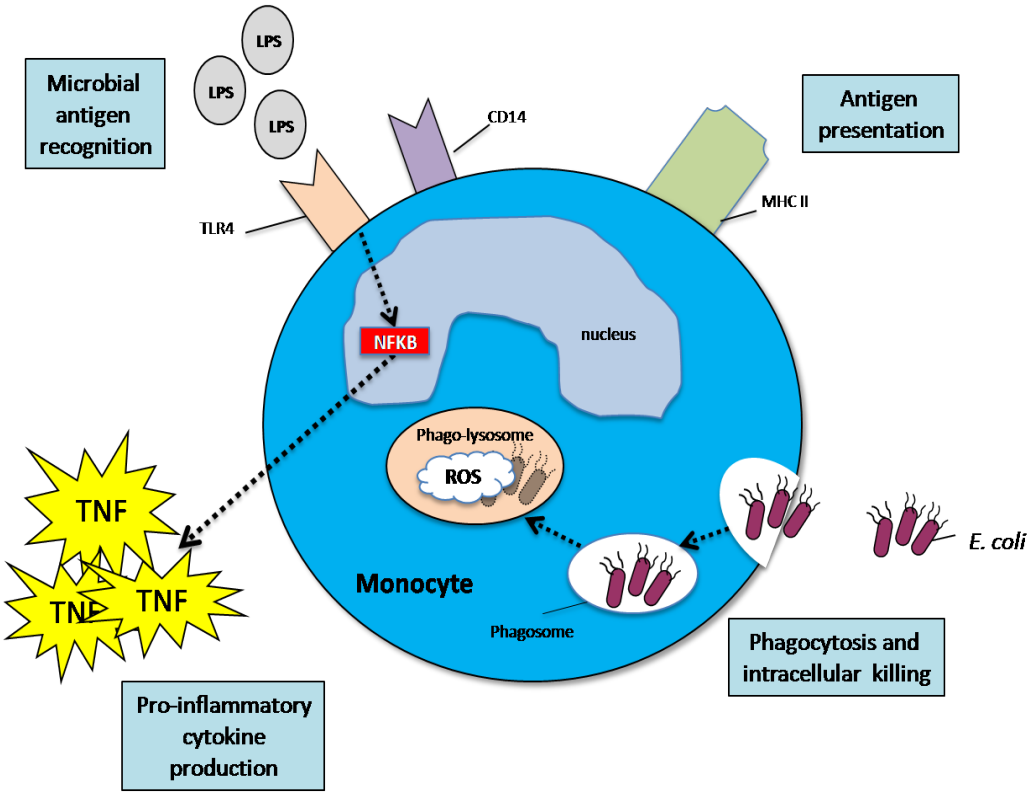

Figure 1-4. Effector functions of monocytes.

Monocytes are capable of numerous effector functions and contribute to immune surveillance in the peripheral blood. They express numerous pattern recognition receptors, including TLR4 (shown), TLR2, TLR5, TLR6, and TLR8 (found on intracellular endosomes). Upon TLR ligand binding, a signal cascade is initiated leading to activation of the transcription factor NFκB, a master regulator of inflammation and immune responses. This leads to the production of pro-inflammatory cytokines such as TNF-α. Monocytes are also capable of antigen presentation on MHC II molecules, and are able to kill microbes through phagocytosis and the production of reactive oxygen species (ROS).

The role of monocytes and macrophages in lentiviral pathogenesis has not been fully elucidated. Studies in rhesus macaques have shown that the blood monocyte count increases slightly after SIV infection and that monocyte turnover is predictive of disease progression (162). However, a similar association has not been found in HIV-infected patients (163). In HIV patients, both monocytes and macrophages have been reported to harbor low levels of virus, with fewer than 1% of cells containing integrated proviral DNA (164). Despite the low frequency of infection, monocytes and macrophages are generally considered to be important long-lived viral reservoirs in infected patients, due in part to a low susceptibility to viral cytopathic effects. In particular, infected circulating monocytes can enter the central nervous system or gastrointestinal tract, where they differentiate into resident tissue macrophages (164). In the brain, these cells have very long half-lives of months to years, thus comprising a potential source of latent virus which can reactivate in response cellular activation such as in the presence of opportunistic infections (164). Moreover, the presence of activated and infected macrophages in the brain leads to the development of neurologic disorders including HIV-associated dementia (163). These cells may increase in the brain due to trafficking of peripheral monocytes that are activated by elevated levels of LPS in the plasma, a factor associated with immune activation and disease progression (165).

Functionally, monocytes and macrophages undergo changes during pathogenic infections. Early findings demonstrated that monocytes isolated from late-stage HIV patients spontaneously secrete elevated levels of TNF-α (166, 167). In contrast, other studies have found that HIV infection is associated with a decrease in TNF-α production in both *ex vivo*

stimulated cells and *in vitro*-infected macrophage cell lines (118, 168). These conflicting findings may result from differences in the disease state of volunteers (asymptomatic vs. AIDS) or from variations in experimental conditions, including cell isolation, duration and concentration of stimulation, and method of TNF-α detection. However, the fact that several groups have reported elevated levels of TNF-alpha in the serum of HIV-infected patients suggests that innate immune cells such as monocytes may produce higher levels of this inflammatory cytokine during pathogenic infection (169-171). Monocytes and monocyte-derived macrophages from HIV-infected patients have also been reported to have a lowered ability to phagocytose multiple antigens/pathogens, including zymosan, *S. aureus*, and Mycobacterium (172-174). In addition, *in vitro* HIV-infected macrophages show an impaired ability to kill *T. gondii (175).* Taken together, these studies suggest that monocytes and macrophages undergo multiple functional alterations during pathogenic infections which may contribute to increases in immune activation, including an inability to clear bacterial infections and a propensity to secrete high levels of pro-inflammatory cytokines.

Little is known about the function of monocytes and macrophages during nonpathogenic SIV infections of natural hosts. Similar to pathogenic infection, very little viral replication (1-8%) occurs in monocyte and macrophages in SIV-infected sooty mangabeys (84). Nonetheless, selective depletion of SIV-infected macrophages in sooty mangabeys undergoing ARV treatment resulted in a delayed viral rebound upon ARV treatment interruption, confirming that active viral replication does contribute to the overall viral load (48). Monocytes of natural host monkeys express lower levels of the viral coreceptor CCR5 relative to

non-natural hosts (91). This phenotype is similar, though less dramatic, than that observed on CD4+ T cells of natural hosts and may contribute to restricting viral entry into target cells (91). Dendritic cells experimentally derived from AGM monocytes were found to have a naturally higher anti-inflammatory profile relative to human cells, but it is not yet known whether monocytes or myeloid dendritic cells maintain an anti-inflammatory profile during nonpathogenic SIV infection (176). While it is logical to propose that cells which promote inflammation such as monocytes and macrophages may be regulated in SIV-infected natural hosts (which display low levels of inflammation), further studies are required to assess the potential contribution of monocytes and macrophages to the overall low levels of immune activation in natural hosts.

Summary

Studies performed to date have focused largely on the role of CD4+ T cells during HIV/SIV infection. While these cells clearly contribute in numerous ways to the pathogenesis of the disease, including serving as the major source of target cells for viral replication, it is now evident that they are not solely responsible for the complex events leading to AIDS. Numerous groups have identified chronic immune activation as the single most predictive element of HIV/SIV disease progression (11, 64, 92, 98). Understanding the virologic and immunologic events leading to increases in immune activation may prove to be one of the most important challenges in the field of HIV research. A clearer understanding of this disease correlate will lead to the design of novel immunotherapeutic compounds and vaccines for HIV infection.

The studies presented here were designed to assess the role of chronic immune activation in HIV/SIV disease pathogenesis and to elucidate the contributions of a specific immune cell subset, monocytes, to the development of immune activation. The natural host models of nonpathogenic SIV infection are an invaluable tool for studying the immunologic parameters of SIV infection. These animals allow a detailed assessment of immune cells in the context of high levels of viral replication but preserved levels of CD4+ T cells and low levels of immune activation. Moreover, the identification of SIV-infected sooty mangabeys which experience a rare decline in CD4+ T cells to levels comparable to AIDS patients provides an unprecedented opportunity to assess the contribution of viral load, CD4+ T cell decline, and immune activation to disease progression (86). The studies outlined here utilize the CD4-low and CD4-healthy SIV-infected sooty mangabey models to investigate the following questions:

1. Does a rapid CD4+ T cell decline in three SIV-infected sooty mangabeys result in any increase in chronic immune activation?
2. Can CD4-low SIV-infected sooty mangabeys mount immune responses to SIV and/or a neo-antigen?

Moreover, these studies expand upon current knowledge of the mechanisms by which SIV-infected sooty mangabeys avoid increases in immune activation regardless of CD4+ T cell levels via an assessment of the functional profile of monocytes, an understudied immune cell subset in the field of lentiviral pathogenesis. These questions include:

3. Does SIV infection of CD4-low and CD4-healthy sooty mangabeys impact the ability of monocytes to respond to TLR stimulation?
4. Does SIV infection of CD4-low and CD4-healthy sooty mangabeys impact the ability of monocytes to perform phagocytosis and intracellular killing of gram-negative bacteria?
5. Can monocytes contribute to maintaining low levels of immune activation during nonpathogenic SIV infection of sooty mangabeys?

The events driving the suppressed immune activation observed during the chronic phase of SIV infection in natural hosts are likely the result of a long evolutionary struggle enabling these monkeys to live in symbiosis with their specific SIV strains. If elucidated, the lessons they have learned from evolution may inform the next generation of therapies and vaccines for HIV patients.

CHAPTER TWO

METHODOLOGY

Human Subjects

HIV-negative blood samples utilized in this study were obtained voluntarily from donors in accordance with Institutional Review Board guidelines at the University of Texas Southwestern Medical Center at Dallas and Seattle Biomedical Research Institute. HIV-infected patient samples were obtained from consenting donors from the University of Texas Southwestern Medical Center AIDS Clinic or the University of Washington Center for AIDS Research Clinic in accordance with Institutional Review Board approval. Patients enrolled in this study ranged between 20 and 55 years of age.

Animals and viral infection

SM1 and SM2 were between 3 and 5 years of age when infected with SIV (86). They were inoculated intravenously with 1 ml of plasma transferred from mangabey FQi, a naturally SIVsmm-infected mangabey at the Yerkes primate colony. The infection occurred in October 2000 and has previously been described (86). Before SIVsmm infection, virologic assessment determined that none of the mangabeys contained simian T cell lymphotropic virus or simian retrovirus. Furthermore, antisera analysis determined that all mangabeys except SM2 were seropositive for CMV/simian agent 6 and herpesvirus B.. Passage of virus from SM2 to

three additional SIV-negative mangabeys—SM7, SM8, and SM9—was performed by intravenous inoculation of 1.5 ml of plasma obtained from SM2 at 303 weeks post infection in October 2007. The viral inoculum contained SIV that were multi-tropic, able to use multiple coreceptors including CCR5 and CXCR4 (86). Additional sooty mangabeys were either SIV-uninfected or naturally infected at the Yerkes National Primate Research Center (Atlanta, GA). Rhesus macaques were housed at the Oregon National Primate Research Center (Beaverton, OR) and were infected with SIVmac239 i.v. using 5 ng equivalents of SIV p27 (1.0×10^5 infectious centers). Pig-tailed macaques were housed at the Washington National Primate Research Center (Seattle, WA) and were infected intrarectally with 1 mL of undiluted stock of SHIV 1157 measuring 95 ng/ml and an *in vitro* infectivity as determined by 50% tissue culture infectious doses ($TCID_{50}$) of 10^6 per ml as titrated in TZM-bl cells (177). Local animal care and use committee and National Institutes of Health protocols were strictly followed in the maintenance of the animals at all National Primate Research Centers.

Blood collection, lymph node biopsies, rectal biopsies and bronchoalveolar lavage

Blood was collected in vaccutainers with EDTA or heparin anticoagulant. Non-human primate blood samples were shipped overnight from national primate centers and blood collected from human donors was held overnight (mock-shipped) to control for changes due to shipping/time post venipuncture. Lymph node (LN) (axillary or inguinal) and rectal mucosal biopsies, as well as bronchoalveolar lavages (BALs), were obtained from

SIV-infected sooty mangabeys SM7, SM8, and SM9. Rectal mucosal biopsies were obtained using a sigmoidoscope with forceps and mononuclear cells were isolated by collagenase digestion (two sequential 30-min incubations at 37°C in RPMI 1640 containing 0.75 mg/ml collagenase). The digested suspension was passed through 70-μm cell strainers and then enriched for lymphocytes by Percoll density gradient. For BALs, a fiberoptic bronchoscope was placed into the trachea after local anesthetic was applied to the larynx. Four 35-ml aliquots of warmed saline were injected into the right primary bronchus and collected by aspiration before a new aliquot was instilled (86).

Viral load analysis

Viral RNA from plasma was extracted with the QIAamp Viral RNA kit (Qiagen, Valencia, CA) and subjected to reverse transcriptase PCR using random hexamers to prime reverse transcription (Invitroge, Carlsbad, CA). SIVsmm plasma viral load was determined by real-time PCR using primers and probe targeting a highly conserved region of the *gag* gene (178). The primers used were: S-GAG03 (5'-CAG GGA Aii AAG CAG ATG AAT TAG-3') and S-GAG04 (5'-GTT TCA CTT TCT CTT CTG CGT G-3' 13); the probe sequence was: 5'-FAM-ATT TGG ATT AGC AGA AAG CCT GTT GGA G-TAMRA-3'. SIV RNA copy number was determined by comparison to an external standard curve consisting of virion-derived SIVmac239 RNA.

Immunophenotyping of T cell subsets

Immunophenotypes of total PBMC and the assessment of cellular proliferation were performed by flow cytometric analysis with antibodies from BD Biosciences (clone numbers indicated in parentheses). Phenotypic analysis was undertaken utilizing: anti-CD3-allophycocyanin-cyanine-7 (APC-Cy7) (SP34), anti-CD8-peridinin chlorophyll protein-cyanine 5.5 (PerCP-Cy5.5) (SK1), anti-CD4-allophycocyanin (APC) (SK3), anti-panγδ-fluorescein isothiocyanate (FITC) (5A6.E9), and anti-Vα24-phycoerythrin (PE) (6B11, courtesy of Lena Al-Harthi, Rush University Medical Center). Naïve and memory CD4+ T cells were defined utilizing anti-CD8 PerCP-Cy5.5 (SK1), anti-CD4 FITC (SK3), anti-CD28-PE (CD28.2), anti-CD95-APC (DX2), and anti-CCR7- phycoerythrin-cyanine-7 (PE-Cy7) (3D12). Cellular proliferation was assessed by intracellular staining with anti-Ki-67-FITC (B56). All cells were stained with LIVE/DEAD fixable dead cell stain in violet (Invitrogen, Carlsbad, CA), fixed in 2% paraformaldehyde in PBS and collected on an LSRII (BD Biosciences, San Diego, CA) and analyzed with FlowJo software (Tree Star, Inc., Inc., Ashland, OR).

Assessment of SIV-specific T cell and antibody responses

SIV-specific peripheral blood CD8+ responses were assessed utilizing SIVmac239-specific peptide pools for Gag, Env, Pol, Nef, and Gag con (a consensus sequence generated from *gag* clones of SIVsmm subtype-1 and -2) Peptide pools consisted of 15-mer peptides overlapping by 11 amino acids and spanning the entire sequence of each viral protein. The number of peptides per pool was as follows: Gag (3 pools of 41 peptides);

Env (2 pools of 54 peptides and 2 pools of 55 peptides); Pol (2 pools of 52 peptides and 3 pools of 53 peptides); and Nef (1 pool of 64 peptides). PBMC samples obtained at 279 wpi (SM1 and SM2) or 52 wpi (SM7, SM8, SM9) were stimulated for 6 hours with 2-5 μg/mL of peptide and the resulting production of TNF-α and IFN-γ by T cells was assessed by intracellular cytokine staining. SIV-specific antibody titers were determined as previously described(86). Briefly, detergent-disrupted SIV envelope proteins from SIVsmB7 captured on the Con A plate were exposed for 1 h at room temperature to plasma Abs, mAbs, or plasma from SIV-negative control mangabeys. To determine end point titers, the plates were washed with PBS and developed using peroxidase-labeled goat anti-monkey IgG Ab and TM blue (Serologicals) as the substrate. End point titers represent the last 2-fold dilution with an OD450 twice that of SIV_ control animals.

Influenza vaccination and assessment of influenza-specific antibody responses SMs 1, 2, 7, 8, and 9 received two 0.5 ml doses (15 μg of hemagglutinin per dose) of a trivalent, inactivated influenza vaccine comprised of A/Solomon Islands/3/2006 (H1N1)-like virus, A/Wisconsin/67/2005 (H3N2)-like virus, and B/Malaysia/2506/2004-like virus via intramuscular injection. Dose 1 was administered in November 2007 and dose 2 was given in September 2008. Influenza-specific antibodies were detected in plasma samples by microneutralization (MN) and hemagglutination inhibition (HI) assays performed by the CDC (179). Briefly, for MN assays, sera were heat inactivated for 30 min at 56°C, and twofold serial dilutions were performed in a 50-μl volume of Dulbecco's modified Eagle's medium containing 1% bovine serum albumin and

antibiotics (V diluent) in immunoassay plates. The diluted sera were mixed with an equal volume of V diluent containing influenza virus at 2×10^3 $TCID_{50}$/ml. Four control wells of virus plus V diluent (VC) or V diluent alone (CC) were included on each plate. After a 2-h incubation at 37°C in a 5% CO_2 humidified atmosphere, 100 µl of MDCK cells at 1.5×10^5/ml was added to each well. The plates were incubated for 18 h at 37°C and 5% CO_2. The monolayers were washed with PBS and fixed in cold 80% acetone for 10 min. The presence of viral protein was detected by ELISA with a monoclonal antibody (A-3) to the influenza A nucleoprotein. The endpoint titer was expressed as the reciprocal of the highest dilution of serum with A_{490} value less than X, where X = [(average A_{490} of VC wells) − (average A_{490} of CC wells)]/2 + (average A_{490} of CC wells). HI assays were performed in V-bottom 96-well microtiter plates (Corning Costar Co., Cambridge, Mass.) with 0.5% turkey erythrocytes, as previously described (180). MN and HI titers greater than or equal to four times baseline titers were considered positive for seroconverion.

Determination of coreceptor usage and env sequence analysis

The coreceptor specificities of SIVsmm viruses were evaluated using methodology from the Trofile coreceptor tropism assay (Monogram Biosciences) as previously described (181, 182). Briefly, full-length envelope (*env*) genes were amplified from plasma samples by RT-PCR and were used to generate Env expression vector libraries. Env expression vector libraries were cotransfected into 293 cells together with an *env*-deleted luciferase-reporter HIV vector to generate SIVsm Env pseudoviruses. Luciferase-reporter pseudoviruses were added to

U87/CD4 cells expressing either CCR5 or CXCR4, in the absence and presence of coreceptor ligands as specificity controls. Infection of target cells was assayed by the addition of luciferase substrate and quantitation of luminescence, measured as relative light units (RLUs). Specific infection of CCR5-positive cells only indicates that the viral population is R5 tropic. Specific infection via CXCR4 indicates exclusive X4 tropism, whereas infection via both CCR5 and CXCR4 indicates that the viral population is dual/mixed (D/M) tropic.

Assessment of occludin expression

Total RNA was extracted from the mucosal and lymph node biopsies utilizing mechanical homogenization, followed by Trizol extraction (183). Real-time PCR utilizing gene-specific primer/probes was performed on an ABI 7700 or ABI 7300 (Applied Biosystems) analyzer, utilizing the default settings. Changes in expression of occludin and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were calculated utilizing delta cycle threshold (ΔCT) values (described in detail in "Quantitative real-time PCR" section below).

Whole blood stimulation and staining for flow cytometry

Whole blood was aliquoted in 96-well U-bottom plates or 5 mL polystyrene culture tubes in 100 µL volume. Samples were stimulated with 10 µg/mL lipopolysaccharide (LPS; Sigma Aldrich, St. Louis, MO), 200 µg/mL lipoteichoic acid (LTA; Sigma Aldrich, St. Louis, MO), or 5 µg/mL single-stranded RNA (ssRNA; Invitrogen, Carlsbad, CA) for 1 hour at 37°C 5%

CO_2. Following the addition of the golgi-blocker brefeldin A (Sigma Aldrich, St. Louis, MO), stimulations were continued for 5 hours at 37°C 5% CO_2. Cells were stained for 30 minutes on ice with anti-CD3-FITC (SP34), anti-CD14 PE-Cy7 (M5E2), anti-CD16-Alexafluor 647 (3G8), and Live/Dead Aqua (Invitrogen, Carlsbad, CA). Following a wash in PBS + 2% FBS, cells were fix/permed in FACS Juice (BD FACS Lysing solution + Tween 20) and stained with anti-TNF-PE (MABII). Cells were collected on a BD LSRII and analyzed with FlowJo software (Tree Star, Inc. Inc., Ashland, OR).

Quantification of cytokine secretion from whole blood cells

Whole blood was aliquoted in 96-well U-bottom plates or 5 mL polystyrene culture tubes in 100 µL volume. Samples were stimulated with 10 µg/mL lipopolysaccharide (LPS; Sigma Aldrich, St. Louis, MO) for 6 hours at 37°C 5% CO_2. 100 µL phosphate buffered saline (PBS) was added, cells were pelleted, and the supernatant was collected and maintained at -80°C until further use. Cytokine secretion was measured by cytometric bead array (BD Biosciences, San Diego, CA) according to the manufacturer's recommendations. Briefly, cell supernatants were diluted 1:2 with PBS and incubated for three hours at room temperature with capture beads and PE detection reagent. Following one wash in wash buffer, samples were collected on a BD LSRII (BD Biosciences, San Diego, CA) and analyzed with CBA analysis software (BD Biosciences, San Diego, CA).

Enrichment of monocytes and CD8+ T cells by magnetic bead sorting

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll-Hypaque gradient. 30,000 PBMCs were incubated with

anti-CD14 or anti-CD8 microbeads (Miltenyi, Auburn, CA) for 15 minutes at 4°C, washed with bead buffer (PBS + 2 mM EDTA + 0.5% BSA, pH 7.2) and collected by magnet associated cell sorting (MACS) on a large selection (LS) column (Miltenyi, Auburn, CA). Purity was assessed by flow cytometry and was on average 55% (monocytes) or 80% (CD8+ T cells). For analysis of cytokine mRNA expression, 100,000 enriched monocytes were plated on 24-well flat-bottom polystyrene plates in 0.5 mL complete RPMI and stimulated with 1 µg/mL LPS for 18 hours.

Total RNA isolation, cDNA synthesis, and quantitative real-time PCR for immune modulators

Cell lysates were collected in RLT buffer (Qiagen, Valencia, CA) and total RNA was isolated using an RNAeasy kit (Qiagen, Valencia, CA). Briefly, RNA was bound to the RNA spin column, washed and eluted in RNase-free water. The concentration of total RNA was quantified with a NanoDrop ND-1000 spectrophotometer (Fisher Scientific, Wilmington DE) and used for complementary DNA (cDNA) synthesis, utilizing the Super Script First Strand cDNA Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). The synthesis was performed following the company's instructions. Briefly, total RNA was mixed with random hexamers, deoxynucleotides triphosphates (dNTPs), and diethyl-pyrocarbonate (DEPC) treated water and incubated at 60 °C for 5 minutes. Next, magnesium chloride ($MgCl_2$), reverse transcriptase (RT) buffer, dithiothreitol (DTT), RNaseOUT, and SuperScript II RT was added and incubated at room temperature for 10 minutes, followed by 42 °C for 50 minutes (to allow the RT to synthesize the cDNA) and 70 °C for 15 minutes (to inactivate the enzyme). After heating, RNaseH was added and

incubated at 37 °C for 20 minutes. Finally, the samples were diluted with DNase/RNase free water so that 1 µL contained the cDNA equivalent to 1 – 2 µg total RNA. Quantitative real-time PCR was utilized to determine changes in gene expression of immune modulators including TNF-α, IL-6, IL-10, IL-8, and TLR4. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene. Briefly, gene-specific primers and probes or TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA) (Table 2-1) were mixed with TaqMan® Universal PCR Master Mix, (Applied Biosystems, Foster City, CA) and nuclease-free water and run on a 7500 Real-time PCR system (Applied Biosystems, Foster City, CA). Relative expression or fold changes in expression of these genes in SIV-infected SM cells compared to uninfected SM cells or in stimulated cells compared to unstimulated controls was calculated utilizing delta cycle threshold (ΔCt) values (183, 184). Briefly, the GAPDH Ct value was subtracted from the Ct value of the target gene generating a ΔCt value, which was used as a measure of relative expression of the gene. To determine the fold change of gene expression in LPS-stimulated samples compared to unstimulated samples, the ΔCt values for unstimulated samples was subtracted from the ΔCt value of the same gene for stimulated samples to achieve the ΔΔCt value. Fold induction for that gene was then determined by the following formula: $2^{-\Delta\Delta Ct}$ (185).

Table 2-1. Primer and probe sequences for quantitative real-time PCR.

Gene	Oligo	Sequence (5'-3')
IFN-γ	Forward primer	GAA AAG CTG ACC AAT TAT TCG GTA A
	Reverse primer	AGC CAT CAC TTG GAT GAG TTC A
	Taqman Probe	5'-FAM TGA CTC GAA TGT CCA ACG CAA AGC AGT A-TAMRA-3'
IL-4	N/A	Taqman® Gene Expression Assay
IL-6	N/A	Taqman® Gene Expression Assay
IL-8	Forward primer	AAG GAA CCA TCT CAC TGT GTG TAA
	Reverse primer	TTA GCA CTC CTT GGC AAA ACT G
	Taqman Probe	5'-FAM- CTG CCA AGA GAG CCA CGG CCA G -TAMRA-3'
IL-10	Forward primer	ACC CAG ACA TCA AGG AGC AT
	Reverse primer	CCA CGG CCT TGC TCT TGT T
	Taqman Probe	5'-FAM-TAC GGC GCT GTC ATC GAT TTC TTC-TAMRA-3'
TLR4	N/A	Taqman® Gene Expression Assay
TNF-α	Forward primer	GGC TCA GGC AGT CAG ATC AT
	Reverse primer	GCT TGA GGG TTT GCT ACA ACA
	Taqman Probe	5'-FAM-TCG AAC CCC AAG TGA CAA GCC TGT AGC-TAMRA-3'
GAPDH	Forward primer	GCA CCA CCA ACT GCT TAG CAC
	Reverse primer	TCT TCT GGG TGG CAG TGA TG
	Taqman Probe	5'-FAM- TCG TGG AAG GAC TCA TGA CCA CAG TCC – TAMRA-3'

Quantification of Monocyte Phagocytosis and Oxidative Burst

100 µL whole blood (collected in heparin vaccutainers) was aliquoted in 5 mL polystyrene tubes and incubated with 10 µg FITC-labeled *E. coli* particles (Invivogen, Carlsbad, CA) for 10 minutes in a 37°C water bath, then moved at once to an ice bath. 100 µL ice-cold trypan blue was added to quench the FITC fluorescence of remaining *E. coli* particles not ingested by phagocytes. For oxidative burst quantification, blood was incubated with 5 µg unlabeled heat-killed E. coli lysate for 10 minutes in a 37°C water bath, then 5 µg/mL of the superoxide-sensitive probe dihydroethidium (DHE) was added and samples were returned to a 37°C water bath for 10 minutes. The fluorescence of DHE is changed from blue (λ_{em}: 420 nm) to red (λ_{em}: 605 nm) upon reduction by superoxide and we measured the MFI of the red DHE signal in monocytes. Control samples were kept at 4°C on ice. All cells were washed with PBS and red blood cells were lysed with FACS lysing buffer (BD Biosciences, San Diego, CA). Results were measured on a BD LSRII (BD Biosciences, San Diego, CA) and analyzed with FlowJo software (Tree Star, Inc. Inc., Ashland, OR). Briefly, monocytes were gated based on forward and side scatter characteristics, and the geometric mean fluorescence intensity (MFI) of FITC (phagocytosis) or oxidized DHE (oxidative burst) was calculated and used to generate the ΔMFI for each sample, where $\Delta MFI = MFI^{37°C} - MFI^{4°C\ controls}$. Reported ΔMFI values are averages from assays performed in triplicate.

TNF-α blocking

One million PBMCs were plated in flat-bottom polystyrene 24-well plates in 500 µL complete RPMI. Cells were stimulated with 20 µg/mL LPS in the presence of 0, 2.5, or 10 µg/mL anti-TNF (clone MABII, BD Biosciences, San Diego, CA) for 40 hours at 37°C 5% CO_2. As a control, 200,000 purified CD8+ T cells were plated on the same plate and stimulated with 20 µg/mL LPS or 4 µg/mL phytohaemagglutinin (PHA) for 40 hours at 37°C 5% CO_2. CD8+ T cell activation was measured by flow cytometry utilizing the following antibodies from BD Biosciences (unless indicated otherwise): anti-CD38-FITC (AT-1), anti-CD8-PE (SK1), anti-HLA-DR-PE-Cy7 (L243), anti-CD4-APC (SK3), anti-CD3-V450 (SP34), and Live/Dead Aqua (Invitrogen, Carlsbad, CA).

Statistical Analyses

Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA). A Mann-Whitney U test (nonparametric, two-tailed or one-tailed, and unpaired) was performed for all assays. Statistical significance is identified as *P* values less than 0.05 (95% confidence interval).

CHAPTER THREE

MULTI-TROPIC SIV PASSAGE RESULTS IN RAPID CD4+ T CELL DEPLETION BUT NOT AIDS IN SOOTY MANGABEYS

Introduction

Disease progression during pathogenic HIV/SIV infection has been defined historically by two parameters: plasma viral load and peripheral CD4+ T cell levels. However, it is becoming increasingly clear that HIV/SIV pathogenesis results from more than continuous virus replication and a coincident decline of target cells. For example, CD4+ T cells in mucosal compartments are severely depleted very early after infection and yet progression to AIDS is significantly delayed (65, 66, 117, 186, 187). Furthermore, the elevated levels of generalized immune activation observed during pathogenic infections are more predictive of disease progression than viral load (92, 103, 188). The identification of natural host species such as sooty mangabeys and African green monkeys that replicate SIV to high levels but generally do not exhibit clinical signs of AIDS has been invaluable. Through comparison of pathogenic and nonpathogenic infections, we can infer the important pathogenic factors. For example, SIV-infected natural hosts experience an early and rapid depletion of mucosal CD4+ T cells to a similar extent as HIV patients (132, 189), and ongoing studies suggest that the functionality of these remaining CD4+ T cells may be most important (89, 96). Also, natural hosts mount

SIV-specific antibody and CD8+ T cell responses comparable to pathogenic HIV/SIV infections, indicating that the lack of disease in these animals is not due to an increased adaptive immune response (139, 142). One striking distinction of the natural SIV infections is the maintenance of low levels of generalized immune activation during the chronic phase of the infection (after 28 days) (78, 92, 122, 188). Despite acute phase increases in immune cell activation and increased levels of plasma cytokines and chemokines, the transition to chronic infection in natural hosts is characterized by a resolution of immune activation (122-125).

We previously observed that two SIV-infected sooty mangabeys infected by SIVsmm via plasma transfer (October 2000) exhibited a dramatic CD4+ T cell decline (to <100 cells/μl of blood) yet maintained low levels of immune activation during chronic infection(86). The CD4-low phenotype in these mangabeys was associated with the presence of a multitropic (R5/X4/R8-using) SIVsmm and the CD4+ T cell depletion occurred within all tissue samples examined (86). Importantly, these mangabeys have remained free of clinical signs of simian AIDS for the past 9 years. The studies herein expand upon our previous observation and identify potential mechanistic explanations for how SIV-infected mangabeys can remain free of disease, i.e. opportunistic infections/cancers, despite AIDS-defining CD4+ T cell levels. Assessment of this novel cohort of SIV-infected natural host monkeys has uncovered insights into mechanisms of SIV pathogenesis that are also applicable to pathogenic SIV/HIV infections.

Results

Passage of multi-tropic SIVsmm results in rapid depletion of CD4+ T cells

To further investigate the CD4-low phenotype observed in two SIV-infected sooty mangabeys (86), we passaged SIVsmm from the previously identified CD4-low mangabey SM2 through intravenous transfer of plasma to three additional mangabeys, SM7, SM8, and SM9 (pre-infection CD4+ T cell levels ranged 600 – 1200 CD4+ T cells/μL blood (Fig. 3-1a) and continued monitoring these animals for several years. Inoculation of the multi-tropic SIVsmm resulted in a peak plasma viral load between 10^6 – 10^8 copies of viral RNA/mL of plasma (Fig. 3-1b), and coincided with a dramatic decline in peripheral blood CD4+ T cells (Fig. 3-1a). Within 14 – 21 days post-infection (dpi), CD4+ T cells had declined to < 200/μL of blood and established a CD4 set-point below 50 CD4+ T cells/μL blood by one month post-infection (Fig. 3-1a). A viral set-point ranging from 10^3 – 10^4 copies of viral RNA/mL of plasma was established around three months post-infection and was maintained throughout the study period (Fig. 3-1b). CD8+ T cells ranged from 1000 – 2000 CD8+ T cells/μL blood prior to infection and exhibited a transient decline after SIV infection, then subsequently returned to near baseline levels (Fig. 3-1c). The rapid depletion of CD4+ T cells in SM7, SM8, and SM9 suggested that the depletion was virally mediated, and indeed following plasma transfer the SIV replicating in these mangabeys retained the ability to utilize both CCR5 and CXCR4 (Fig. 3-2).

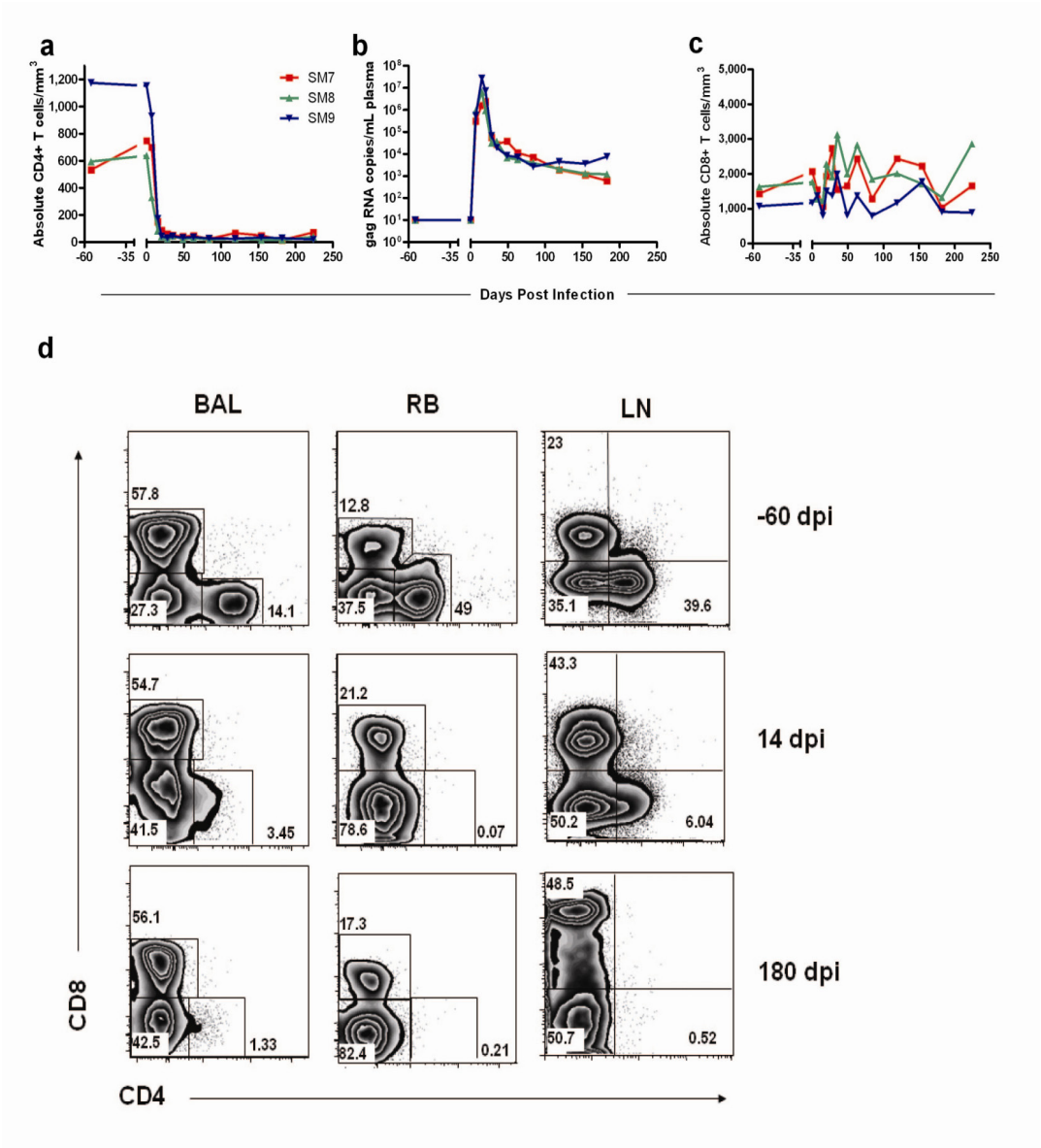

Figure 3-1. Passage of multitropic SIV results in dramatic CD4+ T cell depletion in multiple tissue compartments.

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Figure 3-1 (previous page).

(**a**) Absolute numbers of peripheral blood $CD4^+$ T cells prior to and following passage of multitropic SIV from one CD4-low SIV+ mangabey to SM7, SM8, and SM9. (**b**) Plasma viral loads following passage as measured by the number of copies of *gag* RNA per mL of plasma. (**c**) Absolute numbers of peripheral blood $CD8^+$ T cells following SIV infection. (**d**) Representative flow data showing the percentage of $CD4^+$, $CD8^+$, and DN T cells (indicated by boxes in lower right, upper left and lower left section of flow plots respectively) in brocheoalveolar lavage (BAL), rectal biopsy (RB), and lymph node (LN) samples at -60, 14, and 180 days post infection in SM7. Events shown were gated through live/dead and $CD3^+$ gates.

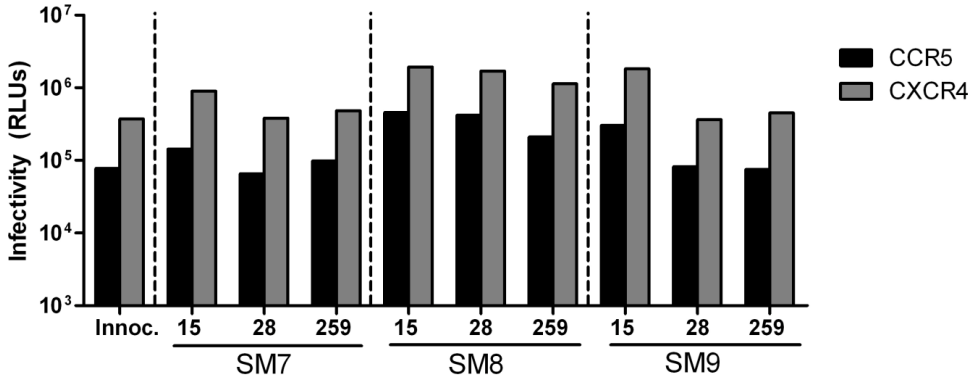

Figure 3-2. SIVsmm multitropism is maintained following passage to SM7, SM8, and SM9.

Plasma from one CD4-low SIV+ mangabey (innoc.) to SM7, SM8, and SM9. SIVsmm tropism for the coreceptors CCR5 and CXCR4 as measured by a pseudovirus assay utilizing cloned viral *env* genes from each animal at 15, 28, and 259 days post passage.

Generalized depletion of CD4+ T cells from multiple sites in CD4-low mangabeys

Infection of rhesus macaques with an X4-tropic SIV or SHIV results in a rapid depletion of naïve CD4+ T cells from blood and peripheral lymphoid tissue, and a gradual loss of mucosal CD4+ T cells (190). Assessing the kinetics of CD4+ T cell depletion in the three CD4-low mangabeys derived from viral passage identified a rapid depletion of both peripheral blood (Fig. 3-1a) and mucosal CD4+ T cells (Fig. 3-1d). The depletion of CD4+ T cells was observed in the rectal mucosa, bronchoalveolar lavage (BAL) and lymph nodes as early as 14 dpi. By 180 dpi the CD4+ T cells comprised < 1% of T cells in the rectal mucosa and lymph nodes and 1 – 2% in the BAL (Fig. 3-1d). In the peripheral blood, naïve CD4+ T cells underwent rapid and sustained depletion by 21 dpi (Fig. 3-3a). The few remaining CD4+ T cells displayed a memory phenotype, expressing the CD95 surface marker (Fig. 3-3b,c). Two of the mangabeys (SM7, SM8) exhibited a dramatic reduction of central memory CD4+ T cells (CD95+/CD28+), and therefore the few remaining CD4+ T cells had an effector memory profile (CD95+/CD28-) (Fig. 3-3b,c). In contrast, SM9, maintained its central memory CD4+ T cells in the peripheral blood, although the numbers of CD4+ cells were dramatically reduced (Fig. 3-3b,c). Within the CD8+ T cells, SM7, SM8 and SM9 each exhibited a lower level of naïve cells compared to pre-infection (Fig. 3-3d), although the proportion of central and effector memory CD8+ T cells was similar to those observed prior to infection (Fig. 3-3e,f).

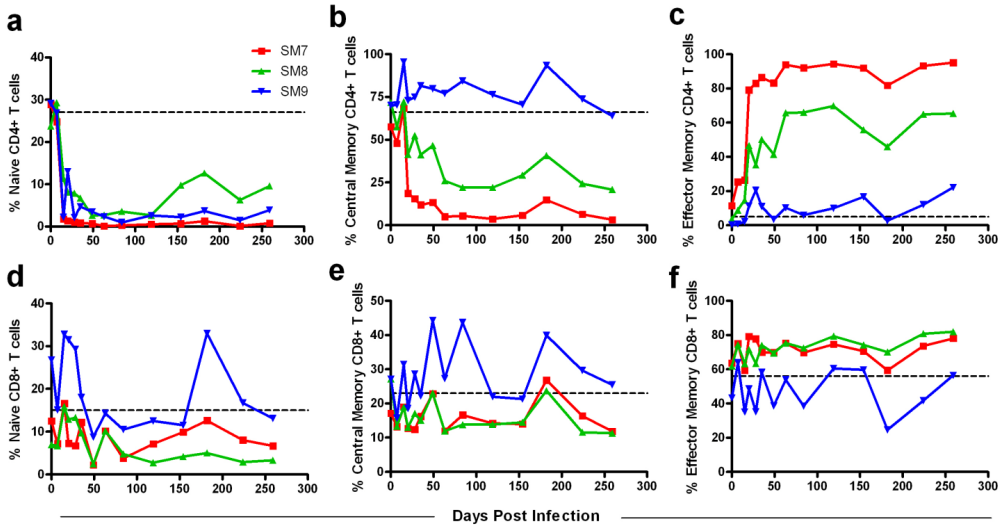

Figure 3-3. Rapid decline of peripheral blood CD4+ T cells impacts the phenotypic distribution of CD4+ T cells.

The percentage of peripheral blood CD4+ T cells (**a, b, c**) and CD8+ T cells (**d, e, f**) expressing naïve, central memory, and effector memory phenotypic markers following passage of multitropic SIV to SM7, SM8, and SM9. Naïve cells were identified as $CD28^{+}/CD95^{neg}$; memory cells were gated as CD28neg/CD95+/CCR7neg (effector memory) or CD28+/CD95+/CCR7+ (central memory). Dotted lines represent the average of all animals at baseline.

CD4 depletion in SIV+ mangabeys is not associated with increased immune activation

Both HIV and pathogenic SIV infections result in severe and persistent mucosal CD4+ T cell depletion as well as elevated immune activation, a finding that led to the bacterial translocation hypothesis and suggested a role for bacterial products in perpetuating HIV-associated immune activation (11, 191). In contrast, SIVsmm infection of sooty mangabeys results in only transient increases in immune activation (generally measured by Ki67 expression in T cells) (86, 121, 132), despite a coincident depletion of gut CD4+ T cells (85). In the CD4-low mangabeys studied here, Ki-67 staining also identified a transient increase in CD4+ and CD8+ T cell proliferation that was limited to the first four weeks post-infection (Fig. 3-4a,b). An assessment of plasma LPS found that all five CD4-low mangabeys, including SM1 and SM2 that had been CD4-low for nine years, maintained low plasma LPS concentrations (3-20 pg/ml), similar to uninfected and SIV-infected CD4-healthy mangabeys (Fig. 3-4c). These findings differed from previous reports assessing pathogenic SIV infection in rhesus macaques which have elevated plasma LPS levels(191) (Fig. 3-4c). To further investigate mucosal integrity, the expression of occludin, a key molecule in tight junction formation was assessed in rectal biopsies of SM7, SM8, and SM9. We observed that all three mangabeys maintained normal or slightly elevated expression of occludin in both acute and chronic infection (Fig. 3-4d). In contrast, occludin expression in a group of SIV+ rhesus macaques was decreased by between 10- to 84-fold compared to uninfected macaques at both acute and chronic time points (Fig. 3-4e). Taken together, these data suggest that CD4-low mangabeys may maintain mucosal integrity through preserved or increased occludin expression, thereby preventing increases

in plasma LPS and generalized immune activation despite the depletion of CD4+ T cells at the intestinal mucosa. In contrast, loss of occludin expression in pathogenic SIV infection may permit bacterial translocation that is associated with chronic immune activation in this model.

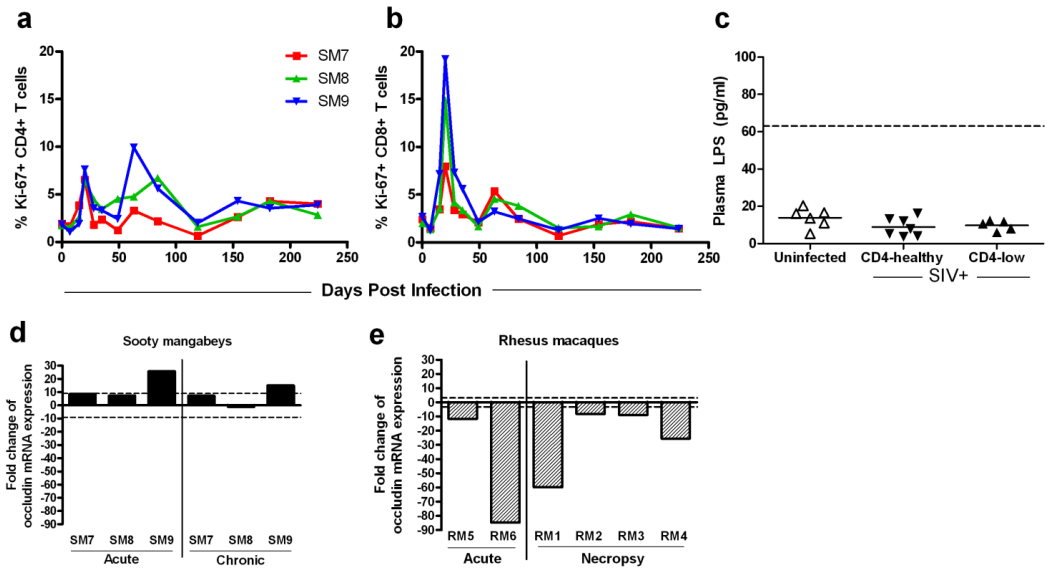

Figure 3-4. Levels of immune activation remain low during chronic SIV infection od CD4-low sooty mangabeys.

(**a, b**) Percentage of proliferating (Ki67+) CD4+ T cells and CD8+ T cells following passage of multitropic SIV. (**c**) Comparison of plasma LPS levels in uninfected and chronically SIV-infected sooty mangabeys with absolute CD4+ T cell levels greater than 200 cells/mm^3 blood (CD4-healthy) or less than 200 cells/mm^3 blood (CD4-low; SM1, SM2, SM7, SM8, SM9 shown). Dotted line represents the average of ten chronically SIV-infected rhesus macaques[23]; Uninfected rhesus macaques have an average of 15 pg/mL LPS. (**d, e**) Fold change of occludin mRNA expression in SM7, SM8, and SM9 or RMs 1-6 during acute (2-15 dpi) and chronic (184 dpi) infection or at time of necropsy. Fold change was determined relative to uninfected control animals; the fold change is considered increased or decreased when it is greater than two standard deviations from the mean expression in uninfected animals (represented by the area between the dotted lines).

Preserved adaptive immune function in CD4-low SIV+ mangabeys

We assessed the ability of the immune systems in the CD4-low mangabeys to mount an adaptive immune response to both SIV and a neo-antigenic challenge, influenza vaccine. Despite the rapid and severe CD4 depletion in SM7, SM8, and SM9, SIV Env-specific antibodies were detectable by 28 days post-infection (Fig. 3-5a). Furthermore, following SIV-specific peptide pulsing and intracellular cytokine staining, CD8+ T cells from all five CD4-low mangabeys were capable of producing TNF-α or IFN-γ (Fig. 3-5b, c) at levels similar to those previously reported for CD4-healthy SIV-infected mangabeys(144). In addition, all five CD4-low mangabeys received a multivalent vaccine containing both an A/Solomon Islands/3/2006 (H1N1)-like virus and an A/Wisconsin/67/2005 (H3N2)-like virus. Upon intramuscular injection of the first influenza vaccine, all five CD4-low mangabeys exhibited increased CD8+ T cell proliferation and four of five exhibited increased CD4+ T cell proliferation (Fig. 3-6a & b). This proliferative response was transient, as proliferation levels returned to baseline by 21 days post-vaccination. Following a second influenza vaccination (10 months after the first) an influenza-specific antibody response was detected in all five CD4-low mangabeys as measured by micro-neutralization for A/Solomon Islands/3/2006 and four of five for A/Wisconsin/67/2005 (Table 3-1). These data indicate that CD4-low SIV-infected mangabeys maintained the ability to mount adaptive immune responses to both SIV and to neo-antigenic stimulation.

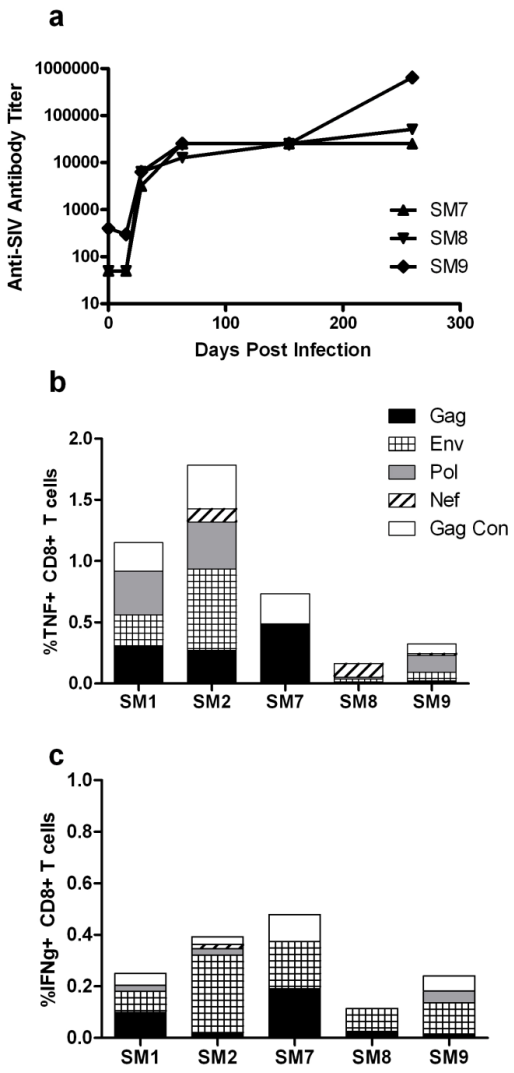

Figure 3-5. SIV-specific adaptive immune responses are present in SIV+ mangabeys with dramatic CD4+ T cell depletion.

(**a**) Reciprocal endpoint titers of SIV-specific antibodies following passage of multitropic SIV to SM7, SM8, and SM9. (**b, c**) Percentages of CD8+ T cells producing TNF-α (TNF) (**b**), or Interferon-gamma (IFNg) (**c**), in response to the SIV peptides Gag, Env, Pol, Nef, and Gag consensus peptides (Gag Con) during chronic infection (279 wpi for SM1 and SM2; 52 wpi for SM7, SM8, and SM9).

Table 3-1. Neutralizing antibody responses following influenza vaccination of SIV+ CD4-low sooty mangabeys. Antibody titers to the vaccine components A/Wisconsin/67/2005 (WI) and A/Solomon Islands/3/2006 (SI) are shown. Values in bold are considered seropositive ≥4 (-fold increase over baseline titers).

Days post 2nd vacc.	SM1		SM2		SM7		SM8		SM9	
	WI	SI	WI	SI	WI	SI	WI	SI	WI	SI
-7	5	5	5	5	10	20	5	5	5	40
0	5	5	5	5	10	20	5	5	7	28
7	**160**	**80**	5	**40**	**640**	**453**	**40**	**80**	**160**	**1280**
14	**320**	**226**	5	**80**	**2560**	**>1280**	**320**	**226**	**>1280**	**2560**
21	**160**	**113**	5	**40**	**1280**	**>1280**	**160**	**113**	**640**	**2560**
28	**80**	**80**	5	**40**	**640**	**>905**	**80**	**80**	**320**	**1810**

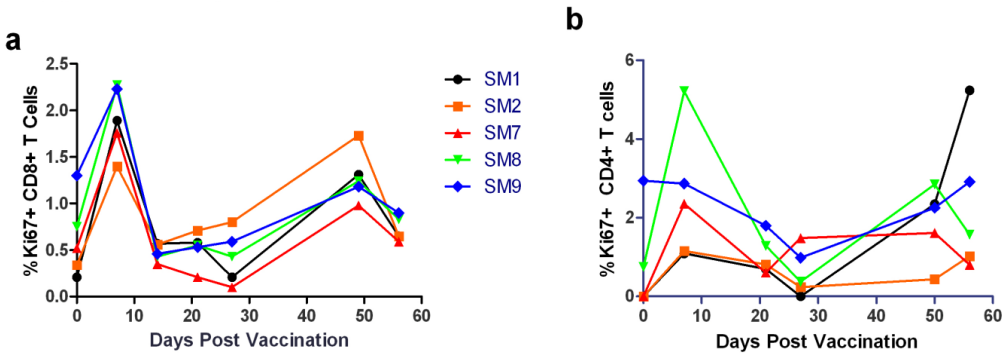

Figure 3-6. Influenza vaccination results in transient increases in T cell proliferation in five CD4-low SIV+ mangabeys.

(**a, b**) Proliferation of CD4+ T cells (**a**) or CD8+ T cells (**b**) as measured by Ki67 expression following the first influenza vaccination in SM1, SM2, SM7, SM8, and SM9.

Discussion

Our previous discovery of two CD4-low SIV+ mangabeys replicating a multi-tropic SIV (CCR5/CXCR4 and CCR8) identified a novel cohort of SIV-infected natural host monkeys which could provide the potential to decipher the contribution of CD4 depletion and other immunologic sequela to AIDS progression (86). Indeed, these first two mangabeys have remained clinically healthy for the past nine years, despite having CD4+ T cell levels that are clinically 'AIDS defining' in humans. The passage of this multi-tropic virus to three additional mangabeys induced a rapid depletion of CD4+ T cells in the blood, lymph nodes, and mucosal tissues along with a sustained expanded coreceptor usage by the virus, and no evidence of elevated immune activation. Despite very low levels of CD4+ T cells, each animal displayed evidence of immune function, including cellular and humoral SIV-specific responses and the ability to respond to a neo-antigenic challenge. Taken together, these data demonstrate that in the absence of chronic immune activation, CD4-low SIV-infected mangabeys are able to respond to foreign antigens while remaining free of clinical signs of AIDS.

The identification of CD4-low mangabeys provides additional evidence for the importance of the low levels of immune activation in preventing the onset of simian AIDS in the natural host species (78, 89, 192). These studies provide keys to unraveling the complexities of HIV-induced disease progression and for understanding a subset of HIV-infected long-term nonprogressors, termed viremic nonprogressors, which display an immunologic profile similar to the natural hosts of SIV (low immune activation despite persistently high viral replication) (193). Immune activation is the strongest correlate of disease progression in

progressive HIV/SIV infection(103, 194, 195) and several models have been suggested to identify the key elements driving the elevated immune activation (108),(111, 112, 191). One model associates rapid disease progression in the SIV-infected macaques with the depletion of CD4+ memory T cells from intestinal mucosa (196, 197) and the inability to replenish these cells results in translocation of commensal organisms. It is therefore difficult to reconcile how our cohort of CD4-low mangabeys could sustain extensive depletion of mucosal CD4+ T cells (for up to 9 years in two mangabeys) without concomitant increases in plasma LPS or immune activation, arguing that the bacterial translocation hypothesis is more complex than just memory CD4+ T cell depletion. It may be that mangabeys utilize other cell populations for maintaining intestinal tight junctions, or that the absence of pro-inflammatory cytokines such as IL-1β and TNF-α in the mangabeys allows for the maintained expression of occludin and other tight junction molecules (197),(198).

Data from HIV-infected elite controller individuals indicates that delayed HIV disease progression is associated with superior control of viral replication by the host's adaptive immune responses. The SIV-infected natural hosts do not fit within this paradigm, as disease progression is averted in the presence of relatively high levels of viral replication and detectable, but not superior, adaptive immune responses(199). As CD4+ T cell help is required for the optimal activation and early clonal expansion of cell-mediated and humoral responses (200, 201) it was initially perplexing that the CD4-low mangabeys were able to develop both SIV-specific antibody and T cell responses as well as flu-specific antibodies. Indeed, HIV-infected patients with CD4 counts below 100 cells/μL have difficulties developing influenza specific antibodies

following an influenza vaccination (202). Moreover, the CD4-low SIV+ mangabeys have maintained immune health and immune function, as no evidence of opportunistic infections or cancers have been observed. One explanation for this outcome is that sooty mangabeys have evolved the potential to rely on a very small number of CD4+ T cells. Alternatively, our results suggest that a non-CD4+ immune cell subset may be compensating for the loss of CD4+ T cells. One potential immune cell subset capable of filling this role is the CD3+/CD4-/CD8- double negative (DN) T cells. Recently Beaumier et al. described a CD3+CD4- T cell subset in SIV-infected AGMs that is resistant to SIV infection, MHC class II restricted, and maintains effector functions normally attributed to CD4+ T cells(93). Ongoing studies in the Sodora lab have found that the DN T cells in our CD4-low SIV infected mangabeys are primarily central memory αβ-TCR+ and their central memory phenotype as well as their absolute numbers are maintained throughout the course of infection (data not shown). These studies are currently assessing the ability of DN T cells from CD4-low mangabeys to produce both Th1 and Th2 cytokines following stimulation as well as their ability to proliferate early in response to SIV infection. We hypothesize that these cells may be capable of performing multiple functions, including CD4 helper-like functions which may have enabled an influenza antibody response in the absence of CD4+ T cells. Interestingly, DN T cells have also been identified in humans, and have been shown to primarily express a memory phenotype, and possess T cytotoxic, T helper, as well as regulatory T cell functions(203),(204),(205). Taken together, these data suggest DN T cells may play an important role in helping SIV-infected mangabeys avoid disease progression by providing a memory T cell repertoire that is

resistant to SIV-infection and remains functional during the SIV-induced CD4+ T cell loss.

Prior to recent publications (85, 86, 189), it was thought that the maintenance of healthy levels of CD4+ T cells was critical for the nonpathogenic outcome observed in SIV-infected sooty mangabeys. More recently, it has been shown that, in the majority of naturally SIV-infected SMs with healthy CD4+ T cell counts, reduced CCR5 expression on activated CD4+ Tcm results in lower levels of infection of these cells in vivo, thus leading to a better preservation of the overall CD4+ T cell pool. Our cohort of SIV-infected sooty mangabeys that has maintained immunologic health and resisted clinical disease progression despite three or nine years of AIDS-defining CD4+ T cell levels demonstrates that there are likely multiple mechanisms contributing to nonpathogenic SIV infection. The main lesson we have learned to date from this novel cohort of SIV-infected CD4-low mangabeys may be that managing immune activation and bolstering the function of non-target T cells through better vaccines and therapeutics has the potential to contribute to preserved immune function and a nonprogressive outcome in HIV infection.

CHAPTER FOUR

NONPATHOGENIC SIV INFECTION OF SOOTY MANGABEYS INDUCES AN LPS-SPECIFIC DECLINE IN MONOCYTE-MEDIATED INFLAMMATION

Introduction

The role of immune activation in lentiviral pathogenesis is well characterized. Numerous groups have reported a higher incidence of immune cell proliferation, activation, and apoptosis in HIV-infected subjects (98) and the levels of activation provide a stronger correlate of HIV disease progression than even viral load (103). Moreover, African natural host monkeys, including sooty mangabeys, which do not progress to AIDS maintain low levels of chronic immune activation despite viral loads rivaling those in HIV-infected subjects (88, 92, 115). Despite the widely accepted contribution of immune activation to HIV/SIV disease, the precise causes of elevated immune activation remain elusive. They are likely multifactorial, including the direct effects of chronic viral replication and the antiviral immune responses that follow, immune cell stimulation by bacterial products translocating from the gut, and the inability of HIV to downmodulate the TCR/CD3 complex from the surface of infected cells (98, 112, 118). Moreover, it is unclear how SIV-infected natural hosts, which do not progress to AIDS, maintain low levels of immune activation during chronic infection despite high viral loads, initial acute phase increases in immune activation, comparable anti-viral immune responses, and in some cases, systemic CD4+ T cell depletion (86, 92, 98). Interestingly, many of the immunologic events thought to propagate

lentiviral-associated immune activation originate with the activity of innate immune cells capable of copious pro-inflammatory cytokine production, such as monocytes/macrophages, plasmacytoid dendritic cells, and myeloid dendritic cells (111, 118, 157). For example, translocated bacterial products in the plasma are recognized by pattern recognition receptors on monocytes, leading to the production of cytokines and chemokines capable of promoting the activation and apoptosis of additional immune cells (11, 118). Indeed, innate immune cells are highly activated during pathogenic HIV/SIV infections (104, 155, 206). Moreover, a primary indicator of immune activation is increases in the levels of plasma pro-inflammatory cytokines, such as TNF-α (18, 169, 171). We chose to study monocytes, effector cells that specialize in detecting and responding to microbial antigens, because they are among the most abundant innate immune cells in the peripheral blood capable of promoting immune activation and apoptosis. Because SIV-infected sooty mangabeys are able to maintain low levels of chronic immune activation, we hypothesized that monocytes must be tightly regulated during this nonpathogenic infection. To this end, we assessed the ability of monocytes to produce pro-inflammatory cytokines in response to multiple toll like receptor (TLR) ligands and to ingest and destroy microbes via phagocytosis and oxidative burst in hosts of progressive and non-progressive lentiviral infections.

Results

Down-modulation of TNF-α production following SIV infection of sooty mangabeys but not HIV/SIV infection of non-natural hosts

To assess the inflammatory response to a microbial antigen during nonpathogenic SIV infection, we stimulated whole blood obtained from SIV-negative or SIV-infected sooty mangabeys with the gram negative bacterial cell wall component, LPS. The resulting secretion of multiple pro-inflammatory cytokines, including TNF-α, IL-6, and IL-8, was measured in cell supernatants by cytometric bead array (Fig. 4-1). We found that whole blood cells from SIV-infected sooty mangabeys secreted significantly less TNF-α than those from uninfected mangabeys (300 pg/mL +/-188 compared to 700 pg/mL +/-517; Fig. 4-1a). In contrast, when we compared the *ex vivo* LPS response of HIV-infected patients to healthy control volunteers, we found no significant difference in the secretion of TNF-α (1000 pg/mL +/-565 compared to 770 pg/mL +/-823; Fig. 4-1b). The production of IL-6 and IL-8 was maintained regardless of infection status in both mangabeys and humans. However, sooty mangabeys secreted overall lower levels of IL-6 compared to humans (Fig 4-1 c-f).

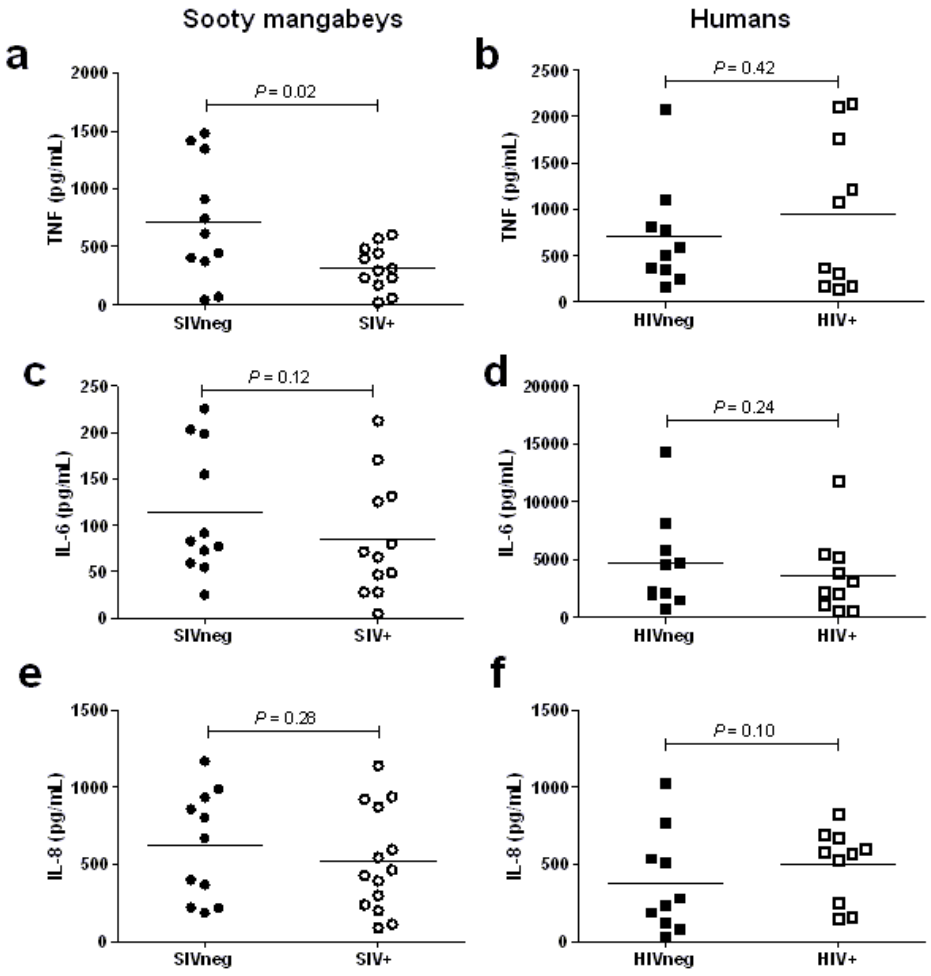

Figure 4-1. Inflammatory cytokine secretion following LPS stimulation of whole blood in sooty mangabeys and human donors.

Whole blood from sooty mangabeys (a, c, e) or human donors (b, d, f) was stimulated with LPS and the resulting secretion of the inflammatory cytokines TNF-α (a,b), IL-6 (c, d), and IL-8 (e, f) was quantified in culture supernatants.

To further investigate the innate immune response to microbial stimulation during both nonpathogenic and pathogenic lentiviral infections, we chose to focus on monocytes, the peripheral blood precursors of tissue macrophages. Monocytes specialize in detecting microbial antigens through the expression of numerous pattern recognition receptors, including the LPS receptors CD14 and TLR4. Utilizing intracellular cytokine staining (ICS), we assessed the monocyte TNF-α response to LPS stimulation in whole blood samples obtained from sooty mangabeys, rhesus macaques, and human volunteers. In SIV-negative mangabeys, TNF-α was detected in an average of 20% (+/-13.77%) of monocytes (identified as CD14+) following *ex vivo* LPS stimulation (Fig 4-2a). In contrast, SIV-infected mangabeys mounted a significantly reduced LPS response with an average of 7% (+/-5.19%) of monocytes producing TNF-α (Fig 4-2a). This reduced monocyte LPS response was not observed in hosts of a pathogenic infection, as both HIV-infected humans and SIV-infected rhesus macaques generated a monocyte LPS response that was comparable to uninfected controls (Fig 4-2b, c). Taken together, these results indicate that sooty mangabeys but not rhesus macaques or humans experience a specific reduction in the LPS response following lentiviral infection.

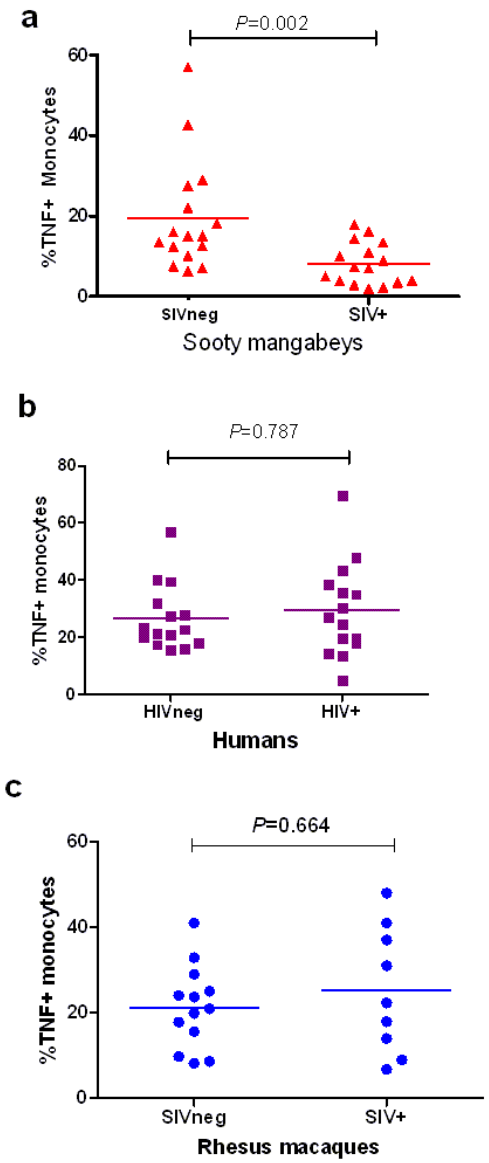

Figure 4-2. Monocyte TNF-α production in response to LPS stimulation in sooty mangabeys, human donors, and rhesus macaques.

Whole blood from sooty mangabeys (a), human donors (b), and rhesus macaques (c) was stimulated with LPS and the resulting TNF-α production by monocytes was quantified by intracellular cytokine flow cytometry.

Rapid reduction in monocyte LPS response following SIV infection in sooty mangabeys

We previously described a cohort of SIV-infected sooty mangabeys that developed a multitropic SIV infection resulting in a dramatic decline of CD4+ T cells to less than 50 cells/μL blood yet remained free of AIDS and opportunistic infections (86). Passage of plasma from one SIV-infected CD4-low mangabey to three additional mangabeys, SM7, SM8, and SM9, initiated a rapid, systemic depletion of CD4+ T cells within 21 days post infection. To assess the impact of virally-mediated CD4+ T cell depletion on innate immune function in SIV-infected sooty mangabeys, we performed a longitudinal analysis of monocyte LPS responses in whole blood obtained from SM7, SM8, and SM9. Prior to infection, 15-30% of whole blood monocytes produced TNF-α upon *ex vivo* LPS stimulation, comparable to what was observed in other SIV-negative sooty mangabeys (Fig. 4-3a and Fig 4-2a). In contrast, as early as 7 days post infection (dpi), the fraction of monocytes producing TNF-α after LPS stimulation declined to 2-7% and remained in this lower range throughout the study period (250 dpi; Fig. 4-3a). Interestingly, the decline in monocyte LPS responses at 7 dpi preceded the decline in peripheral CD4+ T cells, which occurred between 15-21 dpi. Importantly, despite persistent viral replication and the rapid decline in both monocyte LPS responses and CD4+ T cells, SM7, SM8, and SM9 maintained low levels of immune activation during chronic infection and remained free of opportunistic infections. Similar kinetics of the decline in monocyte LPS responses may occur in nonpathogenic SIV infection regardless of CD4+ T cell levels, as analysis of three SIVsmm-infected sooty mangabeys that maintained healthy peripheral CD4+ T cell counts revealed a dramatic decrease in

TNF-α secretion from whole blood cells by 10 days post infection (personal communication from S. Bosinger).

To examine the longitudinal changes in monocyte LPS responses during pathogenic lentiviral infection, we studied a small group of pig-tailed macaques infected with a pathogenic chimeric SIV/HIV (SHIV). Despite a similarly rapid decline in CD4+ T cells (177), SHIV-infected pig-tailed macaques maintained an *ex vivo* LPS response of 10-55% of monocytes producing TNF-α throughout 300 days of observation (Fig. 4-3b). A similar trend was observed in a group of three rhesus macaques infected with SIVsmm (Fig. 4-3d), suggesting that non-natural hosts of SIV do not experience a decline in monocyte LPS responses even when infected with the same virus (SIVsmm).

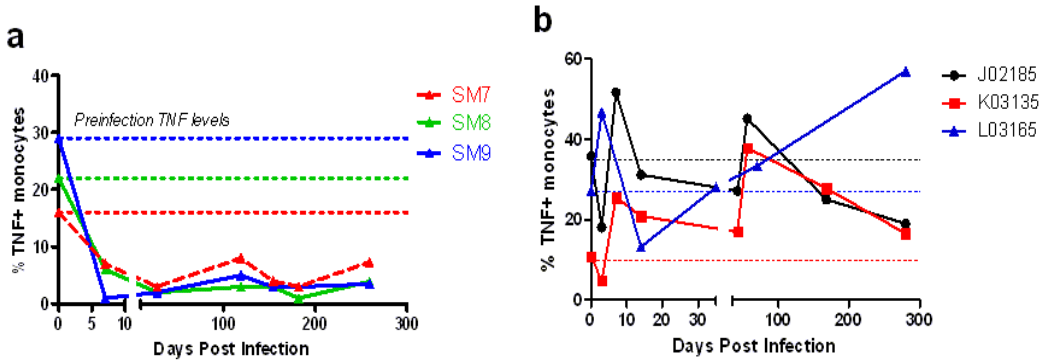

Figure 4-3. Longitudinal analysis of the LPS response in SIV-infected sooty mangabeys and macaques.

The monocyte LPS response was quantified by intracellular cytokine flow cytometry in whole blood obtained from sooty mangabeys infected with a multitropic, CD4-depleting SIV (a) and in pig-tailed macaques infected with a pathogenic SHIV (b).

The monocyte response to lipoteichoic acid and single-stranded RNA is maintained in SIV-infected sooty mangabeys

We next sought to assess the ability of monocytes to respond to additional TLR ligands during nonpathogenic SIV infection. Whole blood obtained from SIV-negative and SIV-infected sooty mangabeys was stimulated *ex vivo* with the TLR2 agonist lipoteichoic acid (LTA) or the TLR8 agonist single-stranded RNA (ssRNA) and the resulting monocyte TNF-α response was quantified by ICS. In contrast to the observed decline in the response to LPS, we found preserved responses to LTA and ssRNA in SIV-infected mangabeys, with an average of 4-5% of monocytes producing TNF-α (Fig. 4-4a). A similar frequency of TNF-α+ monocytes was seen in SIV-negative mangabeys, although the frequency of monocytes responding to LTA and ssRNA was lower than that observed for LPS in these animals (Fig. 4-4a and Fig 4-2a). Overall, these data suggest that the decline in monocyte TNF-α production observed in response to LPS in SIV-infected sooty mangabeys is not recapitulated with additional tested TLR ligands.

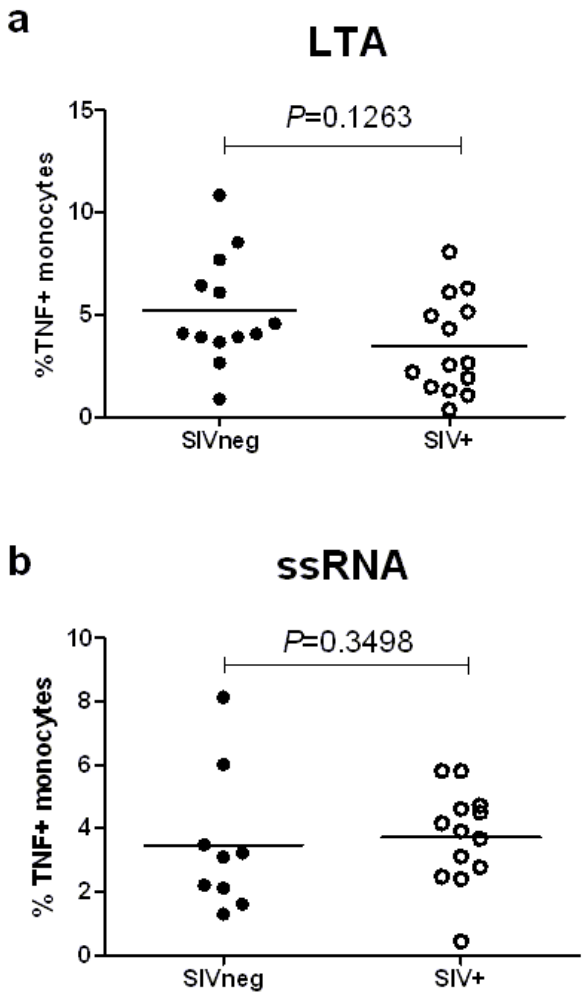

Figure 4-4. TNF-α production by monocytes stimulated with lipoteichoic acid (LTA) and single-stranded RNA (ssRNA) in SIV-negative and SIV-infected sooty mangabeys.

Whole blood obtained from SIV-negative and SIV-infected sooty mangabeys was stimulated with the TLR2 agonist LTA (a) or the TLR8 agonist ssRNA (b) and the resulting TNF-α production in monocytes was quantified by intracellular cytokine flow cytometry.

Phagocytosis and oxidative burst are not altered during nonpathogenic SIV infection of sooty mangabeys

To determine if nonpathogenic SIV infection impacts monocyte effector functions beyond inflammatory cytokine production, we assessed the ability of monocytes to take up gram-negative bacteria by phagocytosis and generate an oxidative burst necessary to kill ingested bacteria. Whole blood from SIV-negative and SIV-infected sooty mangabeys was incubated with FITC-labeled *E. coli* particles at 37°C and flow cytometry was utilized to quantify the geometric mean fluorescence intensity (MFI) of the FITC signal in monocytes (gated based on side scatter and forward scatter properties; Fig. 4-5a). This MFI was normalized to sample-matched controls, which were incubated at 4°C, thus generating a ΔMFI for each sample [$\Delta MFI = MFI^{37°C} - MFI^{4°C}$] (Fig. 4-5). We found that monocytes from SIV-negative and SIV-infected sooty mangabeys had comparable capacities to phagocytose *E. coli*, with a mean ΔMFI for each group of approximately 2000 ΔMFI units (Fig. 4-5b). A similar assay was utilized to assess the oxidative burst capacity of monocytes from SIV-negative and SIV-infected mangabeys. In this assay, a dihydroethidium (DHE) probe that emits a red fluorescent signal when specifically reduced by superoxide, one of the primary reactive oxygen species produced by phagocytes, was used to quantify the monocyte oxidative burst in response to *E.coli*. This analysis revealed that nonpathogenic SIV infection does not impact the ability of monocytes to produce reactive oxygen species in response to *E. coli*, as the mean ΔMFI of DHE for both SIV-infected and SIV-negative mangabeys was approximately 1000 ΔMFI units (Fig. 4-5b). Therefore, despite the observed decline in TNF-α production by monocytes exposed to LPS, the ability of these cells to

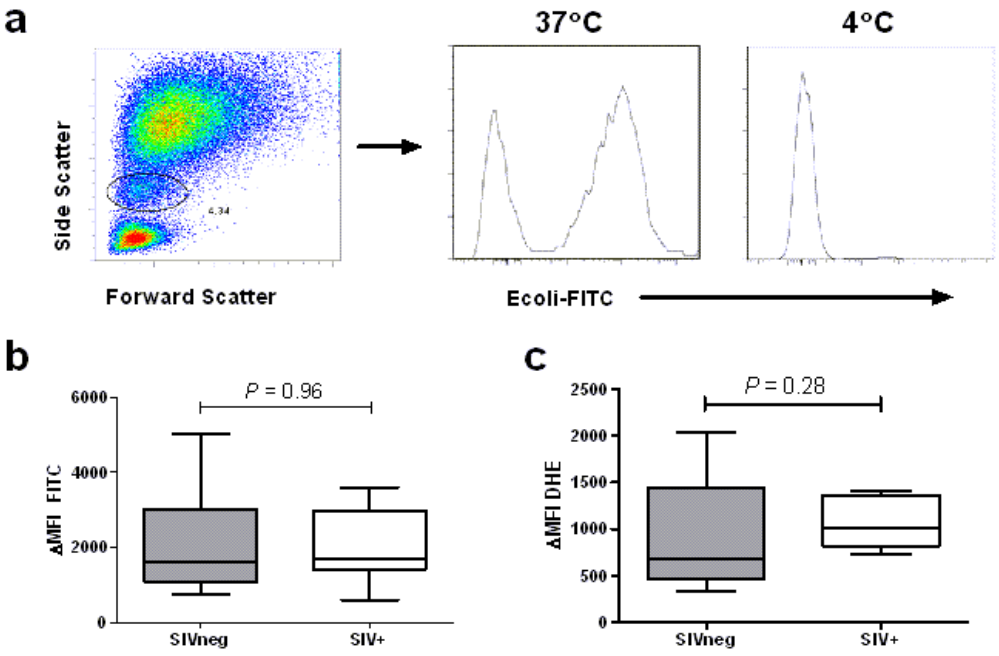

Figure 4-5. Monocyte phagocytosis and oxidative burst in SIV-negative and SIV-infected sooty mangabeys.

(a) Representative flow cytometry data and gating strategy for phagocytosis assay. Oxidative burst assays were gated in a similar strategy, measuring the fluorescence of the DHE probe rather than the E.coli-FITC. (b, c) Phagocytosis capacity (b) and oxidative burst capacity (c) in response to E. coli in monocytes from SIV-negative and SIV-infected sooty mangabeys. ΔMFI represents the average of triplicate assays in which the MFI of experimental samples (37°C) was normalized to the MFI of control samples (4°C). Box plots represent n=8.

phagocytose and kill *E. coli* is preserved during SIV infection of sooty mangabeys.

The decline in monocyte LPS responses in SIV-infected mangabeys is not due to decreased TLR4 or increased IL-10 expression.

To examine the potential mediators of the decline in TNF-α production by monocytes exposed to LPS in SIV-infected mangabeys, we quantified the expression of two molecules involved in the LPS response pathway, TLR4 and IL-10. Because TLR4 is the extracellular receptor for LPS, one potential mechanism by which monocytes may experience a decline in LPS responses is through decreased sensing of LPS as a result of reduced TLR4 expression. To assess this possibility, we utilized real-time PCR to quantify the transcription of TLR4 in enriched monocyte populations obtained from SIV-negative and SIV-infected mangabeys. By normalizing the amount of TLR4 mRNA detected to that of a housekeeping gene, GAPDH, we calculated the relative expression of TLR4 and found no discernable difference in TLR4 mRNA expression in monocytes from either group of mangabeys (Fig. 4-6a). We next examined the expression of IL-10 in monocytes exposed to LPS. Because IL-10 is an anti-inflammatory cytokine capable of acting in an autocrine and paracrine manner to prevent the production of TNF-α and other pro-inflammatory cytokines, we hypothesized that monocytes from SIV-infected mangabeys may produce higher levels of IL-10 in response to LPS, resulting in reduced expression of TNF-α. However, quantitative real-time PCR of enriched monocytes stimulated with LPS determined that mangabey monocytes produce comparable levels of IL-10 mRNA regardless of SIV status (Fig. 4-6b). Taken together, these results

suggest that the reduced monocyte LPS response in SIV-infected sooty mangabeys is not associated with alterations in mRNA expression of TLR4 or IL-10.

Interestingly, however, transcription of the pro-inflammatory cytokines TNF-α, IL-6, and IL-8 was preserved in enriched monocytes from SIV-infected mangabeys (Fig. 4-6c). This result was surprising based on the previous finding of reduced TNF-α production by both ICS and cytometric bead array (Fig. 4-1a and Fig. 4-2a). We would have predicted that the mRNA levels of TNF-α would be reduced in SIV-infected mangabey monocytes following LPS stimulation. The preserved mRNA levels of TNF-α in SIV-infected mangabey monocytes suggests two possibilities: (1) The reduction in monocyte TNF-α production is regulated post-transcriptionally, or (2) extracellular factors present in whole blood which are absent in enriched monocyte cultures contribute to the reduction in monocyte TNF-α production.

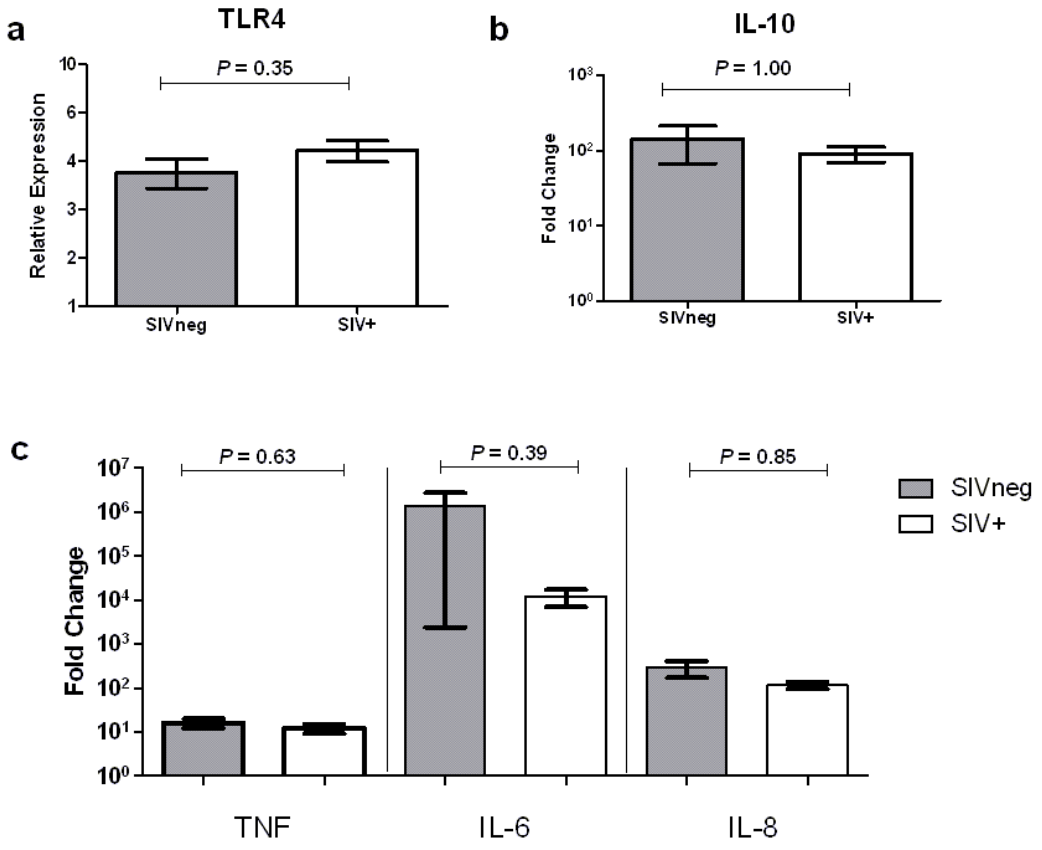

Figure 4-6. Expression of TLR4 and LPS-induced cytokines in monocytes obtained from SIV-negative and SIV-infected sooty mangabeys.

(a) The relative expression of TLR4 was quantified by real-time PCR in cultures of enriched monocytes obtained from SIV-negative and SIV-infected sooty mangabeys. Values shown are normalized to the housekeeping gene GAPDH in the same sample. (b, c) Enriched monocyte cultures were stimulated with LPS and the resulting expression of IL-10 (b) or TNF-α, IL-6, and IL-8 (c) was quantified by real-time PCR. Fold change was calculated by comparing cytokine expression between LPS-stimulated and unstimulated matched samples. Columns represent the mean and standard error of n=8-10.

LPS-induced TNF-α contributes to activation of sooty mangabey T cells

We hypothesized that SIV-infected sooty mangabeys might benefit from reducing monocyte TNF-α production by preventing downstream activation of other immune cells by TNF-α. To explore this idea, we assessed the impact of TNF-α on T cell activation in SIV-negative sooty mangabeys. PBMCs were stimulated with LPS in the presence of a TNF-α blocking antibody (0, 2.5, or 10 μg/mL) and the resulting activation of CD8+ T cells was measured by flow cytometry. LPS stimulation alone (without TNF-α blocking antibody) led to a 5% increase in the frequency of CD8+ T cells expressing the activation markers CD38+ and HLA-DR+ compared to unstimulated controls (Fig. 4-7a). However, CD8+ T cell activation decreased in a dose-dependent manner in the presence of a TNF-α blocking antibody, suggesting that exposure to TNF-α leads to increases in CD8+ T cell activation in sooty mangabeys (Fig. 4-7a). In contrast, when enriched CD8+ T cells were stimulated directly with LPS, there was only a 1% increase in activation (Fig. 4-7b). Whereas stimulation with the mitogen phytoheamagglutinin (PHA) (positive control) led to a 5.5% increase in activation of enriched CD8+ T cells (Fig. 4-7b). These data suggest that in the peripheral blood, TNF-α originating from non-CD8+ T cells can augment the activation status of CD8+ T cells. Therefore, the reduction in monocyte TNF-α responses to LPS may contribute to overall lower levels of CD8+ T cell activation in SIV-infected sooty mangabeys.

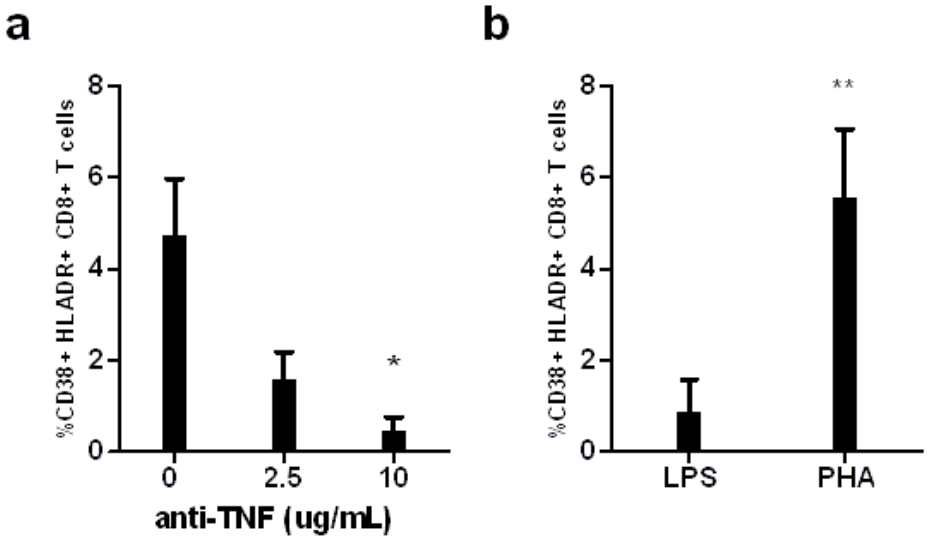

Figure 4-7. The impact of TNF-α blocking antibodies on LPS-induced CD8+ T cell activation in sooty mangabey PBMCs.

(a) PBMCs obtained from SIV-negative sooty mangabeys were stimulated with LPS in the presence of 0, 2.5, or 10 µg/mL anti-TNF-α and the resulting activation of CD8+ T cells, as determined by expression of CD38 and HLA-DR, was quantified by flow cytometry. * denotes *P*=0.004 compared to 0 µg/mL. (b) Enriched cultures of CD8+ T cells obtained from SIV-negative sooty mangabeys were stimulated with LPS or PHA and the resulting expression of the activation markers CD38 and HLA-DR was measure by flow cytometry. ** denotes *P*=0.001 compared to LPS stimulation. Each column represents the mean and standard error of n=6.

Discussion

HIV-associated immune activation is the strongest correlate of disease progression and is characterized by increases in pro-inflammatory cytokines and immune cell activation (11, 98). In contrast, SIV-infected natural hosts maintain low levels of chronic immune activation and do not progress to AIDS (92, 207). Here we show for the first time that monocytes play a role in the ability of SIV-infected sooty mangabeys to resist increases in global immune activation. The data presented demonstrate that in sooty mangabeys, SIV infection induces a rapid and dramatic decline in the monocyte pro-inflammatory cytokine response to LPS. This response is specific to LPS, as the inflammatory cytokine response to additional TLR ligands is preserved (Fig. 4-5), as is the ability to perform additional effector functions, including phagocytosis and oxidative burst (Fig. 4-5). A reduction in monocyte LPS responses was not observed in hosts undergoing a pathogenic lentiviral infection, including HIV-infected patients, SIV-infected rhesus macaques, and SHIV-infected pig-tailed macaques (Fig. 4-1, 4-2, 4-3). Thus, the observation that SIV-infected sooty mangabeys experience a decline in monocyte LPS responses suggests that this phenotype represents a specific adaptation by these natural hosts to regulate pro-inflammatory responses to a ubiquitous bacterial antigen during lentiviral infection.

Our studies indicate that the regulation of monocyte LPS responses in sooty mangabeys occurs rapidly, within 7-10 days following SIV infection (Fig. 4-3). It is interesting that this response declines while other immune activating events are increasing in response to viral infection, including T cell proliferation, type-I IFN production, the expression of numerous interferon-stimulated genes (ISGs) and genes involved in

initiating adaptive immune responses (120, 121, 123-126, 134). Similar to the decline in monocyte LPS responses, however, these initial acute phase immune activation events ultimately resolve during the transition to chronic infection (125, 126). Indeed, maintaining low levels of aberrant immune activation during chronic infection are critical for natural hosts to resist progression to AIDS regardless of CD4+ T cell levels (208). The rapid regulation of monocyte LPS responses in SIV-infected sooty mangabeys suggests that monocytes, which are powerful contributors to inflammation, must be tightly regulated to avoid tipping the balance toward immune activation and disease progression.

Importantly, our data demonstrate that in SIV-negative mangabeys, the magnitude of the LPS response is comparable to that in healthy humans and rhesus macaques (with the exception of IL-6; Fig. 4-1 and 4-2); it is only upon infection with SIV that mangabeys experience a decline in this response. Hence, the regulation of monocyte LPS responses is triggered by the presence of SIV, though the precise mechanism of this regulation remains unknown. Our findings suggest that it does not occur through perturbations in TLR4 or IL-10 expression and may occur post-transcriptionally. Indeed, studies in a mouse model of LPS-mediated septic shock revealed that the production of TNF-α, but not IL-6, was regulated post-transcriptionally by Interleukin-1 Receptor-associated Kinase 2 (IRAK2) (209). The decline in monocyte LPS responses is unlikely to result from direct infection of monocytes by SIV, as we found no evidence of SIV RNA in purified monocytes from sooty mangabeys (data not shown). Alternatively, the regulation of monocyte LPS responses may occur via extracellular mediators present in the plasma, including SIV antigens and soluble LPS binding proteins. However, we found no

change in the plasma concentration two of these proteins, soluble CD14 and LPS binding protein (LBP), after SIV infection of SM7, SM8, and SM9 (data not shown). Other possible mediators include plasma lipoproteins, which act as a sink for LPS in the plasma, and epinephrine, which can induce LPS tolerance in a human model of sepsis (210, 211). Further studies are required to assess the potential contribution of these and other factors in the regulation of monocyte LPS responses in SIV-infected sooty mangabeys.

The regulation of this response in SIV-infected mangabeys may be limited to TNF-α production, as the secretion of IL-6 and IL-8 were maintained in whole blood cultures exposed to LPS (Fig 4-1). A specific regulation of TNF-α production may be beneficial, as TNF-α is one of the earliest and most abundant pro-inflammatory cytokines produced in response to LPS (210). Moreover, TNF-α is a major contributor to apoptosis and the physiological outcomes of LPS exposure, as evidenced by studies demonstrating that TNF-α blocking antibodies can alleviate the symptoms of septic shock while administration of TNF-α can recapitulate septic shock in both animal models and human subjects (212, 213). The observation that LPS-induced TNF-α can contribute to increased CD8+ T cell activation in sooty mangabey peripheral blood cells (Fig 4-7) illustrates the importance of regulating monocyte LPS responses during nonpathogenic SIV infection. It may be that an early reduction in monocyte TNF-α production in SIV-infected mangabeys prevents tipping the balance of the delicate immune environment toward a pro-inflammatory and pro-apoptotic landscape during acute infection. Indeed, it has been demonstrated that natural hosts establish an anti-inflammatory environment during acute infection by increasing IL-10 and TGF-β

production and have lower levels of peripheral T cell apoptosis during both acute and chronic infection (124-126). Moreover, the LPS-specific nature of the observed decline in monocyte TNF-α response may indicate that gram-negative bacteria were a strong driving force in the evolution of sooty mangabeys with their respective SIV. The contribution of LPS to lentiviral pathogenesis is demonstrated by the correlation between elevated plasma LPS concentrations, increased immune cell activation, and advanced disease progression in HIV-infected subjects and SIV-infected macaques (118, 119). Sooty mangabeys seem to have evolved multiple mechanisms to avoid the detrimental effects of LPS during SIV infection, including suppressing monocyte LPS responses and preventing microbial translocation during chronic infection via the selective preservation of mucosal Th17 cells (95). While mangabeys have normal levels of plasma LPS during chronic SIV infection (118), Gordon et al. found that some sooty mangabeys experience increases in plasma LPS above normal levels during acute SIV infection which are resolved during chronic infection (132). A rapid regulation of monocyte LPS responses could prevent increases in TNF-α production during this early phase of SIV infection which may include transient increases in plasma LPS. Alternatively, because the monocyte response to LPS is robust in healthy mangabeys, it may warrant specific regulation, whereas the response to other microbial antigens (LTA and ssRNA) is relatively more subdued. The latter explanation suggests that during nonpathogenic SIV infection, there is a desirable threshold of inflammatory cytokine production which would preserve innate immune responses without promoting widespread increases in immune activation. Indeed, we found that monocytes from SIV-infected mangabeys maintain the ability to phagocytose and kill ingested *E.coli* despite simultaneously down-modulating the inflammatory

response to LPS. Hence, innate effector functions that contribute to clearing opportunistic infections (phagocytosis and oxidative burst) are maintained while those with the capacity to promote immune activation (robust TNF-α production) are held in check.

Overall, these findings further support the importance of controlling immune activation for a nonpathogenic disease outcome and suggest that immunotherapeutic compounds capable of specifically regulating monocyte TNF-α and/or plasma LPS may benefit HIV-infected patients. Moreover, these data demonstrate that monocytes contribute to resolving immune activation during the acute-to-chronic transition phase of nonpathogenic SIV infection. Therapeutic compounds capable of down-modulating monocyte inflammatory responses administered alone or in combination with anti-retroviral therapy may benefit HIV-infected patients by inducing a similar resolution of immune activation regardless of CD4+ T cell status and the duration of infection. This type of therapy may enable HIV-infected patients to live normal, healthy lives free from opportunistic infections similar to the natural hosts of SIV.

CHAPTER FIVE

FINAL DISCUSSION AND FUTURE DIRECTIONS

The global HIV pandemic has claimed an estimated 30 million lives to date and an additional 33 million are currently living with the infection (1). These staggering numbers illustrate the urgency of anti-viral efforts, including education, prevention, and intervention. However, the holy grail of a vaccine for HIV is likely unachievable without a better understanding of the correlates of protection from HIV disease pathogenesis. Recent vaccine trials have offered little hope, serving only to substantiate the need for expanded research efforts aimed at understanding the viral and immunologic contributions to disease. The non-human primate SIV models have proven very useful for studies of lentiviral pathogenesis. In particular, the natural hosts of SIV, which undergo a non-progressive disease course despite infection with a similar virus, offer valuable insights into the contributions and consequences of multiple immune parameters to the overall disease outcome. The studies described here utilized the sooty mangabey model to examine the contribution of CD4+ T cell depletion and monocyte function to the non-pathogenic outcome observed in SIV-infected mangabeys. These studies illustrate for the first time that in SIV-infected sooty mangabeys: (1) a rapid, systemic depletion of CD4+ T cells does not result in increases in chronic immune activation or the inability to respond to immune challenge; and (2) monocytes suppress the inflammatory response to LPS, which likely contributes to lower levels of immune activation.

Part One: The role of CD4+ T cell depletion in nonpathogenic SIV infection

The studies presented here demonstrate for the first time that a rapid virally-mediated depletion of CD4+ T cells does not lead to increases in chronic immune activation and does not prevent the initiation of adaptive immune responses in a group of sooty mangabeys. The ability of these CD4-low SIV-infected mangabeys to maintain low levels of immune activation (like their CD4-healthy counterparts) is likely critical for the preserved function of all immune cells, including the very few remaining CD4+ T cells. Interestingly, SM7, SM8, and SM9 experienced an acute phase increase in immune activation, as measured by CD8+ T cell proliferation (Fig 3-3). However, as in typical nonpathogenic SIV infections of CD4-healthy hosts, all three animals resolved this increase to baseline levels during the transition from acute to chronic infection (20-50 days post infection) and maintained low levels of activation throughout chronic infection (Fig. 3-3). Remarkably, the decline in immune activation in SM7, SM8, and SM9 occurred simultaneously with the depletion of CD4+ T cells, suggesting that healthy CD4+ T cell levels are not necessary for establishing low levels of immune activation during the acute-to-chronic transition phase.

The detection of cellular and humoral immune responses to both SIV and influenza vaccination in the CD4-low SIV-infected mangabeys suggests that extreme depletion of CD4+ T cells in the peripheral blood, LN, and mucosal compartments does not prevent the initiation of adaptive immune responses in SIV-infected sooty mangabeys. These results are counterintuitive, as CD4 help is necessary to initiate both antibody and cytotoxic T cell responses, and raise two possible explanations for the

preserved immune competence in these animals. First, the very few remaining CD4+ T cells may be sufficient to promote the proliferation and differentiation of CD8+ T cells and B cells. In this scenario, however, one might hypothesize that the extreme CD4 lymphopenia in this cohort would result in a lower magnitude of adaptive immune responses. Indeed, HIV-infected patients with CD4 levels below 500 cells/μL blood have reduced antibody responses to influenza vaccination (202). However, the CD4-low mangabeys mounted SIV-specific CD8+ T cell responses that were comparable in magnitude to those previously published for CD4-healthy SIV-infected mangabeys (83), and the influenza-specific antibody titers in our cohort were similar to titers previously reported in SIV-negative rhesus macaques of a similar age (214). A second possibility is that other immune cells are capable of compensating for the lack of CD4+ T cell help in these animals. An immune cell that would stand in for CD4+ T cells must be capable of performing helper-like function, including producing T-helper cytokines (Th1, Th2, Th17). Two potential immune cells with this capacity are the γδ T cells and DN T cells.

γδ T cells are a subset of T cells present in the blood and mucosa that are capable of bridging innate and adaptive immunity through the recognition of non-classical MHC, direct MHC-independent binding of bacterial phosphoantigens, and the production of cytokines including TNF-α, IFN-γ, IL-17, and IL-10 (146, 215). Interestingly, γδ T cells are present at higher frequencies in the peripheral blood of sooty mangabeys compared to humans, and mangabeys naturally posses a higher proportion of γδ T cells expressing the vδ1 receptor that is capable of binding to non-classical MHCs on the surface of virally-infected cells (146, 152) . It is possible that during nonpathogenic SIV infection, γδ T cells

respond to viral antigens (and other foreign antigens) presented by non-classical MHC on the surface of infected cells by producing cytokines that are capable of promoting antigen-specific CD8+ T cell and/or B cell differentiation. These cells are likely capable of responding to antigens and influencing adaptive immune responses regardless of CD4 status, however, in the context of extreme CD4+ T cell depletion, their preserved function may be critical for maintaining immune competence. Indeed, γδ T cells increase in the LN during pathogenic SIV infection of rhesus macaques (216), which is an ideal location to assist in directing adaptive immune responses. The preserved function of γδ T cells during nonpathogenic infection is likely contingent upon low levels of immune activation. Indeed, a recent analysis of γδ T cells in SIV-infected AGMs found no increase in γδ T cell proliferation in the peripheral blood during this natural, nonpathogenic infection (217). This outcome may be due to the lack of microbial translocation in natural hosts, as SIV-infected macaques undergoing a pathogenic infection experienced increases in γδ T cell proliferation that correlated with increased *E. coli* in the LN (217). However, this study did not examine γδ T cells in the LN of SIV-infected AGMS and it is unclear whether a lack of cell proliferation in the blood is indicative of reduced immune activation (allowing for preserved cell function in lymphoid sites) or a lack of γδ T cell responses to SIV antigens.

There is currently no empirical evidence regarding the ability of γδ T cells to provide helper function during nonpathogenic SIV infection. Future studies to address this possibility should examine in detail the distribution of γδ T cells in SIV-infected and SIV-negative sooty mangabeys, including in blood, LN, and mucosal sights such as rectal mucosa and BAL. This analysis would confirm whether γδ T cells

redistribute in response to nonpathogenic SIV infection as they do during pathogenic SIV infection (216). Moreover, it would be useful to identify what, if any, cytokines are produced by γδ T cells during nonpathogenic SIV infection. This could be done by sorting γδ T cells from the LN and peripheral blood and performing real-time PCR to quantify the mRNA levels of multiple cytokines, including IL-2, IL-4, IL-17, IL-10, IFN-γ, and TNF-α. Similar assays could be performed following *ex vivo* stimulation with common mitogens and would provide an informative comparison with previous reports that γδ T cells from rhesus macaques produce both IFN-γ and IL-17 regardless of SIV status (217). Finally, *in vitro* assays to assess the ability of γδ T cells from sooty mangabeys to promote CD8+ T cell or B cell proliferation upon antigen exposure would show whether these cells display the capacity to provide helper function. Specifically, purified populations of γδ T cells would be co-cultured with purified autologous monocytes (to provide a source of antigen presentation), CD8+ T cells or B cells from CD4-healthy and CD4-low SIV-infected sooty mangabeys. These co-cultures would be exposed to SIV peptides, and intracellular cytokine flow cytometry would be utilized to measure the cytokine response of γδ T cells as well as the proliferative response of CD8+ T cells and B cells. Additionally, if γδ T cells are found to produce specific cytokines upon peptide exposure, similar experiments could be performed in which these cytokines are inhibited by the addition of blocking antibodies. The resulting impact on CD8+ T cell and B cell proliferation would again be measured by flow cytometry. This type of experimentation would identify which cytokines are most critical for γδ T cell helper function. It is possible that these experiments will need to be repeated by co-culturing γδ T cells with SIV-infected CD4+ T cells rather than performing SIV peptide stimulation, as γδ T cells are capable of

responding to peptides presenting by non-classical MHC on the surface of virally infected cells (146). These studies could reveal whether γδ T cells are capable of responding to SIV and subsequently directing adaptive immune responses during nonpathogenic SIV infection.

A second immune cell subset with the potential to compensate for the very low levels of CD4+ T cells in the CD4-low mangabeys is DN T cells. These CD3+ cells express the αβ TCR but lack both the CD4 and CD8 molecules and have been found to have a primarily memory phenotype and effector profile in AGMs, humans, and mice (93, 205, 218). We previously reported that DN T cells are present at higher levels in sooty mangabeys compared to humans and that these levels are maintained during nonpathogenic SIV infection (86). However, further investigation is warranted to determine if the DN T cells present at high levels in sooty mangabeys are capable of functioning as helper cells.

Ongoing studies in the Sodora lab are focused on characterizing the phenotype and function of DN T cells in sooty mangabeys (unpublished data). These studies have found that, similar to AGMs, sooty mangabey DN T cells are primarily memory cells, expressing the markers CD28 and CD95. In addition, some cells express CCR7 while others are CCR7-negative, suggesting that they are both central (CCR7+) and effector (CCR7-negative) type memory cells. The majority of DN T cells do not express the invariant Vα24 chain, suggesting that they are not NKT cells. Sooty mangabey DN T cells are capable of producing both Th1 (TNF-α, IFN-γ) and Th2 (IL-4) cytokines in response to stimulation with the TCR agonists anti-CD3/anti-CD8. Moreover, following *ex vivo* stimulation with SIV peptides, DN T cells from CD4-low SIV-infected mangabeys produced both TNF-α and IFN-γ. These studies strongly

suggest that DN T cells possess effector functions that are capable of influencing adaptive immune responses. However, further studies are necessary to demonstrate the functional role of these cells during nonpathogenic SIV infection. We would predict that DN T cells are capable of providing helper function regardless of SIV status and CD4+ T cell counts, but that this function is critical for maintaining immune competence when CD4+ T cell numbers are limiting. One useful experiment would be to assess the ability of DN T cells from SIV-infected and SIV-negative sooty mangabeys to promote B cell antibody class switching in response to stimulation. To this end, purified populations of B cells and DN T cells would be stimulated separately with pokeweed mitogen (B cells) or anti-CD3/anti-CD28 (T cells). The stimulated cells would then be co-cultured for 3-5 days and the resulting proportion of B cells expressing surface IgG and IgM would be quantified by flow cytometry at multiple time points. In addition, the secretion of IgG and IgM antibodies could be measured in culture supernatants by ELISA. If DN T cells are able to provide B cell help, we would expect that B cells would undergo a measurable change in antibody production from primarily IgG to IgM in the presence of DN T cells. However, when cultured alone, B cells would continue to produce primarily IgG antibodies in response to stimulation.

Additional assays would be useful to evaluate the helper potential of DN T cells in SIV-infected sooty mangabeys, including a detailed assessment of DN T cell distribution during SIV infection of sooty mangabeys. Data presented here show that they are present in the BAL, rectal mucosa, and LN throughout infection in CD4-low SIV-infected mangabeys (Fig 3-1). However, further analysis is necessary to

investigate the localization of DN T cells when CD4+ T cells are not limited. It will also be necessary to determine whether DN T cells in SIV-infected sooty mangabeys are vulnerable to SIV infection. We would predict that they are not infected, as they lack the CD4 molecule. Preliminary analysis has determined that DN T cells from CD4-low SIV-infected mangabeys do not harbor detectable SIV RNA (data not shown). However, a more detailed analysis will be required to determine the infection status of DN T cells from CD4-healthy mangabeys, as well as an assessment of the susceptibility of these cells to *in vitro* infection. We would predict that because DN T cells lack the CD4 receptor necessary for SIV entry, they will be resistant to infection and depletion, a hypothesis that is supported by the observation that the absolute levels of peripheral DN T cells remain constant even while CD4+ T cells are depleted in SM7, SM8, and SM9 (Fig 3-5). However, it is possible that sooty mangabey DN T cells arise following the down-regulation of CD4 expression, similar to AGMs (93). In this case, the cells would have been previously susceptible to infection while expressing CD4 on the surface and may continue to harbor proviral DNA after becoming DN. An assessment of viral DNA will be necessary to rile out this possibility.

Overall, future studies assessing the function of γδ T cells and DN T cells may shed light on the mechanisms by which CD4-low SIV-infected mangabeys resist progression to AIDS and opportunistic infections. It is possible that both cell types are capable of providing helper function, providing a form of functional overlap that is often seen in nature and indicative of the importance of preserving a particular function. Yet while both γδ T cells and DN T cells might exhibit the capacity to promote adaptive immune responses, we might postulate that DN T cells are more

likely to functionally compensate for CD4+ T cells, as they are both more abundant than γδ T cells in the peripheral blood and more similar to CD4+ T cells. Specifically, in sooty mangabeys DN T cells comprise approximately 20% of T cells in the blood (Fig 3-1) compared to 12% for γδ T cells (152). Like CD4+ T cells, DN T cells express the αβ TCR, the most abundant TCR with a broad capacity for diversity. In contrast, the γδ TCR is subject to strong selective pressures that limit the diversity of circulating cells (146). Thus, DN T cells are plentiful and capable of recognizing a large spectrum of epitopes, making them a cell type of great interest in the context of CD4+ T cell depletion. Of course, a key experiment would be to deplete DN T cells in the CD4-low mangabeys and assess their ability to respond to neo-antigenic stimulation. However, there is currently no selective marker for depleting DN T cells. The story may be different at mucosal surfaces, where γδ T cells are generally more enriched. We did not assess the mucosal frequency of γδ T cells prior to infection in SM7, SM8, and SM9, but in humans and mice they can comprise up to 50% of T cells in intestinal and reproductive tissue (146). In contrast, we found that DN T cells make up 20-35% of T cells present at the rectal mucosa and BAL (Fig 5-1). It is possible that γδ T cells are more apt to compensate for CD4+ T cells at mucosal surfaces, whereas DN T cells are more likely to provide helper function in the blood. The fact that both γδ T cells and DN T cells are present at higher levels in sooty mangabeys than in humans may represent an evolutionary advantage allowing these natural hosts to maintain immune competence in the context of chronic SIV infection and, in some cases, extreme CD4+ T cell depletion. Elucidating the potential helper functions of these and other immune cells during nonpathogenic infection may lead to novel

therapeutics aimed at bolstering the function of these cells in HIV-infected patients.

Part 2: Role of monocytes in nonpathogenic SIV infection

The preceding discussion focused on which immune cells might be have the potential to functionally compensate for the very low levels of CD4+ T cells in the CD4-low mangabey cohort. A central underlying feature of this discussion is that the remaining immune cells retain their functional capacity during nonpathogenic infection, in stark contrast to the dysfunction reported for numerous immune cells during pathogenic HIV/SIV infections (98, 219). While the cause and effect relationship is unclear, there is unmistakable evidence that immune cell dysfunction, apoptosis, and immune activation go hand-in-hand during pathogenic infections (103, 195, 220-226). It follows that the ability of SIV-infected natural hosts to sustain immune cell function is intimately linked to the low levels of immune activation observed in these non-progressing hosts (92, 115). However, the mechanisms by which natural hosts resolve the initial acute phase increase in immune activation and later resist chronic phase increases in activation are unclear. The studies presented here offer new insight into the role of monocytes in both contributing to the acute-to-chronic phase resolution of immune activation and preventing increases in immune activation during the chronic phase of infection (Fig. 5-1). By down-modulating the inflammatory response to a ubiquitous microbial antigen, LPS, monocytes avert increases in plasma pro-inflammatory cytokines, a key marker of immune activation. Furthermore, the data presented demonstrate that reducing the bioavailability of TNF-α translates to lower levels of CD8+ T cell activation, another important marker of immune activation. We also demonstrate for the first time that

monocytes from SIV-infected mangabeys maintain the functional capacity to respond to microbes via phagocytosis and oxidative burst. Thus, during nonpathogenic SIV infection, monocytes specifically regulate the release of TNF-α in response to LPS but remain functionally competent to clear gram negative bacteria from the peripheral blood through phagocytosis and intracellular killing. This functional profile simultaneously combats opportunistic infections without promoting global increases in immune activation (Table 5-1).

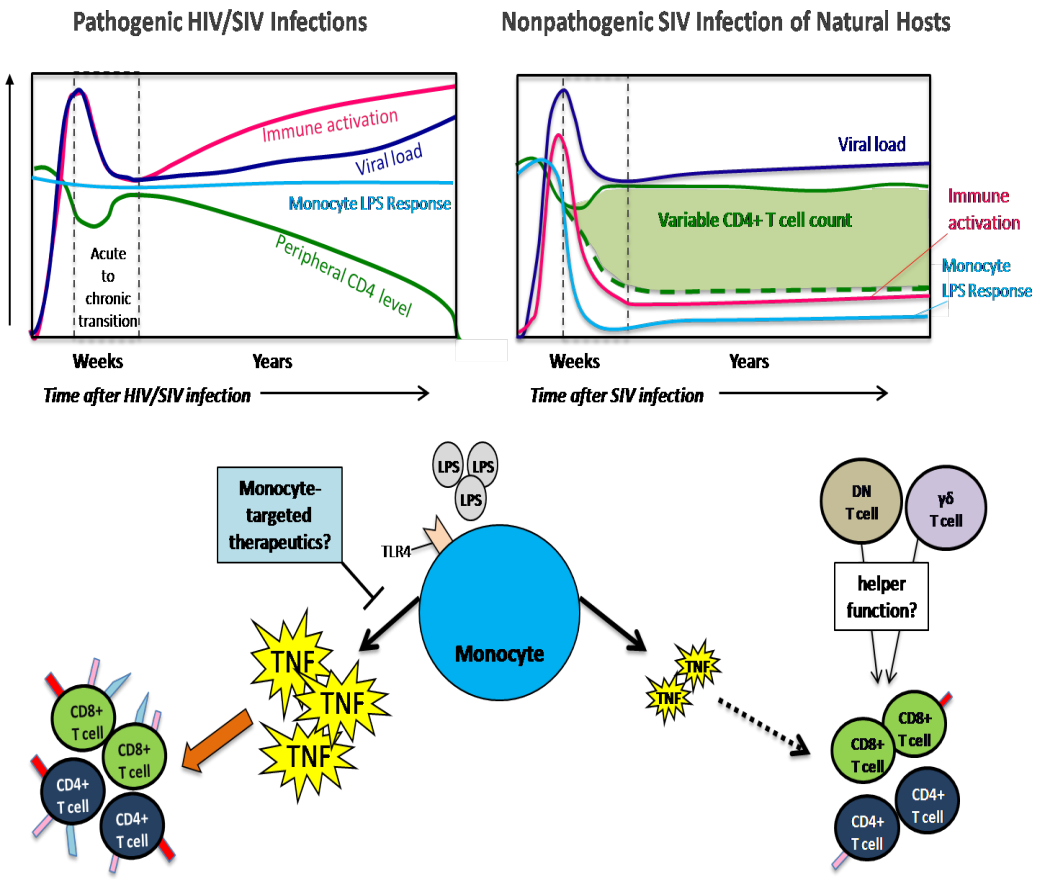

Figure 5-1. Model of the functional contribution of monocytes to pathogenic HIV/SIV infections and nonpathogenic SIV infections of natural hosts.

Figure legend on following page.

Figure 5-1. Model of the functional contribution of monocytes to pathogenic HIV/SIV infections and nonpathogenic SIV infections of natural hosts. *(previous page)*

Figure summarizes the differences between pathogenic HIV/SIV infections and SIV infection of natural host monkey species, including the contribution of monocyte function following an SIV or HIV infection. Within the graphs the dark blue line represents typical viral load levels, the green line and shaded green area represent typical CD4 levels, the red line represents immune activation, and the light blue line represents the monocyte LPS response (as measured by TNF-α production). Vertical dotted lines indicate the time of acute-to-chronic transition which generally occurs between approximately 21 and 40 days. The LPS response of monocytes during pathogenic or nonpathogenic infection is depicted below the graphs, where TNF-α is produced and has downstream impacts on CD4+ and CD8+ T cell activation (red, pink, and blue shapes represent activation markers on the T cell surface such as CD38 and HLD-DR). Additionally, during nonpathogenic infection, the potential helper function of DN and γδ T cells is depicted.

Table 5-1. Summary of monocyte function and resulting impacts on immune activation and disease progression during pathogenic HIV/SIV infections and nonpathogenic SIV infections.

Pathogenic HIV/SIV Infections	Nonpathogenic SIV Infection
Maintained monocyte LPS response	Down-modulated monocyte LPS response
Impaired monocyte phagocytosis and oxidative burst (15, 93)	Maintained monocyte phagocytosis and oxidative burst
Elevated pro- inflammatory cytokines	Low levels of pro- inflammatory cytokines
Elevated immune cell activation and apoptosis	Low levels of immune cell activation and apoptosis
More target cells for infection	Fewer target cells for infection

The data presented confirm that monocytes play a role in limiting immune activation during nonpathogenic infection and suggest that during pathogenic infections they may contribute to harmful increases in immune activation. Previous studies have found that monocytes obtained from HIV-infected patients secrete elevated levels of TNF-α spontaneously in culture (167). Other groups have reported a spectrum of monocyte/macrophage TNF-α responses in HIV-infected donors, including decreases, increases, or no change compared to uninfected controls (118, 166, 170, 171, 227). The variability in these findings likely results from the vast array of experimental procedures utilized, including examining isolated monocytes versus whole blood or PBMCs, extended culture of monocytes, the use of *in vitro* cultured monocyte-derived macrophages, and diverse methods of cytokine detection (ELISA, flow cytometry, real-time PCR). The studies presented here derive their power from the ability to assess the functional responses of monocytes in whole blood, which is as close to the *in vivo* environment as possible, and allows for neighboring immune cells and plasma factors to exert their natural influence on monocytes during stimulation. Moreover, the results obtained from flow cytometric analysis, in which intracellular TNF-α was quantified, agree with cytokine measurements in the supernatant of whole blood by cytometric bead array, confirming that the blood monocyte response to LPS is preserved during pathogenic infection but suppressed during nonpathogenic infection. These studies also utilized a two-fold comparative approach in which monocyte function was first compared between infected and uninfected groups of the same species, followed by a secondary comparison between different species which undergo either a pathogenic or nonpathogenic infection. This analysis avoids direct comparison between different species, which can be inherently

misleading. The fact that we found no difference in monocyte TNF-α production compared to uninfected controls in humans and rhesus macaques, both of which undergo a pathogenic infection, elicits confidence in these data.

There are, however, some minor limitations to these studies. The purity of enriched monocytes was not ideal (average 55%). Therefore, the preserved expression of the inflammatory cytokines TNF-α, IL-6, and IL-8 observed in enriched monocytes from SIV-infected sooty mangabeys could be due to contaminating cells capable of responding to LPS such as myeloid dendritic cells (Fig 4-6). Hence, it is also possible that the low purity in these cultures prevented the detection of minor alterations in IL-10 and TLR4 expression which could be contributing to the regulation of monocyte LPS responses in SIV-infected sooty mangabeys. Further analysis will be necessary to confirm the role of IL-10 and TLR4. Additionally, the magnitude of monocyte TNF-α production in response to TLR2 and TLR8 ligands was low compared to the TLR4 (LPS) response (Fig 4-4). It is possible that at this low level of response we were unable to detect a difference between SIV-infected and SIV-negative mangabeys. It would be interesting to confirm these findings by measuring the secretion of TNF-α in whole blood supernatants by ELISA, thereby confirming whether the observed regulation of monocytes is indeed LPS-specific.

While the data presented demonstrate that monocytes have a reduced LPS response during nonpathogenic SIV infection of sooty mangabeys, the mechanisms by which this response is regulated are not

known. We found that SIV infection did not impact the expression of the LPS receptor TLR4 nor the anti-inflammatory cytokine IL-10, two proteins with the potential to regulate LPS responses. Future studies are necessary to assess additional potential contributing factors to this regulation. For example, numerous soluble factors present in the plasma are capable of modulating LPS and/or the response to LPS; it would be interesting to quantify the concentration of several of these factors in both SIV-infected and SIV-negative mangabeys. We previously detected no change in the plasma concentration of LPS binding protein or soluble CD14 following SIV infection of the CD4-low mangabeys SM7, SM8, and SM9 (data not shown), which agrees with previously published data in CD4-healthy SIV-infected mangabeys (118). However, a more exhaustive analysis is necessary to evaluate the contribution of additional factors, including plasma lipoproteins, which are capable of providing a sink for LPS, and hormones such as epinephrine, which can induce LPS tolerance (211). These assays could be performed by ELISA on plasma samples obtained from sooty mangabeys. If either of these factors influence the observed reduction in monocyte LPS responses, we would expect to find elevated concentrations in the plasma of SIV-infected mangabeys compared to SIV-negative mangabeys. In addition, a comparative analysis in hosts of a pathogenic infection, including HIV-infected humans and SIV-infected rhesus macaques, would confirm whether these plasma factors could be contributing to the regulation of monocyte LPS responses in SIV-infected mangabeys.

It is also possible that viral factors are directly or indirectly mediating the regulation of monocytes during nonpathogenic infection, most likely through inducing tolerance to TLR4 stimulation. In this

scenario, we would predict that monocytes obtained from SIV-infected sooty mangabeys would not display a typical pattern of LPS tolerance, as they had been exposed *in vivo* to a putative tolerizing factor. However, we found that the already low response to LPS became even lower when monocytes from SIV-infected mangabeys were exposed *ex vivo* to multiple doses of LPS (data not shown). Moreover, *in vitro* studies suggest that pre-exposure to lentiviruses, including viral RNA and the accessory protein Vpr, generally results in augmented TLR4 responses in monocytes (228, 229). In addition, we found that monocytes from sooty mangabeys mount a low response to TLR8 stimulation (4%) (Fig 4-4). It is unlikely that this relatively subdued response could prime the cells for a muted LPS response. Alternatively, monocyte regulation may occur at the cellular level. To this end, it would be informative to perform microarray analysis of purified monocytes following stimulation with LPS. This genomic approach may identify multiple targets capable of suppressing the production of TNF-α in LPS-stimulated monocytes. After further validation, it is possible that this type of analysis could identify novel therapeutics for HIV and inflammatory disorders, including rheumatoid arthritis and Crohn's disease.

Because we found that monocytes from HIV-infected donors and SIV-infected macaques do not control the inflammatory response to LPS, we would predict that *in vivo,* monocytes continue to produce substantial amounts of pro- inflammatory cytokines in these hosts upon encountering antigens. Indeed, pathogenic infections are associated with increased microbial antigens in the plasma, in addition to high levels of viral antigens that have been shown to activate monocytes (118, 230). Monocytes are also among the most abundant innate immune cells in the blood that are

capable of large scale inflammatory cytokine production. Studies of sepsis have demonstrated that monocytes are capable of affecting great harm to tissues when inflammatory responses are not regulated (210). Together, these findings identify monocytes as prime suspects for propagating inflammation and activation in the peripheral blood during pathogenic infection and suggest that they are potential targets for therapeutic intervention. Caution should be used, however; inhibiting a single compound such as TNF-α on a broad scale is unlikely to be advantageous. This is demonstrated by studies in which HIV-infected patients with rheumatic disorders were treated with a TNF-α-blocking antibody. While the treatment reduced symptoms of rheumatic inflammation, it did not have any beneficial effects on viral load or CD4+ T cell counts, suggesting that blocking inflammation is not a cure-all for HIV pathogenesis. Rather, our data suggest that targeting anti-inflammatory compounds to specific cells, such as monocytes, may be more useful in controlling HIV-associated immune activation. More specifically, our studies suggest that LPS is particularly provocative for promoting immune activation, as sooty mangabeys have evolved to specifically control the inflammatory response to this antigen during SIV infection. This agrees with previous studies reporting a correlation between plasma LPS concentration and immune cell activation (118, 165). Possibly, inhibiting LPS in the plasma of HIV patients by bolstering endogenous endotoxin-core antibody (EndocAb) could help regulate immune activation. However, targeting one specific immune cell or one factor may not be sufficient to control chronic activation and disease progression. This is illustrated by the fact that we found no correlation between monocyte LPS responses and either viral load or CD4+ T cells counts in HIV-infected donors. Thus, there are likely multiple factors contributing to elevated

immune activation during HIV infection. Targeting monocytes may be more likely to contribute to lower levels of activation when viral loads are controlled by ART, thus eliminating viral sources of immune activation (the HIV-infected donors in this study were not on ART).

To further characterize the role of monocytes in nonpathogenic infection, future studies should examine monocyte function in AGMs, an additional model of nonpathogenic infection. It would be interesting to determine if these natural hosts, which also maintain low levels of chronic immune activation, also evolved to regulate monocyte LPS responses. Additionally, the LPS response of additional innate immune cells such as myeloid dendritic cells (mDCs) should be examined in both sooty mangabeys and AGMs. We would predict that other cells would display a phenotype similar to monocytes if the regulation of LPS responses is important for avoiding immune activation and establishing a nonpathogenic disease course. However, it is also possible that monocytes are specifically regulated due to their higher frequency in the peripheral blood. It would also be interesting to examine the fate of monocytes during nonpathogenic SIV infection. Monocytes are precursors for tissue macrophages and mDCs and the differentiation of monocytes into these mature cells is dependent on the environment in which they are activated. For example, mouse models have shown that infection with *Listeria monocytogenes* results in monocyte differentiation into pro-inflammatory mDCs, whereas certain tumors cause monocytes to become primarily anti-inflammatory myeloid suppressor cells (MDSCs) (161). We would predict that because monocytes from SIV-infected sooty mangabeys have a reduced inflammatory response, they will differentiate into mature cells with an anti-inflammatory profile, including anti-

inflammatory “M2” macrophages and MDSCs, which could continue to suppress immune activation at tissue sights. To this end, future studies should utilize immunohistochemistry to assess the phenotype of myeloid cells at mucosal sights, including the intestinal and lung mucosa. Additionally, macrophages isolated from BAL samples could be assessed for their functional responses to TLR stimulation. We would predict that these tissue resident cells would exhibit a limited inflammatory response in SIV-infected mangabeys, similar to blood monocytes.

Another class of tissue macrophages that warrants investigation during nonpathogenic and pathogenic HIV/SIV infection is Kupffer cells, the resident macrophages of the liver. The liver is responsible for detoxifying LPS found in the bloodstream through the activity of Kupffer cells, which recognize LPS via CD14 and TLR4 and remove it from blood percolating through the liver sinusoids (231). Under normal conditions, Kupffer cells exposed to LPS release IL-10, which is thought to prevent inflammatory cytokine production and contribute to the immune-tolerant environment of the liver (232). Thus, unlike monocytes in the blood, Kupffer cells are programmed to avert inflammation. Conditions that result in liver dysfunction, such as cirrhosis, blunt the ability of Kupffer cells and other resident liver cells to detoxify LPS, resulting in increased inflammatory cytokine production in the liver and elevated levels of LPS in the blood (231). Because plasma LPS levels are increased during pathogenic HIV/SIV infections, we would predict that the liver Kupffer cells are dysfunctional and unable to remove LPS from the blood. On the contrary, because SIV-infected sooty mangabeys generally maintain normal levels of plasma LPS, we would predict that Kupffer cells remain functional during nonpathogenic SIV infection. Comparative studies of the

function of Kupffer cells during pathogenic and nonpathogenic HIV/SIV infections could define a role for these specialized cells and the liver in promoting/preventing immune activation as a result of microbial translocation.

There are two potential functional alterations of Kupffer cells which could contribute to LPS-induced immune activation: a reduced ability to detoxify LPS (including recognition, uptake, and intracellular enzymatic degradation) and an augmented pro-inflammatory cytokine response to LPS. Future studies should evaluate these functions in Kupffer cells obtained from hosts of pathogenic and nonpathogenic infections. The non-human primate models would be ideal for performing these studies, as liver immune cells can be obtained via whole liver perfusions. Specifically, the rhesus macaque could be utilized as a model of pathogenic infection and the sooty mangabey and/or African green monkey provide a model of nonpathogenic infection. Cells obtained from liver perfusates of these animals can be assessed *ex vivo* for the expression of TLR4 and CD14 (LPS recognition), the ability to phagocytose LPS, the expression of enzymes responsible for detoxifying LPS, including acyloxyacyl hydrolase (210), and the cytokine production profile following exposure to LPS (pro-inflammatory vs. anti-inflammatory). If Kupffer cell dysfunction is involved in promoting immune activation during pathogenic infection, we would predict that Kupffer cells obtained from SIV-infected rhesus macaques would display alterations in one or several of these effector functions. For example, they may display a reduced ability to phagocytose LPS/E. coli, which would in turn result in elevated levels of LPS in the peripheral blood. It follows that Kupffer cells from SIV-infected sooty mangabeys and African green monkeys would be

expected to display a preserved or enhanced ability to detoxify LPS and/or produce IL-10 in response to LPS. These studies could offer novel insights into the contribution of Kupffer cells and overall liver function to HIV/SIV-associated immune activation and pathogenesis.

Closing Statement

The goal of studying the natural hosts of SIV is to elucidate mechanisms contributing to a nonpathogenic disease course and translate those findings into new therapeutics and vaccine strategies for HIV. The studies presented here expand current knowledge of the non-progressive SIV infection in sooty mangabeys and further highlight the importance of controlling immune activation during lentiviral infection. Specifically, they suggest that HIV-infected patients may be able to remain healthy even in the face of CD4+ T cell depletion. Studies of γδ T cells and DN T cells in nonpathogenic infections may lead to the development of therapeutic compounds capable of bolstering the function of these cells in HIV-infected patients who do not recover CD4+ T cells even after initiation of ART. Additionally, these studies identify monocytes as key immune cells capable of controlling immune activation. Used in combination with ART, therapies that target the inflammatory response of monocytes while preserving phagocytosis and oxidative burst may help regulate immune activation and apoptosis levels in HIV-infected patients. Together, these novel approaches could help patients lead longer, healthier lives regardless of CD4+ T cell counts.

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