

Mechanisms of Mucosal Dysfunction in HIV Infection and Potential Therapeutic Strategies

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Abstract

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HIV and pathogenic SIV infection are characterized by chronic gastrointestinal (GI) mucosal dysfunction, which contributes to morbidities and mortality. This includes damage to the epithelial barrier, loss of Th17 cells, microbial translocation and local and systemic inflammation and immune activation. The mechanisms underlying mucosal damage have not been fully elucidated. The broad purpose of this thesis was to address several unanswered questions relating to mechanisms and potential therapies for GI mucosal dysfunction in HIV infection. We aimed to address three main objectives: 1) longitudinally assess the kinetics of GI neutrophil accumulation and reduced neutrophil antimicrobial function in relation to mucosal dysfunction, immune activation, and Th17 cell depletion in acute SIV infection; 2) investigate increased neutrophil lifespan as a mechanism for GI neutrophil accumulation in chronic, treated HIV infection and a potential role for HIV altered mucosal bacteria; and 3) assess the safety and efficacy of fecal microbial transplantation (FMT) as a potential therapeutic for mucosal dysfunction in SIV infection.

The role of neutrophils in HIV gastrointestinal mucosal dysfunction is one major theme of the studies described herein. Neutrophils are important for containment of pathogens, but can also contribute to tissue damage due to their release of reactive oxygen species and other potentially harmful effector molecules. Here, we demonstrate that immune alterations such as

Th17 loss, prolonged intestinal neutrophil accumulation, and decreased neutrophil functionality occur after the onset of peripheral and mucosal T cell activation and evidence of microbial translocation, and are thus unlikely to be drivers of initial damage. We additionally demonstrate that neutrophils are recruited to the site of lentivirus infection, maintain antimicrobial function in acute infection, and may therefore participate in the antiviral immune response. In chronic treated HIV infection, we report that reduced homeostatic neutrophil apoptosis contributes to neutrophil accumulation in the gastrointestinal tissues, thus implicating neutrophil lifespan as a new therapeutic target for mucosal inflammation in HIV infection.

Microbial dysbiosis in HIV infection is a second major underlying theme of this thesis. During HIV infection, the delicate balance of healthy bacterial communities is perturbed, and this dysbiosis associates with T cell activation and disease progression. Here, we report differential effects of HIV-altered mucosal bacteria on neutrophil survival, suggesting that dysbiosis may contribute to GI neutrophil accumulation in treated HIV. We also provide evidence that *Lactobacillus* species increase neutrophil apoptosis and decrease neutrophil frequency *in vitro*, which could have important therapeutic implications for reducing neutrophil-driven inflammation in HIV and other chronic inflammatory conditions. Finally, we demonstrate the safety of FMT in primates infected with lentivirus and examine the relationships between alterations to the microbiome and immunological parameters. We observed increased Th17 and Th22 cells as well as decreased activation of CD4⁺ T cells post-FMT, and these changes correlated most strongly across all sampling time points with lower abundance genera in the colon. Taken together, the data presented in this thesis improves our understanding of the mechanisms contributing to mucosal dysfunction and potential therapies that can be further investigated to improve overall health in HIV-infected individuals.

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For my mighty girl Mayla.

"...anything's possible if you've got enough nerve."
-J.K Rowling, Harry Potter and the Order of the Phoenix

Chapter I Introduction

The Global HIV/AIDS Disease Burden

Human immunodeficiency virus is the causative agent of acquired immunodeficiency syndrome (AIDS), in which the immune system becomes incapacitated and is unable to protect the body from foreign invaders. The virus accomplishes this by causing the depletion of CD4+ T cells, and a person is considered to have progressed to AIDS when CD4+ T cells drop below 200 cells/ μ l. This renders an individual highly susceptible to opportunistic infections, and an AIDS diagnosis is also made when an HIV infected individual succumbs to one of several AIDS-defining illnesses (ADIs) regardless of CD4 count¹. Common ADIs include infections such as *Pneumocystis jirovecii*, *Mycobacterium avium*, cytomegalovirus, and tuberculosis, and cancers such as Kaposi sarcoma and non-Hodgkin lymphoma².

Although ADIs have decreased dramatically with the widespread use of antiretroviral therapy (ART) to suppress virus replication, HIV infection continues to remain a global pandemic³. In fact, the Global Burden of Disease Study 2015 reported that the number of people living with HIV/AIDS has been steadily increasing since 1980 and had reached 38.8 million people in 2015⁴. This is the result of about 2.6 million new infections globally per year since 2005, and 1.8 million (75.4%) new infections in 2015 were in sub-Saharan Africa. Heterogeneity by region in access to preventative strategies and care creates this disproportionate disease burden on sub-Saharan Africa. While increased access to ART has greatly reduced AIDS-related mortality worldwide since its widespread introduction in 1996, 1.2 million deaths were still recorded in 2015⁴. Additionally, individuals on ART continue to have increased mortality and increased co-morbidities compared to uninfected individuals⁵. Consequently, it has become clear that managing HIV infection and pathogenesis goes beyond the maintenance of CD4+ T cells

and the prevention of opportunistic infections and new therapies to reduce mortality and comorbidities in treated individuals are needed.

Treated HIV Infection: A chronic disease

Before 1996, few ART options existed for HIV infection and treatment consisted mainly of managing AIDs⁶. The advent of triple drug combination therapy termed highly active antiretroviral therapy (HAART) for treatment of HIV greatly reduced morbidity and mortality associated with AIDS⁷. HAART, which consists of three different drugs that act against at least two distinct molecular targets necessary for viral replication, successfully suppresses viral replication and reduces the plasma viral load below the limit of detection for sensitive PCR-based assays, increases CD4+ T cell counts, and recovers CD4 T cell function, even in advanced disease^{8,9}. Because of achievable viral suppression and immune reconstitution, infected individuals on ART are less likely to die of ADIs; however, they are more likely to prematurely die of non-HIV related comorbidities including liver disease, non-AIDS related cancers, lung disease, cardiac disease, and trauma¹⁰. In fact, while advances in ART have greatly increased life expectancy, mortality rates remain 3-15 times higher than that of the general population¹¹.

Additionally, with increased life expectancy and an aging HIV-infected population, it has become clear that individuals living with chronic HIV disease are also living with increased prevalence of other medical disorders, substance abuse disorders, and psychiatric disorders that greatly affect their quality of life¹². There is an increased prevalence of both age related and non-age related medical disorders such as hypertension, diabetes, renal fracture, cardiovascular disease, and bone fractures in HIV-infected individuals on ART¹³. While the role of ongoing HIV-infection in the early onset or exacerbation of comorbidities is complex and continues to be

a topic of ongoing investigation, there is a consensus in the field that systemic immune activation and inflammation resulting from HIV infection contributes to increased morbidities and mortality in chronic HIV disease¹².

Mucosal Dysfunction and Systemic Immune Activation

Gastrointestinal mucosal dysfunction is a defining feature of HIV infection and is characterized by structural damage to the GI epithelial barrier that results in reduced barrier integrity¹². This damage manifests as epithelial structural abnormalities including atrophy and blunting of enterocyte villi, crypt hyperplasia, and breaches in tight junctions that lead to increased intestinal permeability¹⁴. The causes and consequences of mucosal dysfunction are multifaceted and complex and are depicted in Figure 1.1. In short, several putative mechanisms have been shown to contribute to reduced GI barrier integrity in HIV infection. These include 1) death, dysfunction, and abnormal proliferation of enterocytes caused by HIV proteins 2) enterocyte apoptosis and tight junction downregulation caused by inflammatory cytokines such as tumor necrosis factor- α (TNF- α) and IL-1 β and 3) massive CD4+ T cell depletion in the GI in acute infection, including the loss of IL-17 and IL-22 producing T cells known to homeostatically maintain the epithelial barrier^{14, 15, 16, 17, 18, 19}. Dysbiosis of the GI microbiome is associated with downstream effects of barrier damage, which will be discussed later in this chapter, and has also been hypothesized to directly damage the epithelial barrier, although this has yet to be fully examined²⁰. Importantly, despite long-term plasma viral suppression with ART, mucosal dysfunction persists^{21, 22}.

Mucosal dysfunction results in focal breaches in the GI epithelial barrier and contributes to systemic inflammation and immune activation in HIV infection by allowing microbial

products to translocate across the gut barrier and circulate in the blood (microbial translocation)^{22, 23, 24}. In a healthy individual, an intact epithelial barrier retains microbes in the intestinal lumen and microbes that do get through the barrier are contained in the lamina propria by an intact mucosal immune system. However, HIV infection results in dysfunctional macrophage phagocytosis, defective B cell responses with altered antibody production, and reduced antimicrobial functions of neutrophils (discussed later this chapter)^{25, 26, 27}. These immunological abnormalities in addition to the depletion of CD4+ T cells and altered CD8+ T cell functionality create the perfect environment for bacterial escape^{28, 29}. Evidence of microbial translocation in HIV infection is well documented and is most commonly evidenced by increased lipopolysachharide (LPS), increased LPS binding protein (LBP), and/or increased soluble CD14 (sCD14), which is released by activated macrophages, in the plasma of HIV infected individuals^{30, 31}. Over 40 reports of increased microbial translocation in HIV or pathogenic simian immunodeficiency virus (SIV) infection and its association with immune activation have been published^{23, 32}. Although microbial translocation is reduced with suppressive ART, it remains elevated compared to uninfected individuals^{23, 33, 34, 35, 36, 37}.

Ongoing microbial translocation is a central factor in the persistent systemic immune activation and inflammation that occur despite ART and is associated with increased morbidities and mortality in treated patients^{31, 35, 38, 39}. Systemic immune activation in HIV infection is evidenced by increased peripheral and mucosal T cell activation and exhaustion, which are hallmarks of HIV infection that associate with disease progression. Specifically, PD-1, which is an inhibitory surface molecule on T cells that represents T cell exhaustion, is closely linked to disease progression in HIV-1 infection and both PD-1 and HLA-DR, which represents T cell activation, are increased on rectal CD4+ T cells in HIV-infected individuals^{40, 41}. Additionally,

CD4+ and CD8+ T cell activation, as measured by HLA-DR expression, persists in ART-treated infection and is associated with lower CD4+ T cell gains after treatment⁴². Soluble inflammatory and coagulation biomarkers, including IL-6 and D-dimer, are also increased in HIV infection and associate with increased mortality in infected individuals³⁹. Taken together these data provide evidence that gastrointestinal damage and immune dysfunction drive inflammation and immune activation by allowing bacteria and bacterial products to enter the periphery from the intestinal lumen, thus contributing to increased morbidities and mortality in HIV infected individuals, even when virally suppressed by ART.

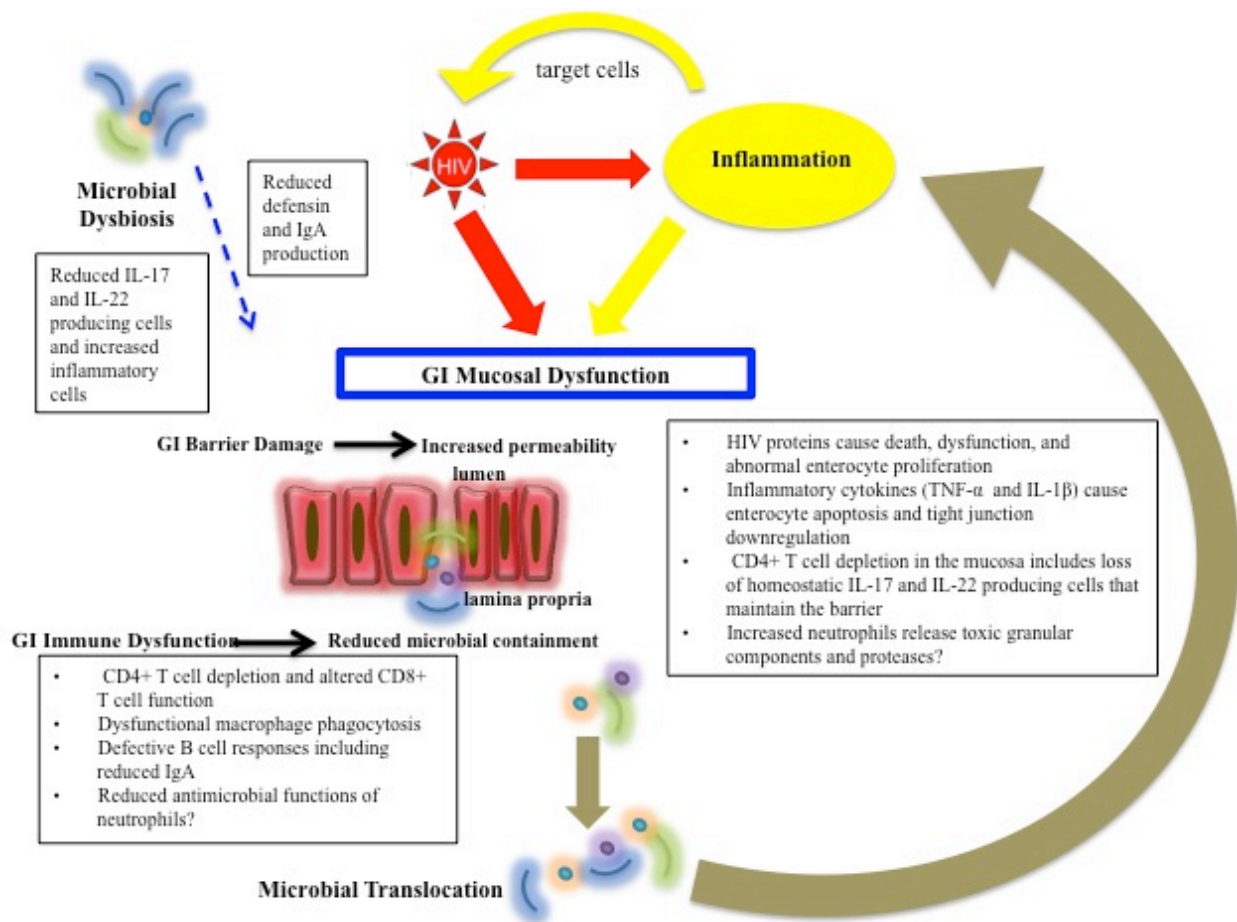


Figure 1.1. Causes and consequences of gastrointestinal mucosal dysfunction in HIV infection. HIV infection causes GI mucosal dysfunction through both viral proteins and the

resulting inflammatory cells that infiltrate mucosal tissues. GI mucosal dysfunction consists of barrier damage resulting in increased permeability and immune dysfunction resulting in decreased microbial containment, and together these permit bacteria to translocate into the intestinal lamina propria and the periphery. Microbial translocation then perpetuates inflammation, resulting in increased target cells for viral replication and increased comorbidities and mortality, even in ART-treated individuals.

Neutrophil Recruitment, Antimicrobial Functions, and Tissue Damage

Neutrophils, the most abundant immune cell, are the first responders to infection and are crucial in the immune response to bacterial and fungal pathogens⁴³. Neutrophils are actively recruited to sites of infection by several different chemotactic factors. These chemoattractants signal to neutrophils that they are needed in the tissue as they roll along the walls of post-capillary venules searching for signs of distress⁴⁴. Specifically, chemoattractants act on endothelial cells to upregulate selectin molecules involved in neutrophil tethering and integrins involved in neutrophil adhesion. The most potent of the host-derived chemoattractants is the chemokine interleukin-8 (IL-8), which is released by monocytes, macrophages, epithelial cells, mast cells, keratinocytes, fibroblasts, endothelial cells, and neutrophils during inflammation⁴⁵. Importantly, IL-8 is increased in HIV-infected patients on ART⁴⁶. High levels of IL-8 causes increased endothelial expression of adhesion molecules and increased leukocyte transmigration, which have been proposed to contribute to increased risk of comorbidities in treated HIV infection⁴⁷. Bacteria also secrete molecules, such as N-formylated peptides, that recruit neutrophils by similarly activating endothelial cells to mediate transmigration into tissues⁴⁸.

Once in the tissue, neutrophils employ several different potent antimicrobial mechanisms to fight invading pathogens. Central to all of these mechanisms are various cytotoxic granules within neutrophils that contain different types of antimicrobial molecules including 1) cationic peptides such as defensins 2) proteases such as cathepsins, lysozyme, gelatinase, and elastase and 3) reactive oxygen and reactive nitrogen species⁴⁹. Degranulation is the exocytosis or release of granular contents into the extracellular matrix to kill pathogens or promote transmigration^{50, 51}. Additionally, through a process known as phagocytosis, neutrophils internalize microorganisms and sequester them within the phagosome, which then merges with granules to kill the pathogen⁵². Finally, neutrophils can release neutrophil extracellular traps (NETs) consisting of DNA to trap microbes followed by killing of the pathogen by granule components⁵³. These antimicrobial functions of neutrophils are mediated through recognition of pathogen-associated molecular patterns (PAMPs). PAMPs interact with pattern-recognition receptors (PRRs) on the neutrophil surface, including toll like receptors (TLRs), peptidoglycan-recognition protein (PGRP), and collectins^{54, 55}. Neutrophils express TLRs 1 and 2 and 4-10, and TLR ligation mediates cell survival, cytokine release, superoxide generation, degranulation, and phagocytosis⁵⁶. Additionally, neutrophils recognize complement-opsonized pathogens by surface receptors such as CD11b/CD18 and CD11c/CD18 and antibody-opsonized pathogens through Fc receptors (discussed further below)⁵⁶.

Importantly, while neutrophils are most often associated with control of bacterial and fungal pathogens, neutrophils also constitute the first and most abundant leukocyte recruited to sites of viral infection and are involved in the antiviral immune response⁵⁷. Additionally, neutrophils and Th17 cells participate in reciprocal recruitment through the production of chemokines and the Th17/neutrophil axis has been well studied in several bacterial and viral

infections^{15, 58, 59}. The potential role of neutrophils in the antiviral response to HIV and SIV and their relationship to the loss of IL-17 producing T cells in the GI is further discussed in Chapter II.

The antimicrobial functions of neutrophils make them a necessary component of the immune system's ability to fight pathogens. However, since their discovery they have been thought of as inflammatory cells that cause destruction in their wake, and collateral tissue damage is often observed as a result of neutrophils' antimicrobial activities⁶⁰. Indeed, excessive host tissue damage can be caused by unregulated control of granule proteases⁶¹. The three most commonly associated with damage and targeted in therapeutics are the serine proteases elastase, proteinase-3 and cathepsin G⁶². Additionally, NETs can trigger antibody-mediated autoimmune responses and organ dysfunction, and the release of toxic reactive oxygen species can cause extracellular matrix damage and tissue necrosis^{63, 64}. Timing is an important determining factor in beneficial vs. detrimental neutrophil responses: neutrophils recruited to the site of infection early may help contain invading pathogens and promote protection while prolonged or untimely neutrophil accumulation may do more harm than good. In Chapter II, I investigate the kinetics of the neutrophil response in acute SIV infection in relation to mucosal dysfunction to inform their potential to contribute to viral containment and/or tissue damage.

Programmed Cell Death of Neutrophils

Because of their propensity for unintended tissue damage, neutrophil clearance is an important mechanism in tissue homeostasis, and neutrophils are generally short-lived, with findings from studies investigating neutrophil lifespan *in vivo* ranging from 8 hours to 5 days⁵⁰. Neutrophils can undergo cell death in one of several ways. Programmed cell death via apoptosis

followed by engulfment by macrophages is the least inflammatory mechanism of neutrophil removal, and neutrophils undergo constitutive apoptosis in order to limit their lifespan in tissues^{65, 66}. Importantly, apoptotic neutrophils remain intact, thus preventing their intracellular constituents from being released⁶⁷. Additionally, apoptotic neutrophils undergo a decrease in cellular function, which as previously described can cause tissue damage, and also stimulate anti-inflammatory factors such IL-10 to be released by macrophages^{68, 69}. Contrarily, necrosis occurs when the plasma membrane is compromised and results in the release of intracellular molecules that act as damage-associated molecular patterns (DAMPs) to activate pro-inflammatory cytokine production by macrophages⁷⁰. Importantly, late or secondary necrosis can occur if removal of the apoptotic cell is delayed and a delayed ability of macrophages to phagocytose apoptotic neutrophils has been observed in HIV infection, which may contribute to inflammation⁷¹.

Apoptosis is mediated by several different pathways that ultimately result in the activation of effector caspases: caspase-3, caspase-8, and caspase-9. Importantly, caspase activation was shown to play a pivotal role in neutrophil regulation in inflammation in a study that demonstrated that individuals with bacterial sepsis have reduced caspase-3 and caspase-9 activities and delayed neutrophil apoptosis⁷². While peripheral blood neutrophils in HIV and SIV infection exhibit increased apoptosis that contributes to neutropenia, neutrophil apoptosis and lifespan in the gut has yet to be examined^{73, 74}. In Chapter III, we investigate reduced caspase-3 activation in mucosal neutrophils as a mechanism for persistent neutrophil accumulation in treated HIV infection.

Neutrophils and HIV Infection

Neutrophil frequencies and functionality could impact HIV infection in many different capacities, and this is depicted in Figure 1.2. Importantly, most of what is known about neutrophils in HIV infection pertains only to peripheral blood neutrophils and there have been no studies directly assessing the role of neutrophils in gastrointestinal immunity or dysfunction in HIV or SIV infection. During chronic HIV infection, neutrophils infiltrate the GI tract at high levels, yet their contribution to the pathology of mucosal dysfunction in GI tissue is unknown²⁵. While neutrophils are critical in protection from infections, aberrant neutrophil responses can also be harmful. Models of inflammatory bowel disease (IBD) suggest that neutrophils in the GI tract may contribute to disease^{75, 76}. However, some IBD models demonstrate that depletion of neutrophils can exacerbate disease, illustrating a controversy over the role of these cells^{77, 78, 79}. In favor of neutrophils contributing to mucosal damage in settings other than HIV are several studies indicating that transepithelial migration of neutrophils creates gaps between epithelial cells, alters levels of tight junction proteins, and increases epithelial permeability^{80,81,82}. The potential role of neutrophil recruitment in inducing mucosal damage could have a critical impact on microbial translocation, systemic immune activation, and the resulting comorbidities in HIV infection.

In the SIV model of infection, increased neutrophil infiltration in the GI tract has been observed in association with increased disease progression and damage to the epithelial barrier, but it is unclear if this infiltration contributes to the barrier damage or is a response to contain microbial products that have translocated to the lamina propria²⁵. In another SIV infection study, neutrophil infiltration coincided with microbial translocation, suggesting that neutrophils may be

responding to the bacterial products but are unable to contain them in the gut⁸³. In rats, increased microbial translocation after neutrophil depletion has been reported, suggesting neutrophils may be critical in clearing translocated bacteria and preventing access of these bacteria and bacterial products to the periphery⁷⁷. We examine the relative kinetics of neutrophil recruitment and microbial translocation in a longitudinal acute SIV study in Chapter II.

Importantly, the inability of neutrophils to contain microbial translocation in the context of SIV and HIV infection may be due to decreased antimicrobial function of the recruited neutrophils. Peripheral neutrophils in HIV-infected individuals have reductions in several functions, including chemotaxis, phagocytosis, bactericidal, and oxidative burst abilities which worsen during the course of infection and are linked to increased risk of secondary infections^{84,85,86,87,88}. In addition, one study reported that ART improves chemotaxis but not the crucial microbe-killing functions of neutrophils, further suggesting that decreased neutrophil function may contribute to microbial translocation in the setting of ART-treated, chronic HIV infection⁸⁹.

One potential mechanism for reduced antimicrobial functions of neutrophils in HIV infection is the reduced expression of Fc receptors, which greatly increase the efficiency with which neutrophils are able to respond to antibody-opsonized pathogens⁹⁰. Previous studies have reported reduced Fc γ RII and Fc γ RIII expression on blood neutrophils in HIV infection, both of which are constitutively expressed on neutrophils and important for phagocytosis and cytotoxic responses to pathogens^{90, 91, 92}. Contrarily, while neutrophils from healthy donors do not express the high-affinity receptor Fc γ RI, studies have shown that Fc γ RI is induced on blood neutrophils in some but not all cases of bacterial infection, in response to cytokine stimulation, and in HIV infection^{93, 94, 95}. Blood neutrophils also constitutively express Fc α RI, the IgA antibody Fc receptor, but the effect of HIV on neutrophil Fc α RI expression has yet to be examined⁹⁶. The

expression of Fc α RI on GI neutrophils could have important implications given that IgA is the most abundant antibody isotype, enriched at mucosal sites, and important for intestinal immune responses and homeostasis⁹⁷.

Neutrophils in the GI tract in HIV infection may also be interfacing with the adaptive immune system and driving dysfunction. Neutrophils that act as granulocytic myeloid-derived suppressor cells (G-MDSCs) have been identified as a specific phenotype of neutrophils with the ability to suppress the adaptive immune response, particularly through suppression of T cell cytokine production and proliferation⁹⁸. A study published in 2014 elucidated a role for suppressive neutrophils in T cell exhaustion and immune suppression in HIV infection⁹⁹. Specifically, the authors found that blood neutrophils in HIV-1 infected individuals have increased PD-L1 expression and suppress T cell function via a mechanism involving reactive oxygen species and PD-L1 interaction with the T cell surface molecule PD-1. Importantly, the functionality of GI tract neutrophils in HIV infection is unknown, and the expression of functional markers such as Fc receptors and PD-L1 has yet to be assessed on GI neutrophils in HIV infection, making this an important area for future study.

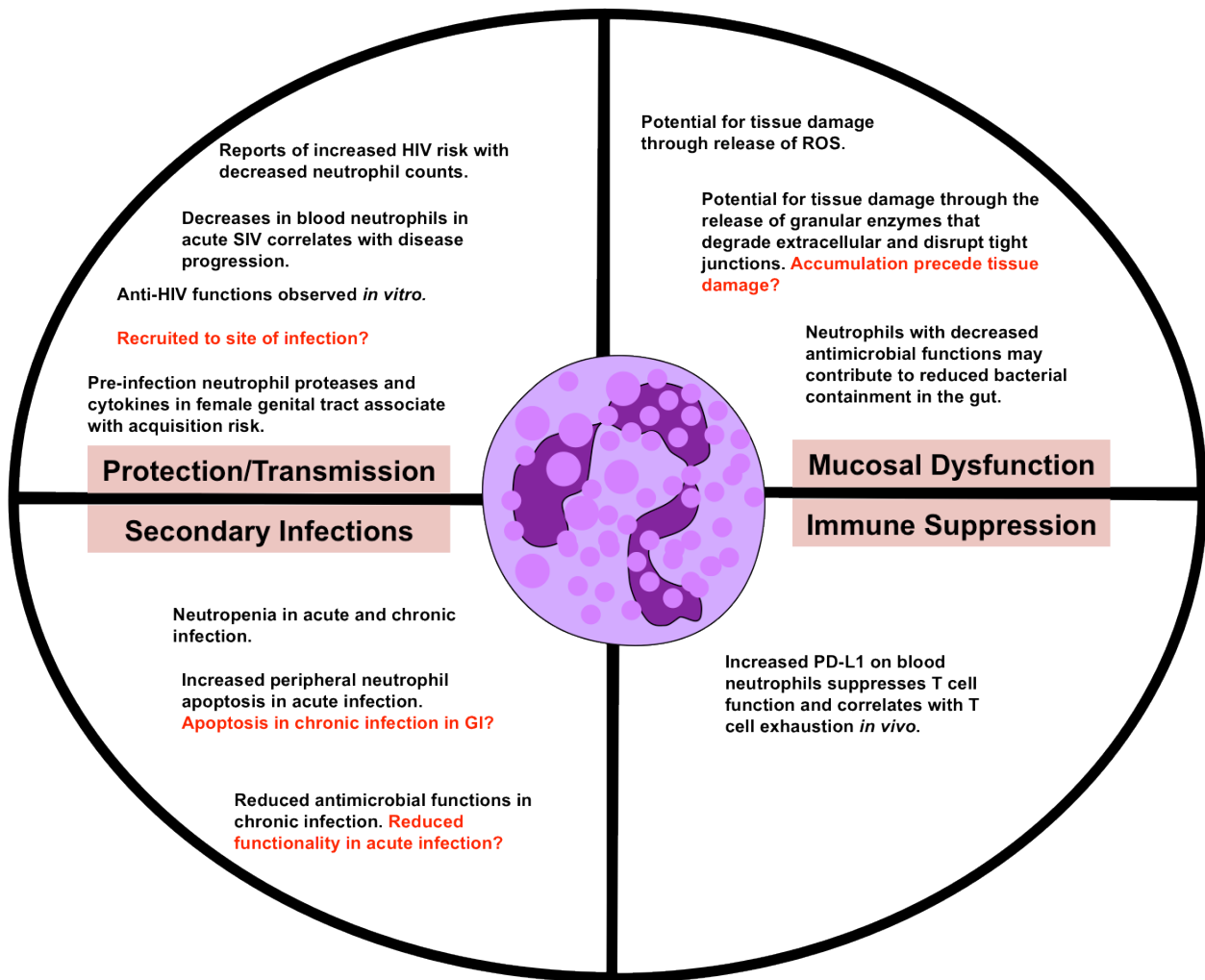


Figure 1.2. Neutrophils in HIV infection. Neutrophils could have a diverse impact on HIV infection depending on their location, timing, and functionality. Important unanswered questions addressed in this thesis are depicted in red. Protection/transmission: *In vitro* studies demonstrate anti-HIV functions of neutrophils and reports correlate peripheral blood neutrophil frequencies with HIV acquisition risk and SIV disease progression *in vivo*. Contrarily, neutrophil factors in the female genital tract pre-infection also associate with HIV acquisition risk *in vivo*. Secondary infections: Neutrophil dysfunction is associated with increased risk of secondary infections in HIV-infected individuals. Mucosal dysfunction: A direct link between neutrophils and mucosal dysfunction has yet to be assessed but it is proposed that neutrophils could contribute to tissue

damage and that neutrophil dysfunction may allow microbial translocation. Immune suppression: Neutrophils can act as gMDSCs that suppress T cell function and increased suppressor activity of peripheral neutrophils was observed in HIV infection.

The Intestinal Microbiome and HIV Infection

The human intestine is home to 10^{15} total microbes composed mostly of bacteria, with each individual harboring at least 160 different species^{100, 101}. This intestinal microbiome contributes to immune homeostasis and an individual's overall health, and alterations in microbiome composition are associated with several diseases including IBD, obesity, and type 1 diabetes^{97, 102, 103, 104}. Interactions between gut microbes and the immune system and their reciprocal regulation and programming are complex, but several key interactions are known and may be important for GI homeostasis and mucosal dysfunction in HIV infection. For example, commensals induce antimicrobial peptide production by paneth cells in the intestinal epithelium¹⁰⁵. Antimicrobial peptides, such as α -defensins limit the number of bacteria contacting epithelial cells at the mucosal surface and prevent microbial translocation into the intestinal lamina propria¹⁰⁶. Commensal bacteria also play a role in maintaining epithelial integrity in the intestines by promoting intestinal epithelial cell proliferation, which is necessary for healing after intestinal injury¹⁰⁷. Additionally, specific bacteria, including segmented filamentous bacteria (SFB), can induce IgA production, which maintains homeostasis by restricting systemic immune activation against commensal bacteria^{108, 109}. Importantly, SFB as well as bacteria from the cytophaga-flavobacter-bacteroidetes group can also induce Th17 cells, which are notably reduced in the absence of commensal flora in murine models^{110, 111}. As previously mentioned, Th17 cells and IgA producing plasma cells are reduced in the gut in HIV

infection and this contributes to GI mucosal dysfunction^{27, 112}. Finally, commensal bacteria including *Bacteroides fragilis* induce IL-10-producing CD4+ T cells, which have a beneficial anti-inflammatory effect¹¹³. Indeed, decreased regulation of inflammation may be associated with disease progression¹¹⁴.

There is much evidence that microbes impact neutrophil survival and function through both direct interactions and by influencing cytokine release by other immune cells. First, TLR-4 activation by bacterial LPS has been shown to increase neutrophil survival both through direct interaction with neutrophils and through the release of TNF- α and IL-1 β by monocytes¹¹⁵. In addition, incubation with the gram-positive bacteria *Streptococcus haemolyticus* also increased neutrophil survival *in vitro*¹¹⁶. Finally, monocytes were shown to secrete G-CSF, a cytokine involved in regulating neutrophil survival, in response to LPS and lectin stimulation¹¹⁷. Previous studies have also demonstrated an impact of microbial interactions and inflammatory cytokines on neutrophil phenotype and functionality whereby stimulation of neutrophils with both LPS and AT-2 inactivated HIV-1 induced expression of PD-L1 on isolated human neutrophils⁹⁹. Additionally, neutrophil priming by cytokines such as IFN γ induces Fc receptor expression and augments cytotoxic killing by neutrophils, which may be affected by the presence of different microbes in the GI environment¹¹⁸. Finally, one study demonstrated that a bacterial peptidoglycan, diaminopimelic acid-type peptidoglycan found predominantly on Gram-negative bacteria, could increase killing capacity of peripheral neutrophils¹¹⁹. In Chapter III, I examine the effects of bacterial dysbiosis on neutrophil survival *in vitro*. The connection between microbiome alterations and neutrophil lifespan and functionality in HIV infection *in vivo* is an important area of future study.

HIV-induced alterations to the intestinal microbiome are well-documented¹²⁰. Briefly, studies report overall decreased diversity and decreased abundances of bacteria in the *Bacteroides*, *Lactobacillus*, *Bifidobacterium*, *Coprococcus*, *Eubacterium*, *Blautia*, and *Ruminococcus* genera^{121, 122, 123, 124}. Genera enriched in HIV include *Prevotella*, *Pseudomonas*, *Acinetobacter*, *Campylobacter*, *Escherichia*, and *Desulfovibrio*^{121, 122, 125}. Dysbiotic bacteria in HIV infection have been termed HIV altered mucosal bacteria (HAMBs)¹²⁶. Individual HAMBs associate with increased mucosal T cell and dendritic cell activation and an overall altered microbiome associates with increased microbial translocation in HIV infection¹²¹. Importantly, dysbiosis remains evident in individuals on ART and associates with disease progression¹²². Finally, it should be noted that a recent study reported dysbiosis often attributed to HIV infection may actually be a result of sexual preference, as men who have sex with men (MSM) had an increased abundance of *Prevotella* independent of HIV status, and the authors observed no HIV-specific dysbiosis when controlling for sexual preference¹²⁷. This highlights the importance of future studies investigating confounding demographic factors in comparisons of HIV infected and uninfected populations.

The SIV Model

Given that many critical events occur within the first weeks of infection when a person is unlikely to know he or she is infected, and that most T cell depletion and immune dysfunction occurs at mucosal tissues that are not easily accessible, animal models of HIV are necessary to fill in timing and sampling gaps and provide insights into pathogenesis and targets for intervention. HIV-1 and HIV-2 both originated from cross-species transmission. HIV-1 is a recombination of chimpanzee and gorilla simian viruses and HIV-2 is a result of several

transmissions from sooty mangabeys¹²⁸. Macaques, which are not naturally infected with SIV, are capable of experimentally being infected with different SIV viruses, and the infection of *Macaca mulatta* (rhesus macaques; RM) and *Macaca nemestrina* (pigtail macaques) have become important models for the study of lentiviral pathogenesis. Indeed, when macaques are infected with SIV, viral pathogenesis closely parallels that seen in HIV infection in humans¹²⁹. Specifically, the following defining characteristics of HIV are also observed in SIV-infected macaques: 1) a failure of immune responses to contain the virus; 2) early CD4+ T cell depletion in mucosal tissues followed by widespread peripheral CD4+ T cell depletion; 2) GI barrier breaches, mucosal dysfunction, and microbial translocation; 3) systemic immune activation and inflammation that predict disease progression and persist despite treatment; 4) progression to AIDS if untreated; and 5) persistent virus in latently infected cells constituting a viral reservoir when treated with antiretroviral therapy^{130, 131}. Additionally important to appropriately modeling HIV infection is that macaques can be infected by different routes to model transmission including vaginal and rectal for sexual transmission of HIV and oral infection to model mother-to-child transmission through breast milk. Repeated low-dose challenges to the vaginal and rectal mucosa have been used to further mimic sexual transmission^{132, 133}.

HIV does not infect macaques, so nonhuman primate models use various SIV viruses or chimeric simian-human immunodeficiency viruses (SHIVs) in which HIV genes are placed into an SIV backbone containing the necessary viral antagonists to block host restriction factors^{134, 135}. Several different SIV viruses have been used to model HIV pathogenesis. SIVmac251 is an uncloned viral isolate from an infected macaque containing genetically heterogeneous virus, which makes it useful for assessment of the transmission and integration of different viral variants within an inoculum¹³⁶. However, comparisons between studies using uncloned viral

isolates can be complicated due to differences between challenge stocks. Therefore, an SIV clone from the same source as SIVmac251 was passaged in macaques to increase pathogenicity and better model disease progression to AIDS¹³⁷. The resulting clonal virus, SIVmac239, has been used in studies of lentiviral transmission, pathogenesis, vaccination, and eradication, and has provided major insights into complex host-pathogen interactions applicable to HIV in humans^{16, 138, 139, 140}. We utilized SIVmac239 infection in macaques to investigate early events in the GI mucosa in Chapter II and to examine the safety and efficacy of fecal microbial transplantation as a therapeutic in lentiviral infection in Chapter IV.

Thesis Objectives

Neutrophils are understudied in HIV infection due to the cells' susceptibility to freezing injury and reduced viability after cryopreservation, which require that they be assessed fresh after isolation from blood or tissue¹⁴¹. For this reason, neutrophils in the gut in HIV infection have previously only been assessed through microscopy staining of MPO. Although these studies suggest neutrophils accumulate in the gut in chronic HIV and SIV infection, no studies have been performed to longitudinally assess neutrophil infiltration or further characterize the cells in the tissues in chronic HIV infection. Given that IL-17 is an important regulator of neutrophil responses, it is possible that early loss of Th17 cells may inhibit the participation of neutrophils in an antiviral response in the gut mucosa. It also remains unclear if neutrophils infiltrate the gastrointestinal tract prior to the onset of mucosal damage. Additionally, HIV induced dysbiosis of the gastrointestinal microbiome is well described and HIV altered mucosal bacteria could impact neutrophil survival and contribute to neutrophil accumulation in chronic HIV infection. Finally, therapies aimed at dysbiosis could reduce mucosal dysfunction and increase health in

infected individuals. This thesis describes studies aimed to address three objectives: 1) longitudinally assess the kinetics of gut neutrophil accumulation and reduced neutrophil antimicrobial function in relation to mucosal dysfunction, immune activation, and Th17 cell depletion in acute SIV infection; 2) investigate increased neutrophil lifespan as a mechanism for gut neutrophil accumulation in chronic, treated HIV infection and a potential role for HIV altered mucosal bacteria; and 3) assess the safety and efficacy of fecal microbial transplantation as a potential therapeutic for mucosal dysfunction in SIV infection.

Chapter II

Longitudinal evaluation of mucosal dysfunction, immune activation, and neutrophil infiltration in SIV infected rhesus macaques

Introduction

The widespread use of ART has reduced progression to AIDS and shifted HIV infection to a chronic disease. In this chronic disease state, treated HIV-infected adults have a higher risk of developing serious non-AIDS-related diseases, including non-AIDS associated cancers as well as cardiovascular, kidney, liver, neurologic, and bone diseases compared to uninfected individuals¹⁴². Gastrointestinal (GI) mucosal damage and immune dysfunction results in microbial translocation and systemic immune activation, which are major contributors to non-infectious comorbidities and mortality^{38, 39, 143, 144, 145, 146 22, 23}. During both treated and untreated chronic HIV infection, neutrophils infiltrate the GI tract at high levels, yet their contribution to the pathology of mucosal dysfunction in GI tissue is unknown¹⁴⁷. Importantly, it is unknown if neutrophil infiltration precedes mucosal damage and systemic immune activation and could therefore be an initial mechanism of mucosal dysfunction.

Loss of IL-17 producing T cells in the mucosa contributes to barrier damage and mucosal dysfunction and correlates with systemic immune activation and disease progression in HIV and pathogenic SIV infection^{148 149, 150}. There is cross-talk between IL-17 producing cells and neutrophils that supports reciprocal differentiation, recruitment, and maintenance, and one of the methods by which Th17 cells support barrier protection is by promoting a neutrophil response to pathogens¹⁵¹. Specifically, IL-17 induces IL-8 and G-CSF production from mucosal epithelial cells to recruit and prime neutrophils¹⁵². While neutrophils are classically considered contributors to immunity against bacterial and fungal pathogens, they also play an important role in the antiviral response to several infections, particularly at mucosal surfaces. Indeed, a Th17 response

and subsequent recruitment of neutrophils have been shown to contribute to protection against a large number of bacterial and viral infections in the gut, lung, and oral mucosa¹⁵. Importantly, protective vaccination against murine rotavirus infection involves the IL-17/neutrophil pathway, demonstrating that this pathway and neutrophil recruitment can contribute to viral containment in the GI mucosa¹²⁸. Th17 cells are preferentially depleted in acute HIV and SIV infection, yet it remains unknown how early this occurs after infection and if very early dysregulation of the IL-17/neutrophil pathway therefore precludes a neutrophil response to the virus. Further, neutrophil recruitment to the initial site of SIV infection and to what extent they may contribute to viral containment has not previously been examined.

In this chapter, we describe a longitudinal study utilizing SIV infection of rhesus macaques, which is a well-characterized infection model and leads to CD4+ T cell depletion in the periphery and mucosa, mucosal dysfunction, chronic immune activation, and progression to AIDS if left untreated¹⁵³. These characteristics parallel HIV pathogenesis and make it an excellent model for studying pathogenic lentiviral infection. Using this model we investigated the kinetics of microbial translocation, immune activation, and Th17 depletion and how these dysfunctions relate to GI neutrophil frequencies after intrarectal SIV infection. We hypothesized that an early loss of Th17 cells would inhibit neutrophil recruitment to the rectum in response to the virus. We therefore additionally hypothesized that neutrophils would accumulate later in infection in response to barrier damage and microbial translocation rather than precede it.

Methods

Study animals and sampling schedule

Animals were housed and cared for in Association for the Assessment and Accreditation of Laboratory Animal Care international (AAALACi) accredited facilities, and all animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) of University of Washington. Six male rhesus macaques (*Macaca mulatta*) were infected intrarectally with 100,000 TCID₅₀ SIVMAC239x, a swarm virus composed of 10 different molecular clones each containing a unique molecular barcode¹³⁹. All six animals became infected after one round of infection (Figure 2.1A). EDTA blood, bone marrow in ACD, peripheral lymph nodes, colon, rectum, and jejunum biopsy samples were collected prior to infection and on days 3, 14, and 28 and 63 days post-infection. Baseline samples prior to infection were taken 63, 49 and 21 days pre-infection, and data from two baseline samples were averaged and graphed as day -49. Animals were then euthanized per the protocol specified experimental endpoint between 101-141 days post-infection. Samples analyzed from individual animal necropsy timepoints were graphed collectively as day 122 post-infection.

Blood and tissue processing

Plasma and peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation, and the plasma was frozen for later analysis. Colon, rectum, and jejunum biopsies were immediately enzymatically digested with RPMI media (GE Healthcare Life Sciences, Logan UT) supplemented with Liberase (40 µg/ml, Sigma-Aldrich, St. Louis, MO) and DNase (4 µg/ml, Sigma-Aldrich) for 1 hour at 37°C with vigorous stirring, then ground through a 70-µm cell strainer into a single cell suspension. Lymph nodes were immediately ground through 70-µm cell strainer into a single cell suspension. PBMCs and single cell suspensions of biopsies and lymph nodes were then analyzed by flow cytometry. Complete blood counts with differential were measured on a Beckman Coulter AC*T 5diff CP hematology analyzer. Viral loads were

determined by real-time reverse transcription (RT)-PCR using primers specific for SIVgag as previously detailed¹⁵⁴.

Flow Cytometry Staining

Surface antigen and intracellular proliferation marker staining:

Whole blood, whole bone marrow, and single cells from lymph nodes, colon biopsies, and rectum biopsies were first stained immediately with LIVE/DEAD Fixable Aqua Dead Cell Stain (ThermoFisher). Cells were then stained using the following surface antigen antibodies with clone denoted in (), from BD biosciences unless otherwise stated: CD45 PerCP (D058-1283), CD3 PE CF594 (SP34-2), CD20 PE Cy5 (2H7), CD4 Brilliant Violet 605 (Biolegend, OKT4), CD8 Brilliant Violet 570 (Biolegend, RPA-T8), CD14 Brilliant Violet 786 (Biolegend, M5E2), HLA-DR Brilliant Violet 711 (G46-6), and CD11b APC-H7 (ICRF44). Following surface staining, cells were permeabilized using Cytotfix/Cytoperm (BD biosciences), and stained for intercellular Ki67 (BD, B56) to assess proliferation. Red blood cells were lysed in whole blood samples using FACs lysing solution (BD). T cells were defined as live/CD45+/CD20-/CD14-/CD3+ and CD4+ and CD8+ T cells were further delineated to assess activation (HLA-DR) and proliferation (Ki67). Neutrophils were defined as live/CD45+/CD3-/CD20-/CD14+/CD11b+/HLA-DR-/High SSC cells (Figure 2.6).

Intracellular cytokine staining:

PBMCs and lymph node, colon, rectum, and jejunum cells were stimulated for 14 hours at 37°C with 10 ng/ml PMA (Sigma-Aldrich) and 1 µg/ml Ionomycin (Life Technologies) in R10 media with 1 mg/ml of Brefeldin A (Sigma-Aldrich). Stimulated cells were then stained with the following surface antigen antibodies from BD biosciences unless otherwise stated: CD45 PerCP

(D058-1283), CD3 PE CF594 (SP34-2), CD4 Brilliant Violet 605 (Biolegend, OKT4), CD8 APC-H7 (SK1), CD14 Brilliant Violet 786 (Biolegend, M5E2). Following surface staining, stimulated cells were permeabilized using Cytotfix/Cytoperm (BD biosciences), and stained using IL-17 PE (eBioscience, ebio64CAP17). T cells were defined as live/CD45+/CD20-/CD14-/CD3+ and CD4+ T cells were further delineated to assess IL-17 production.

Stained samples were fixed in 1% paraformaldehyde and collected on an LSR II (BD Biosciences, La Jolla, California). Analysis was performed in FlowJo (version 9.7.6, Treestar Inc., Ashland, Oregon).

Assessment of neutrophil phagocytosis

To assess the ability of neutrophils to perform phagocytosis, plasma was removed from whole blood through density centrifugation and blood was resuspended in PBS. pHrodo® Red *S. aureus* BioParticles® were preopsonized with combined plasma from 5 healthy rhesus macaques for 30 minutes at 37°C. As a negative control, an aliquot of whole blood was preincubated with the phagocytosis inhibitor cytochalasin D (Sigma-Aldrich) for 30 minutes at 37°C. Blood aliquots (including the negative controls) were then incubated with the opsonized pHrodo® for 2 hours at 37°C. All incubations were done with no CO₂ to reduce potential acidity and background. Samples were washed once with PBS+EDTA and resuspended in FACS lysing solution (BD). Samples were collected on an LSR II and analysis was performed in FlowJo. Neutrophils were defined by their side scatter and forward scatter properties and the percentage of neutrophils exhibiting positive pHrodo® Red fluorescence was determined. Background subtraction was performed by subtracting the percentage of pHrodo+ neutrophils in the cytochalasin D negative control.

Assessment of plasma cytokines and soluble factors

LPS Binding Protein (Biometric, Brixen, Italy) was assessed in plasma via ELISA using a pre-prepared kit. ELISA plates were read using an iMark Microplate Reader (Biorad, Hercules, CA).

Statistical Analyses

Statistical analysis was performed using GraphPad Prism statistical software (Version 6, GraphPad Software, San Diego, CA). Post-infection timepoints were compared to baseline (averaged pre-infection timepoints), and significance was evaluated using a Wilcoxon signed-rank test with an alpha level of 0.05.

Results

Kinetics of plasma viremia and peripheral and mucosal CD4+ T cell depletion following intrarectal SIV infection of rhesus macaques

After one round of intrarectal infection, we observed detectable plasma virus in all six animals beginning 7 days post-infection (Figure 2.1A). Plasma viremia reached an average peak viral load of 6.4×10^7 copies/ml 14 days post-infection and an average viral set point of 3.5×10^6 copies/ml 28 days post-infection. As expected, blood CD4+ T cells dropped dramatically early in infection with significant depletion reached 28 days post-infection, as measured by both complete blood count (CBC) and flow cytometry (Figure 2.1A and Figure 2.1B). Although five of the six animals dropped below 200 CD4+ T cells/ μ l, none had progressed to AIDS by the study endpoint. Significant depletion of CD4+ T cells was observed in the peripheral lymph nodes beginning 14 days post-infection and the bone marrow beginning 28 days post-infection. In mucosal tissues, we observed a significant loss of CD4+ T cells beginning 14 days post-infection in both the colon and rectum (Figure 2.1C). Taken together these data demonstrate that the animals in this study became productively infected and succumbed to early depletion of

peripheral and mucosal CD4+ T cells post SIV-infection, which is a defining characteristic of lentiviral infection.

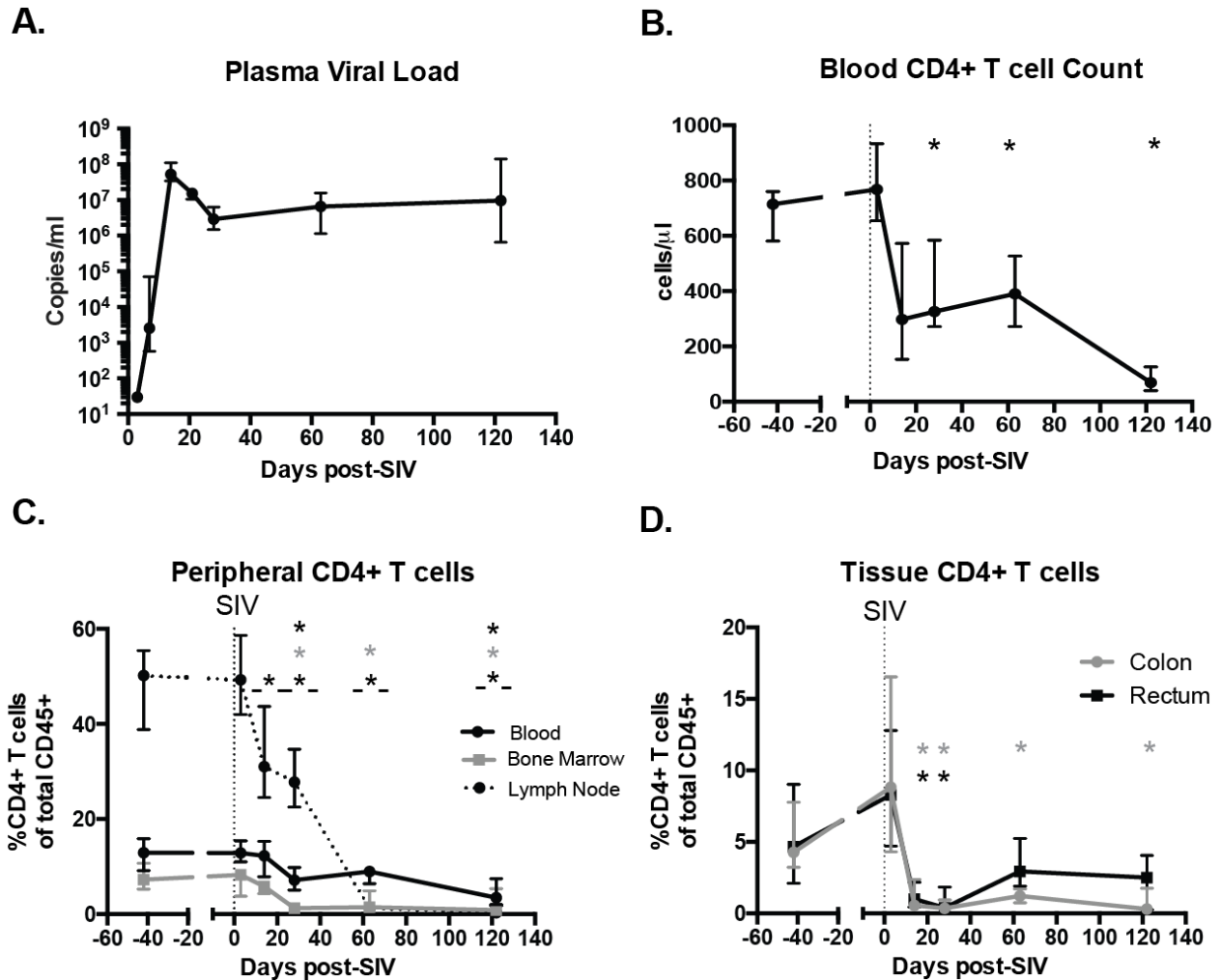


Figure 2.1. Dynamics of viral load and CD4+ T cell depletion in rhesus macaques following intrarectal infection with SIV_{Mac239x}. A.) SIV_{gag} RNA copies/ml measured in plasma. Data reported is median and interquartile range (IQR) for n=6. B.) Median and IQR of blood CD4+ T cell counts as measured by CBC. C.) CD4+ T cells as a percentage of total live, CD45+ leukocytes measured in whole blood (solid black), whole bone marrow (grey), and peripheral lymph nodes (dotted black). D.) CD4+ T cells as a percentage of total live, CD45+ leukocytes isolated from colon (grey) and rectal (black) biopsies. Significant differences determined by

comparing post-SIV timepoints to baseline using a Wilcoxon signed-rank test and a p -value < 0.05 is denoted with *.

Evidence of systemic microbial translocation begins 14 days post-SIV infection

Microbial products in the periphery have been observed in both early/acute and chronic HIV infection and correlated with systemic immune activation³⁰. However, studies longitudinally evaluating microbial translocation in relation to systemic immune activation, Th17 cell depletion, and neutrophil recruitment are lacking, particularly in acute infection. LPS binding protein (LBP) binds to LPS and presents it to CD14 and TLR-4. LBP has been used as an alternative to measuring plasma LPS because LPS detection is technically difficult and often results in contamination and irreproducible data³². We observed a trending increase in LBP beginning 14 days post-infection that remained through day 122 post-infection (Figure 2.2). The increased level of LBP observed in 4 of the 6 animals is similar to what was observed in progressive SIV infection in macaques and HIV infection in humans in previous studies^{30, 155}. In an additional experiment, we observed no changes in plasma LBP 3-7 days post-infection (data not shown). Although statistically insignificant in our small cohort of animals, the increase in LBP observed beginning at day 14 post-infection likely represents measurable systemic microbial translocation that corresponds with the marked depletion of mucosal CD4+ T cells observed at that time.

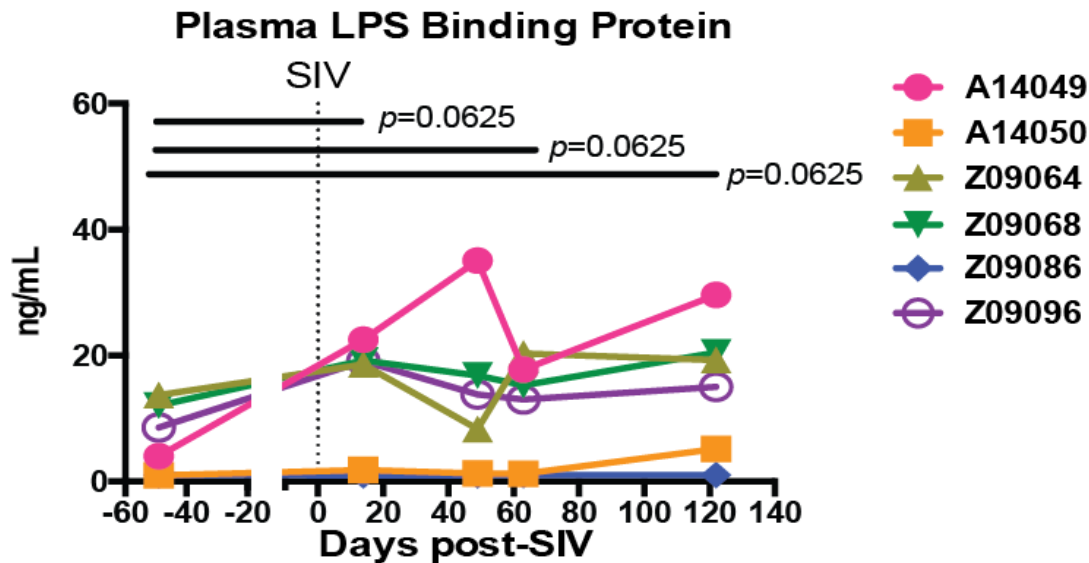


Figure 2.2. Trending increase in LPS binding protein beginning 14 days post-SIV infection. LPS binding protein (LBP) levels in plasma as measured by ELISA. *p*-values determined using a Wilcoxon signed-rank test between post-SIV timepoints and baseline.

Peripheral and mucosal T cell activation and proliferation begin 3-14 days post-infection

Peripheral T cell activation as measured by the expression of activation markers such as HLA-DR was shown to be more predictive of CD4⁺ T cell depletion than viral load and levels of T cell activation correlate with systemic microbial translocation^{30, 156, 157}. However, the relative contributions of virus replication and microbial translocation to early systemic immune activation remain unclear. In order to better elucidate this, we evaluated peripheral and mucosal T cell activation and proliferation longitudinally post-SIV. We observed increased CD4⁺ T cell activation in peripheral lymph nodes beginning at day 14 and blood and bone marrow beginning at day 28 post-SIV (Figure 2.3A). Activation of CD4⁺ T cells in the mucosal tissues began as early as day 3 in the rectum and day 28 in the colon (Figure 2.3C). Therefore, CD4⁺ T cells at the site of infection (rectum) become activated early, and this is followed by increased activation in the lymph node at peak viral load and finally the blood and bone marrow once virus set point

is reached. In contrast, CD8⁺ T cells in the blood and bone marrow become activated 3 days post-infection and remain highly activated throughout infection (Figure 2.3B). Similar to activation of CD4⁺ T cells, activation of CD8⁺ T cells in peripheral lymph node occurs at day 14 at peak viral load. Finally, increased activation of CD8⁺ T cells in mucosal tissues was observed beginning 14 days post-infection. These data demonstrate that CD8⁺ T cells in the periphery become activated earlier than CD4⁺ T cells but that CD8⁺ T cells in the mucosa become activated later. Interestingly, the percentage of activated CD8⁺ T cells in the colon and rectum and CD4⁺ T cells in the colon decrease 3 days post-infection (Figure 2.3C and D). This could be due to an influx of inactivated CD4⁺ and CD8⁺ T cells in the tissues, as we observed a transient increase in the frequency of total CD4⁺ and CD8⁺ T cells in both tissues (Figure 1D and data not shown).

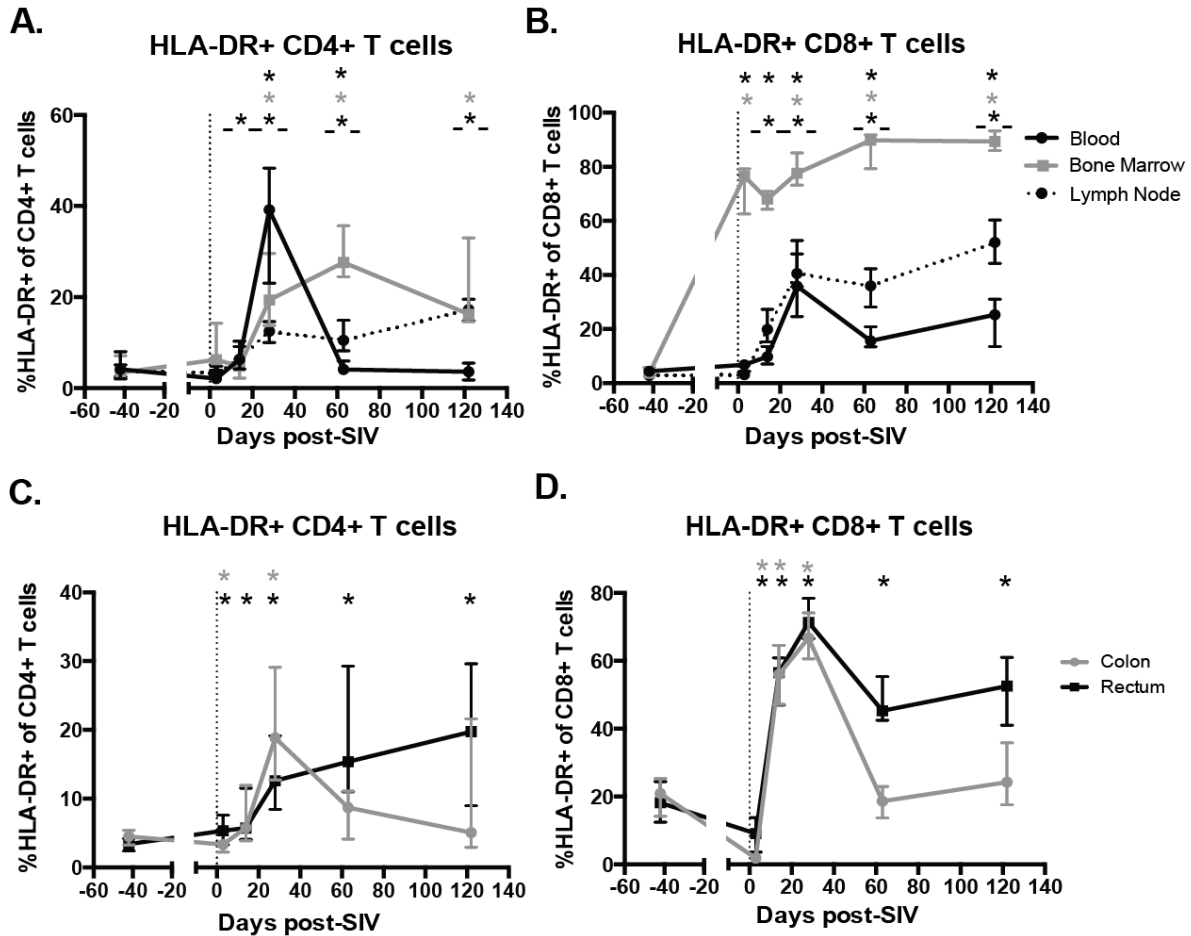


Figure 2.3. Kinetics of peripheral and mucosal T cell activation following intrarectal SIV infection of rhesus macaques. A.) Percentage of HLA-DR+ CD4+ T cells measured in whole blood (solid black), whole bone marrow (grey), and peripheral lymph nodes (dotted black). B.) Percentage of HLA-DR+ CD8+ T cells measured in whole blood (solid black), whole bone marrow (grey), and peripheral lymph nodes (dotted black). C.) Percentage of HLA-DR+ CD4+ T cells measured in leukocytes isolated from colon (grey) and rectum (black) biopsies. D.) Percentage of HLA-DR+ CD8+ T cells measured in leukocytes isolated from colon (grey) and rectum (black) biopsies. Data reported are median and interquartile range (IQR) for n=6. Significant differences determined by comparing post-SIV timepoints to baseline using a Wilcoxon signed-rank test and a p -value < 0.05 is denoted with *.

Proliferation as measured by nuclear antigen Ki67, which is expressed exclusively in cycling cells, is also increased in T cells in HIV and SIV infection^{158, 159}. Importantly, this measure of T cell proliferation has also been shown to predict the rate of progression to AIDS¹⁶⁰. We also examined Ki67 in the periphery and mucosal tissues to better understand the dynamics of T cell turnover in relation to microbial translocation. Increased frequencies of CD4+ T cells expressing Ki67 in the blood, bone marrow, and lymph nodes was observed beginning 28 days post-infection (Figure 2.4A). Increased T cell proliferation was observed in the colon beginning 14 days post-infection and both the colon and rectum had the highest frequency of Ki67+CD4+ T cells 28 days post-infection (Figure 2.4C). Ki67+ CD4+ T cells in the blood and bone marrow returned to baseline by 63 days post-infection, while frequencies dropped but remained elevated in the lymph nodes and mucosal tissues. In contrast, peripheral CD8+ T cells showed increased signs of proliferation 14 days post-infection that remained elevated compared to baseline through the course of infection, especially in the lymph node and bone marrow (Figure 2.4B). Frequencies of CD8+Ki67+ T cells in mucosal tissues also increased 14 days post-infection (Figure 2.4). Taken together these data demonstrate that peripheral and mucosal T cell activation begins as early as 3 days post-infection but widespread systemic T cell activation and proliferation is not established until 14-28 days post-infection.

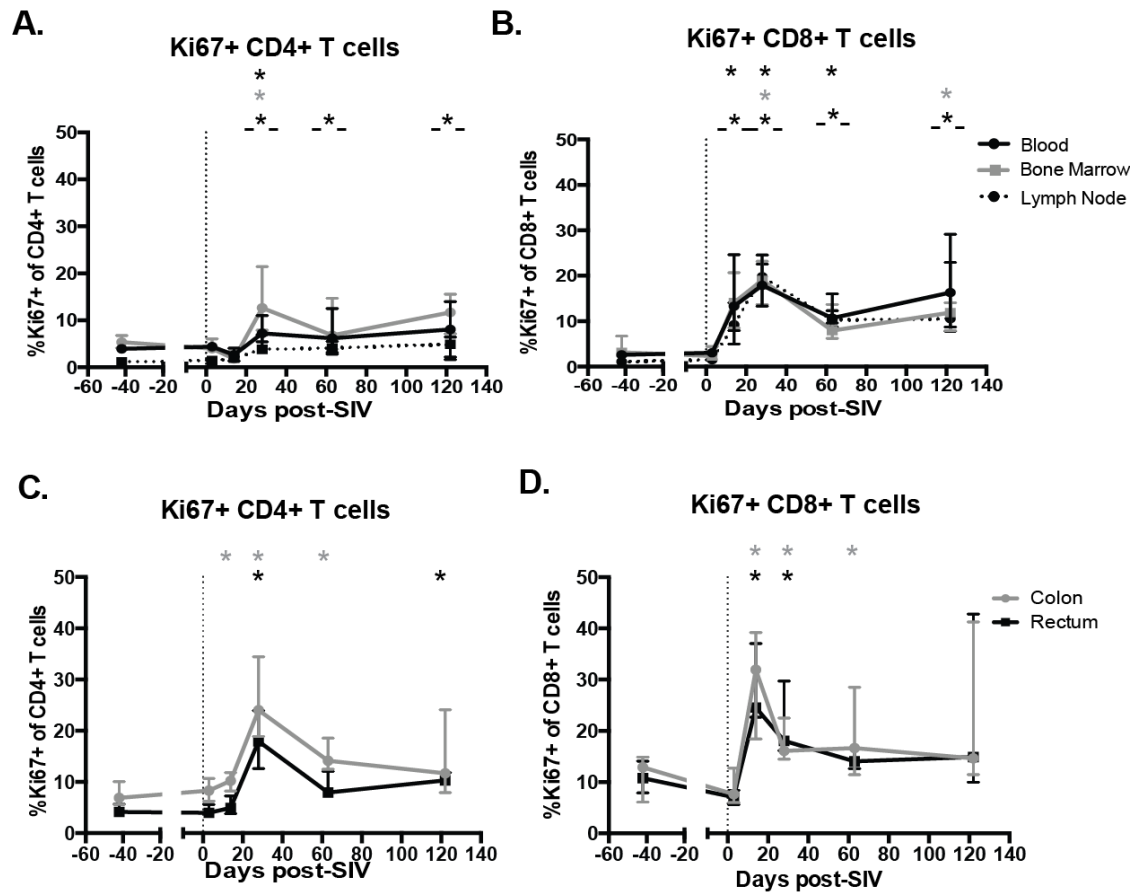


Figure 2.4. Kinetics of peripheral and mucosal T cell proliferation following intrarectal SIV infection of rhesus macaques. A.) Percentage of Ki67+ CD4+ T cells measured in whole blood (solid black), whole bone marrow (grey), and peripheral lymph nodes (dotted black). B.) Percentage of Ki67+ CD8+ T cells measured in whole blood (solid black), whole bone marrow (grey), and peripheral lymph nodes (dotted black). C.) Percentage of Ki67+ CD4+ T cells measured in leukocytes isolated from colon (grey) and rectum (black) biopsies. D.) Percentage of Ki67+ CD8+ T cells measured in leukocytes isolated from colon (grey) and rectum (black) biopsies. Data reported are median and interquartile range (IQR) for n=6. Significant differences determined by comparing post-SIV timepoints to baseline using a Wilcoxon signed-rank test and a p -value < 0.05 is denoted with *.

Loss of peripheral and mucosal Th17 cells begins 14-28 days post-SIV infection

Peripheral and mucosal Th17 cells are preferentially depleted in HIV and SIV infection and their loss is associated with mucosal damage^{19, 112}. Although Th17 cell depletion has been reported as early as 14 days post-infection, no studies have assessed Th17 cell frequencies in mucosal tissues prior to day 14 or the potential reconstitution after their initial depletion in a longitudinal study¹⁵⁰. Therefore, the kinetics of their depletion relative to the onset of mucosal dysfunction remains unknown. Additionally, very early loss of Th17 cells could have an impact on the subsequent neutrophil response to the virus. For these reasons, we assessed Th17 cell frequencies in the periphery and mucosal tissues in our longitudinal study. We found that Th17 cell frequencies in peripheral lymph nodes and blood do not decline until 28 days post-infection (Figure 2.5A). In the mucosa, we saw evidence of Th17 cell depletion beginning 14 days post-infection in the jejunum and colon, but significant depletion was not observed until 28 days post-infection in the jejunum and 63 days post-infection in the colon (Figure 2.5B). Significant depletion in the rectum was not observed until necropsy, which was 122 days post-infection on average. These are the first data suggesting that Th17 depletion does not occur prior to the onset of the microbial translocation and systemic immune activation. Therefore, their loss likely perpetuates mucosal damage later in infection but is not necessarily an initial mechanism of reduced barrier integrity. Further, these data suggest that Th17 frequencies are maintained in early infection and could therefore participate in a neutrophil response to infection. However, the dynamics of Th17 loss in different models should be investigated.

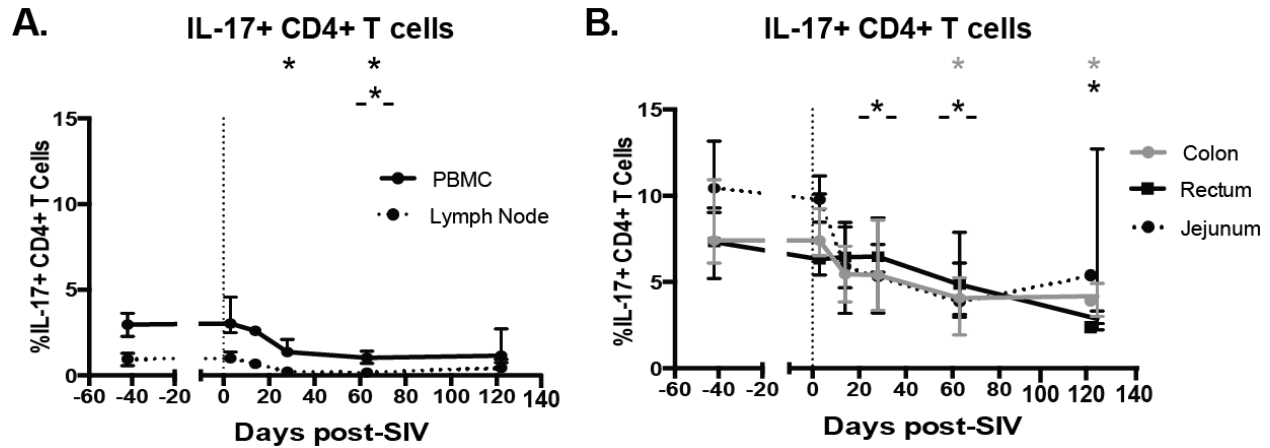


Figure 2.5. Kinetics of Th17 cell depletion in the periphery and mucosa following intrarectal SIV infection. The percentage of total CD4+ T cells expressing IL-17 after 14 hours at 37°C with 10 ng/ml PMA and 1 µg/ml Ionomycin was measured at each timepoint. A.) Percentage of IL-17+ CD4+ T cells in PBMCs (solid black) and peripheral lymph nodes (dotted black) after stimulation. B.) Percentage of IL-17+ CD4+ T cells in leukocytes isolated from colon (grey), rectum (solid black), and jejunum (dotted black) biopsies after stimulation. Data reported are median and interquartile range (IQR) for n=6. Significant differences determined by comparing post-SIV timepoints to baseline using a Wilcoxon signed-rank test and a *p*-value < 0.05 is denoted with *.

Neutrophils transiently respond to infection in the rectum 3 days post-infection but do not perpetually infiltrate GI tissues in early SIV infection

Given our data that mucosal Th17 cells are maintained early after SIV infection and the role that Th17 cells play in the neutrophil response to pathogens, we next investigated the neutrophil response after SIV infection. In order to assess neutrophil frequencies, we developed a flow cytometry panel to distinguish neutrophils in both blood and tissues (Figure 2.6). In short, neutrophils in macaques are defined as CD45+CD3-CD20-CD14+CD11b+HLA-DR-HighSSC cells. Markers often used to distinguish human neutrophils cannot be used in macaque studies

due to lack of cross-reactive antibodies (e.g CD15) or because macaque neutrophils do not express those markers (e.g CD16)¹⁶¹. Additionally, macaque neutrophils express levels of CD14 more similar to monocytes/macrophages than human neutrophils, and therefore HLA-DR and SSC is necessary to distinguish neutrophils from monocytes/macrophages¹⁶². Confirming this distinction between neutrophils and monocytes is the presence of HLA-DR+, low SSC monocytes in lymph node but a lack of cells that we define as neutrophils, as would be expected (Figure 2.6C). Additionally, neutrophil frequencies in the blood obtained using this panel highly correlated with CBC frequencies (data not shown), thus validating our approach.

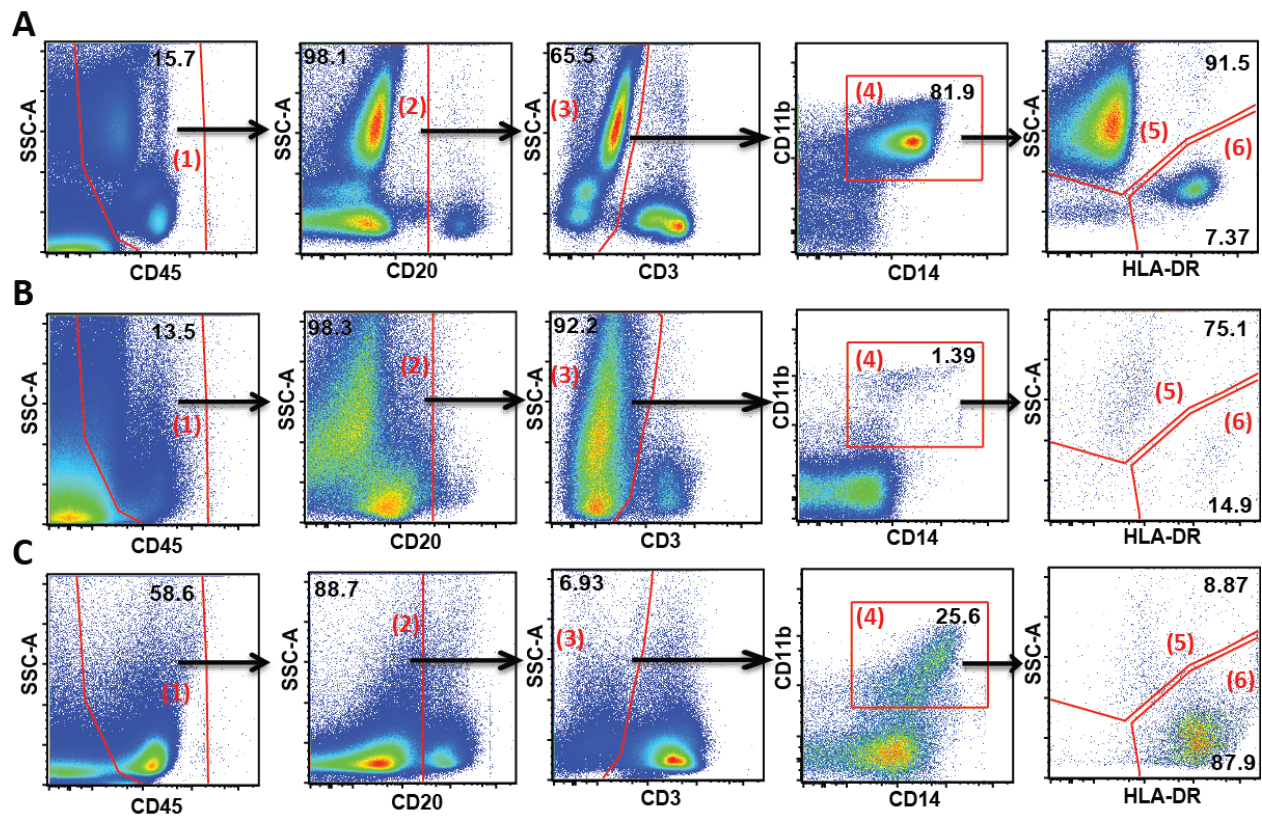


Figure 2.6. Distinguishing neutrophils in blood and tissues using flow cytometry.

Representative plots demonstrating neutrophil gating in blood (A) and leukocytes isolated from rectum biopsies (B) and lymph nodes (C) in rhesus macaques. Live cells are gated for CD45+ leukocytes (1) and CD20+ B cells (2) and CD3+ T cells (3) are excluded. Neutrophils and

monocytes are then gated as CD14+CD11b+ (4). Finally, neutrophils are distinguished as high SSC, HLA-DR low cells (5) while monocytes and macrophages are distinguished as low SSC, HLA-DR+ cells (6).

Importantly, we observed decreased neutrophil frequencies in the blood and bone marrow 28 days post-infection that returned to baseline by day 122 post-infection (Figure 2.7A). This decrease in the blood in acute SIV infection has been reported previously and was attributed to increased neutrophil apoptosis, but this is the first report of a similar occurrence in the bone marrow following infection⁷⁴. In the mucosa, we observed an infiltration of neutrophils into the rectum 3 days post-SIV infection, which returned to baseline by day 14 post-SIV (Figure 2.7B and Figure 2.7C). Interestingly, we observed no sustained accumulation of neutrophils in the GI tissues throughout the course of the study. The transient influx into the rectum but not the colon at day 3 suggests that neutrophils may be recruited in the antiviral immune response to SIV infection, which has not been previously reported.

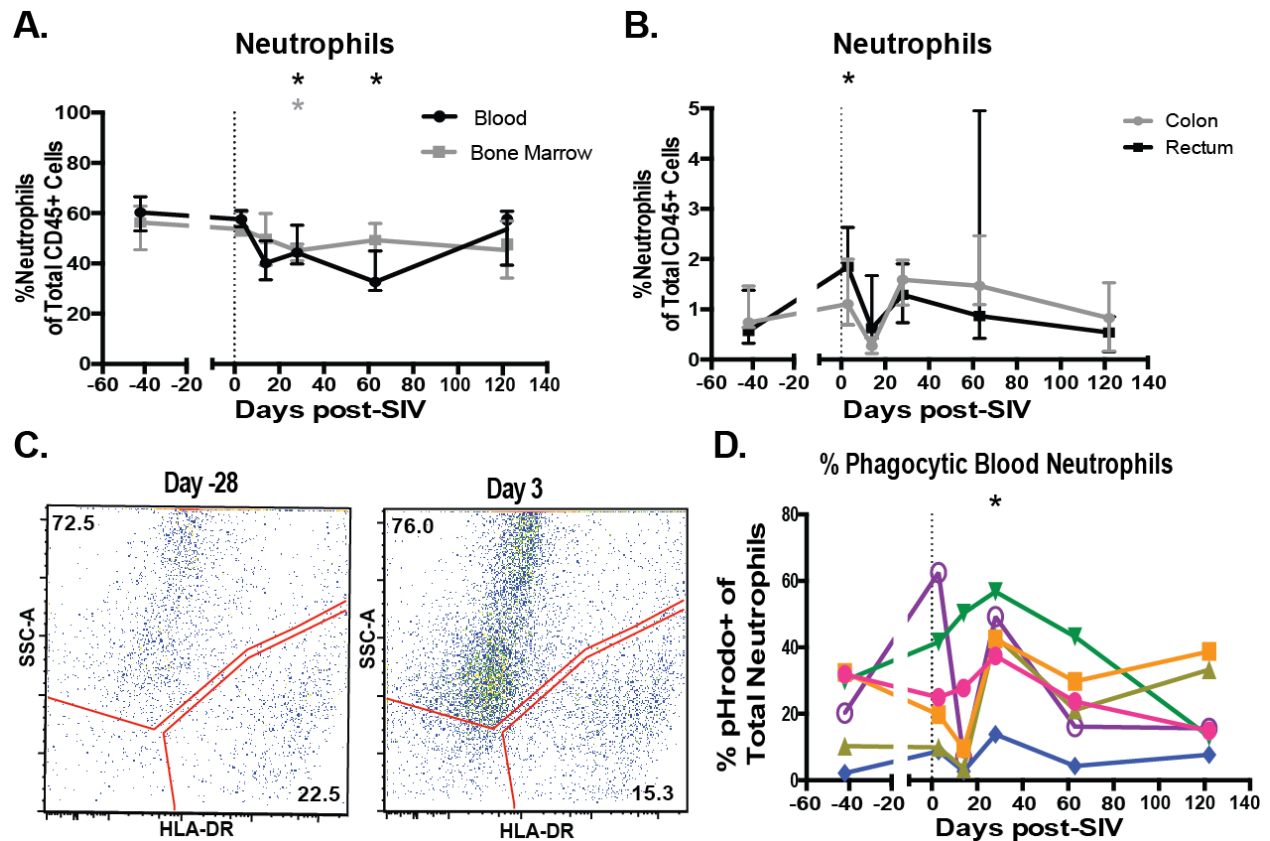


Figure 2.7. The dynamics of peripheral and mucosal neutrophil frequencies and functionality following intrarectal SIV infection of rhesus macaques. A.) Neutrophils as a percentage of CD45+, live leukocytes in whole blood and whole bone marrow. B.) Neutrophils as a percentage of CD45+, live leukocytes isolated from colon (grey) and rectum (black) biopsies. C.) Representative flow plots demonstrating increased neutrophils in the rectum 3 days post-infection. D.) Percentage of whole blood neutrophils performing phagocytosis of plasma-opsinized *S. aureus* bioparticles after a 2-hour incubation. Significant differences determined by comparing post-SIV timepoints to baseline using a Wilcoxon signed-rank test and a p -value < 0.05 is denoted with *.

Neutrophils demonstrate stable phagocytic ability in acute SIV infection

The contribution of neutrophils to antiviral immunity in HIV remains unclear, but neutrophils demonstrate reduced antimicrobial functions including decreased chemotaxis, oxidative burst, and phagocytosis in chronic HIV infection which increases the risk of secondary infections^{88, 163, 164}. When this reduction in function occurs in infection and if neutrophils retain function in acute infection in order to participate in an antiviral response is unknown. We investigated neutrophil functionality using *Staphylococcus aureus* bioparticles labeled with a pH sensitive molecular probe (pHrodo®). We assessed the ability of blood neutrophils to phagocytose plasma-opsonized bioparticles throughout the study. In contrast to the reduction in neutrophil phagocytic ability observed in chronic HIV infection and AIDS, neutrophils from SIV-infected macaques exhibited a transient significant increase in phagocytic ability 28 days post-infection and did not demonstrate a significant decrease in neutrophil functionality throughout the course of infection (Figure 2.7D). These data suggest that neutrophils maintain phagocytic function in acute SIV infection and may contribute to the antiviral response and protection from secondary bacterial infections.

Discussion

The events contributing to systemic immune activation and increased morbidities and mortality in HIV infection are complex and interconnected. While relationships clearly exist between mucosal dysfunction, characterized by barrier breakdown and microbial translocation, and immune activation and disease progression in chronic infection, the kinetics of these events in relation to one other has yet to be examined in a longitudinal study beginning early after infection. Here, we comprehensively examined the kinetics of pathological events in both the

mucosa and periphery in order to better elucidate cause and effect and create a more complete understanding of the onset of important pathologies in lentiviral infection.

First, we observed CD4+ T cell activation in the rectum beginning 3 days post-infection and CD8+ T cell activation in the rectum and colon beginning 14 days post-infection. This early activation in mucosal tissues is likely partially mediated by direct interaction with virus, however it is unlikely that a majority of this T cell activation is stimulated by virus in an antigen-specific way because the frequency of HIV-specific T cells is too small to represent all activated T cells measured¹⁶⁵. Additional mechanisms of T cell activation other than the virus alone is further supported by comparisons to nonpathogenic SIV infection of sooty mangabeys and African green monkeys whereby SIV infection of these natural hosts results in high levels of virus replication yet low levels of immune activation^{159, 166}. It has been hypothesized that the immune response to the virus elicits proinflammatory cytokines that aid in bystander T cell activation in pathogenic infection while this remains limited in nonpathogenic infection^{167, 168}.

It is also possible that early local microbial translocation participates in mucosal T cell activation, as peripheral measurements of microbial translocation also distinguish infection of rhesus macaques and nonpathogenic infection of natural hosts¹⁶⁹. The most compelling evidence of early microbial translocation comes from a recently published study in which the authors observed an increase in 16s ribosomal DNA (rDNA) in the “hyperacute” phase (0-7 days post-infection) in SIV-infected rhesus macaques¹⁷⁰. Although the authors did not measure LBP, they did not observe increases in other peripheral markers of microbial translocation such as soluble CD14 (sCD14), suggesting that measurements of microbial DNA may be a more sensitive approach to assessing systemic microbial translocation. In our study, we observed trending increases in LBP beginning 14 days post-infection and previous studies measuring peripheral

markers of microbial translocation or staining for local LPS in the tissues also report microbial translocation beginning around that timeframe^{25, 171}. Taken together, these data suggest that microbial translocation in the gut begins 0-14 days post-infection and may have an impact on early mucosal and systemic T cell activation.

IL-17 producing T cells are important for homeostatic maintenance of the epithelial barrier and their loss is linked to increased barrier permeability, disease progression, and microbial translocation^{19, 172}. Importantly, Th17 cells are maintained in nonpathogenic SIV infection, further suggesting that their depletion contributes to mucosal dysfunction and immune activation in pathogenic infection¹⁷³. Although several studies have assessed IL-17 producing T cells in cross-sectional studies in chronic infection, here we present the first longitudinal study to demonstrate that Th17 cells are maintained in the periphery and mucosal tissues in acute lentiviral infection, suggesting that they could respond to the virus by recruiting neutrophils and promoting immunity. Indeed, virus specific Th17 cells have been observed in early HIV infection but not chronic infection, further confirming they may be important in an initial antiviral response¹⁷⁴.

One of the main roles by which IL-17 promotes tissue immunity is through the induction of a neutrophil response in both viral and bacterial infections¹⁵. Our data demonstrate that neutrophils are recruited to the rectum 3 days post-infection, but this inflammatory response resolves by 14 days post-infection. These are the first data to suggest that neutrophils are recruited to the site of SIV infection and may contribute to the antiviral response. A role for neutrophils in viral immunity has been more recently appreciated and neutrophils could contribute to an anti-HIV response in several ways. First, neutrophils produce antiviral defensins and human neutrophil alpha-defensin 4 inhibits HIV infection *in vitro*^{175, 176}. Additionally,

neutrophils demonstrate antibody dependent cell-mediated cytotoxic killing of HIV-infected cells and viricidal effects on HIV through the release of myeloperoxidase (MPO) and reactive oxygen species to form hypochlorous acid^{177, 178}. Neutrophil extracellular traps also capture HIV and promote elimination of the virus through defensins and MPO¹⁷⁹. Finally, neutrophils can participate in antibody dependent cellular phagocytosis to clear infected cells and immune complexes and this has been associated with suppressed viremia in elite controllers¹⁸⁰. Importantly, two studies have demonstrated associations between increased risk of HIV acquisition in African women and perinatal HIV infection in newborns with reduced blood neutrophil frequencies, implicating neutrophils in a protective role against HIV *in vivo*^{181, 182}.

While the recruitment of neutrophils to the rectum, studies of anti-HIV functions *in vitro*, and neutrophil frequencies *in vivo* support a potential role for neutrophils in the antiviral response to lentiviruses, neutrophil functionality in acute infection has never been examined. We determined that, in contrast to studies of neutrophil functionality in chronic HIV infection, neutrophils in acute SIV infection demonstrated an increased ability to phagocytose antibody opsonized bacterial particles. Although we did not assess ability to phagocytose virus or virally infected cells, these data more accurately contrast the reduction in function observed in chronic infection as those assessments have only been done with bacterial and fungal pathogens. Therefore, these data suggest that the reduced functionality of neutrophils observed in chronic infection occurs later and neutrophils not only maintain functionality in acute infection but also increase their ability to phagocytose immune complexes.

A previous cross-sectional study evaluating neutrophils in the gastrointestinal tract after intravaginal SIV infection reported increased MPO staining (representing neutrophils) in the tissues in two animals beginning in the late acute stage (14-28 days post-infection) and even

higher levels of MPO in animals with chronic infection and AIDS²⁵. Each of the chronically infected animals in that study had been infected longer than the animals in our study at necropsy and given the apparent increase of neutrophils with disease progression reported, it's likely our animals had not progressed to an adequately advanced stage of infection to demonstrate sustained neutrophil accumulation in the GI tissues. MPO staining of tissues from individuals chronically infected with HIV also demonstrates increased neutrophil infiltration despite ART, and those with lower immune reconstitution on ART had the highest accumulation of neutrophils¹⁴⁷. This further suggests that neutrophil infiltration depends on disease progression and overall state of the immune system, and taken together with our longitudinal, data promotes the hypothesis that neutrophils are transiently recruited to the site of infection, potentially as part of the antiviral response. However, sustained neutrophil accumulation in the gut presents later after prolonged infection, immune dysfunction, and microbial translocation. It's likely that the mechanisms driving neutrophil accumulation in the gut in chronic infection are multifaceted and can be influenced by a wide variety of factors including route of infection, length of infection, CD4+ T cell count, genetic inclination to produce increased inflammatory cytokines, and the host microbiome. In Chapter III, I investigate the role of changes to the gastrointestinal microbiome in the accumulation of neutrophils in the gut in chronic HIV infection.

Chapter III

Increased neutrophil lifespan as a mechanism for gastrointestinal neutrophil accumulation in treated HIV infection

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Introduction

Gastrointestinal (GI) mucosal damage and immune dysfunction drive chronic inflammation and microbial translocation, which predict and likely contribute to non-infectious comorbidities and mortality^{38, 39, 143, 144, 145, 146 22, 23}. Although long-term antiretroviral therapy (ART) partially restores mucosal damage, a degree of mucosal immune dysfunction and inflammation persists^{21, 22, 42}. Improving the understanding of this persistent mucosal dysfunction and inflammation during ART is a major hurdle for the development of targeted therapies that may promote health and decrease morbidities and mortality in HIV-infected individuals. Neutrophils, the most abundant immune cell, are the first responders to infection and are crucial in the immune response to bacterial and fungal pathogens⁴³. However, the role of neutrophils in HIV infection is not well understood. Imaging studies assessing myeloperoxidase, an enzyme produced and secreted by neutrophils, suggest that neutrophils accumulate in the GI tract in treated and untreated infection¹⁴⁷, yet the frequency and functionality of neutrophils in GI tissues during HIV infection has yet to be examined. A better understanding of the mechanisms involved in neutrophil accumulation in the GI in HIV infection is necessary to develop new strategies to alleviate GI inflammation in HIV infection.

While neutrophils are critical in protection from infections, aberrant neutrophil responses can also be harmful. Neutrophil infiltration in the colonic mucosa is a distinguishing

characteristic of inflammatory bowel disease, and neutrophil markers in stool and increased neutrophils in the colon both correlate with disease severity^{183, 184, 185}. Neutrophil lifespan is tightly regulated in order to limit unintended damage to tissues by reactive oxygen species and granular enzymes released by neutrophils that degrade extracellular matrix and disrupt tight junctions during transmigration¹⁸⁶. Increased neutrophil lifespan is observed under inflammatory conditions and has been attributed to both direct interaction with microbes and the release of cytokines known to delay apoptosis from other immune cells^{115 116, 117}. During HIV infection, the delicate balance of healthy bacterial communities is perturbed, resulting in microbial dysbiosis^{121, 122, 123, 124}. Given that TLR activation and cytokine stimulation are important for neutrophil survival, changes in the microbiome composition could affect neutrophil lifespan either directly or indirectly through cytokine production from other immune cells. In this study, we hypothesized that HIV-infected individuals would have increased neutrophil frequencies in the lower GI and reduced neutrophil apoptosis and that bacterial species with altered abundances in HIV infection would differentially contribute to alterations in neutrophil apoptosis.

Methods

Study participants

HIV+ and HIV- study participants were recruited through either the University of Washington Center for AIDS Research, University of California San Francisco SCOPE cohort, Northwestern University, or the University of Washington AIDS Clinical Trials Group. Biopsies were obtained by either colonoscopy or rectosigmoidoscopy as indicated in Table 3.1. Blood samples for bacterial stimulations were collected in EDTA from HIV+ and HIV- participants recruited through the University of Washington Center for AIDS research. All HIV+ participants were on

ART at time of biopsy or blood draw with no detectable viral load and all participants were adults >18 years of age. The appropriate Institutional Review Boards approved all protocols and informed written consent was obtained from all participants. Table 3.1 describes relevant participant demographic information including age, sex, ethnicity, CD4+ T cell count, and time since HIV diagnosis.

Table 3.1. Study Participant Demographics

	HIV+ (ART-Suppressed) N=14	HIV- N=11
Sample Type, No. (%)		
Colonoscopy	5 (35.7%)	1 (9.1%)
Rectosigmoidoscopy	9 (64.3%)	10 (90.9%)
Sex, No. (%)		
Female	4 (28.6%)	7 (63.6%)
Male	10 (71.4 %)	4 (36.4%)
Age, Median (IQR)	52.5 (28.25-55.25)	62 (51.5-69.5)
Race/Ethnicity, No. (%)		
Unknown	1 (7.1%)	2 (18.2%)
White, non-Hispanic or Latino	10 (71.4%)	6 (54.6%)
Black or African American	3 (21.4%)	1 (9.1%)
Latino	0 (0%)	1 (9.1%)
Native American	0 (0%)	1 (9.1%)
Years since HIV diagnosis, Median (IQR)	13.4 (6.5-23.2)	--
CD4+ T cell Count, Median (IQR)	635 (457-700)	--

Sample processing

Gut biopsies were enzymatically digested with media (RPMI 1640 with 2.05mM L-glutamate, 100U/ml Penicillin, 100µg/ml Streptomycin [all from GE Healthcare, Logan, UT]) supplemented with Liberase (40 µg/ml, Sigma-Aldrich, St. Louis, MO) and DNase (4 µg/ml, Sigma-Aldrich)

for 1 hour at 37°C with vigorous stirring, ground through a 70- μ m cell strainer into a single cell suspension, and then analyzed by flow cytometry.

Neutrophil Isolation

Neutrophils were isolated by lysing the red blood cells in whole blood with ACK lysing buffer (ThermoFisher Scientific) and then labeling the leukocytes with CD15 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The labeled cells were then loaded onto a MACS Column, which was placed into a magnetic MACS Separator (both from Miltenyi Biotec). The retained CD15⁺ cells were then washed with ice-cold buffer (PBS with 0.5% BSA and 2 mM EDTA). The column was then removed from the separator and the labeled cells were eluted in ice-cold buffer. Cells were counted and used immediately in bacterial or TLR stimulation experiments.

Flow cytometry

Single cell isolations from biopsies were analyzed by flow cytometry immediately after isolation. Biopsies and blood or isolated neutrophils from the bacteria stimulations were stained using the following surface antigen mouse anti-human antibodies with clone denoted in (), from Becton Dickinson, and Co. (BD) Biosciences (Franklin, NJ) unless otherwise stated: CD45 PE-CF594 (HI30), CD11b APC-Cy7 (ICRF44), CD66b PE (Biolegend, G10F5), CD49d PE/Cy5 (Biolegend, 9F10), CD20 Brilliant Violet 570 (Biolegend, 2H7), CD3 Brilliant Violet 570 (Biolegend, UCHT1), CD16 BV605 (3G8), CD15 BV650 (HI98), and CD14 BV786 (M5E2). Following surface staining, cells were permeabilized using Cytofix/Cytoperm (BD Biosciences). Intracellular active Caspase-3 was stained using a v450-conjugated rabbit anti-human antibody (BD Biosciences, C92-605). Stained samples were fixed in 1% paraformaldehyde and collected

on an LSR II (BD Biosciences, La Jolla, California). Analysis was performed in FlowJo (version 9.7.6, Treestar Inc., Ashland, Oregon).

Bacterial preparation and stimulations

Prevotella stercorea (DSMZ #18206, Braunschweig Germany), *Prevotella copri* (DSMZ #18205), *Bacteroides fragilis* (ATCC #25285, Manassas, Virginia), and *Ruminococcus bromii* (ATCC #27255) were all grown anaerobically in chopped meat broth (Hardy Diagnostics, Santa Maria, CA) supplemented with 1% trace minerals (ATCC), 1% vitamin supplements (ATCC), 0.05% Tween 89 (Sigma-Aldrich, Saint Louis, MO), 29.7 mM acetic acid (Sigma-Aldrich), 8.1 mM propionic acid (Sigma-Aldrich), and 4.4 mM butyric acid (Sigma-Aldrich). *Acinetobacter junii* (ATCC #17908) was grown aerobically in nutrient agar (BD). *Lactobacillus plantarum* (ATCC #14917) and *Lactobacillus rhamnosus* (ATCC #53103) were grown aerobically in MRS broth (BD). All bacteria were counted using CountBright Absolute Counting Beads (Thermo Fisher Scientific, Waltham, MA) and Syto 9 dye (Thermo Fisher Scientific) on the LSR II. Bacteria were frozen as dry cell pellets until reconstituted in PBS for use in stimulations. For stimulations, bacteria were added at 2.5 bacteria per leukocyte to 100 μ l of whole blood or 500,000 isolated neutrophils in 1 ml R10 media (RPMI 1640 with 2.05mM L-glutamate, 100U/ml Penicillin, 100 μ g/ml Streptomycin, and 10% fetal bovine serum [all from GE Healthcare, Logan, UT]) and incubated aerobically for 20 hours at 37°C. Blood or isolated neutrophils from each donor were also incubated with lipopolysaccharide (LPS) or AT-2 inactivated HIV as controls in each experiment as described below. Following the incubation, the supernatant was removed and the blood and isolated neutrophils were washed before being analyzed by flow cytometry.

TLR Control Stimulations

Blood and isolated neutrophils were incubated with the following TLR agonists (all from InvivoGen, San Diego, CA): ultra pure LPS from the *E. Coli* K12 strain, the synthetic tripalmitoylated lipopeptide Pam3CysSerLys4 (Pam3CSK4), and the synthetic lipoprotein Pam2CGDPKHPKSF (FSL-1) at 100 ng/ml concentrations. MN HIV inactivated with aldrithiol-2 (AT-2 HIV) as previously described¹⁸⁷ was generously provided by Julie McElrath and used at 200 ng/ml in incubations. For stimulations, TLR agonists were added to 100 µl of whole blood or 500,000 isolated neutrophils in 1 ml R10 media and incubated aerobically for 20 hours at 37°C. Following the incubation, the supernatant was removed and the blood and isolated neutrophils were washed before being analyzed by flow cytometry.

Statistical analyses

Differences in neutrophil frequencies and active Caspase-3 expression between infected and uninfected individuals were determined by Mann-Whitney test. A paired one-way ANOVA was used to assess differences between groups stimulated with different bacteria or TLR agonists in the *in vitro* experiments followed by a Dunnett's post-hoc analysis for multiple comparisons. *p*-values reported are adjusted *p*-values from the post-hoc analysis. Correlations were assessed using the Spearman's rank correlation analysis. A joint regression analysis was also performed to adjust for the confounding demographic factors of age, ethnicity, and sex by regressing neutrophil frequency or neutrophil active caspase-3 expression on HIV status and the other potential confounders using least squares regression and calculated an adjusted *p*-value for the association with HIV status using a 1 degree of freedom wald test.

Results

Distinguishing neutrophils by flow cytometry

Previous studies reporting neutrophil infiltration in HIV infection and the nonhuman primate model of SIV have relied solely on MPO staining of neutrophils measured via microscopy and reported the percentage of tissue staining positive for the enzyme^{25, 147}. Because MPO secretion is increased upon neutrophil activation, it is unclear if this increase in MPO in the tissues represents increased neutrophil frequency or increased neutrophil activation. In addition, in some cases of inflammation and disease, tissue macrophages also produce MPO and stain positive for the enzyme¹⁸⁸. For these reasons, we used a multicolor flow cytometry based analysis that distinguishes neutrophils in the context of other leukocytes in order to obtain a more quantitative measure of neutrophil frequencies in the GI during HIV infection. Flow cytometric studies on neutrophils are lacking due to the cells' susceptibility to freezing injury and reduced viability after cryopreservation¹⁴¹, which require that they be assessed fresh after isolation from blood or tissues. We have developed a panel to distinguish neutrophils in blood (Figure 3.1A) and fresh GI tissue (Figure 3.1B) to determine what percentage of total live leukocytes are neutrophils, which we report as the percentage of total live CD45+ cells. It should be noted that a significant proportion of CD15+ cells in the colorectal biopsies are CD49d+CD16low eosinophils and therefore assessing cells using CD15 alone is not an accurate representation of neutrophils as is often done in studies on peripheral blood⁹⁹.

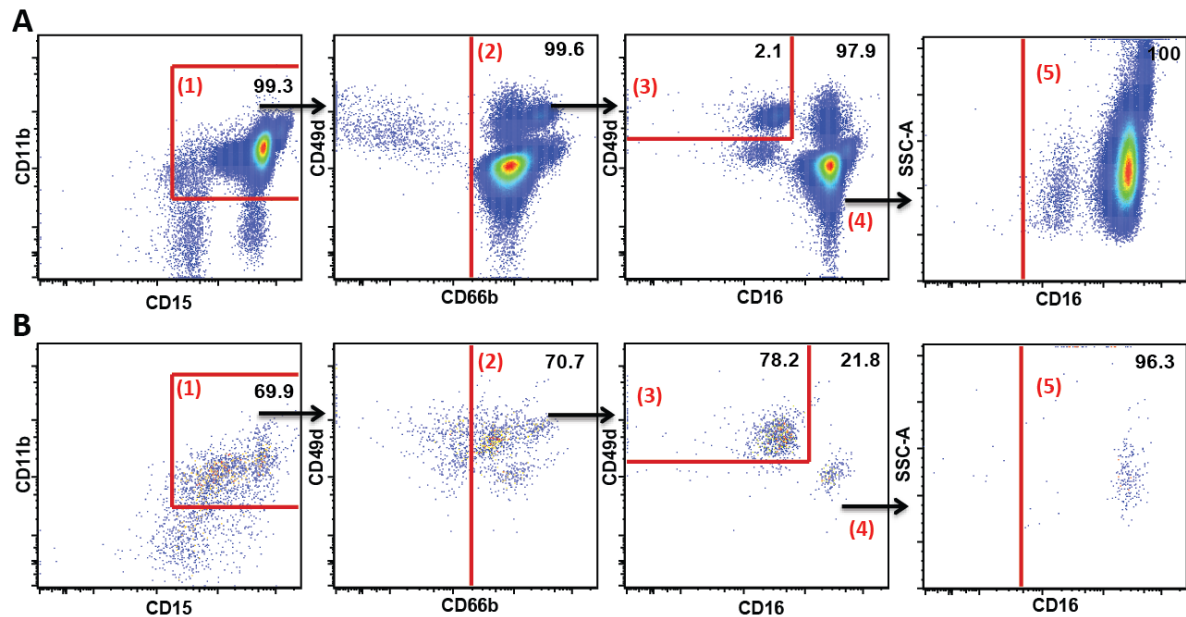


Figure 3.1. Representative flow cytometry gating of blood and GI neutrophils.

Representative plots showing live CD45+CD14-CD3-CD20-HighSSC cells in blood (A) and GI leukocytes (B) from an HIV+ individual further gated to identify neutrophils. Neutrophils and eosinophils are defined as CD11b+CD15+ (1) and CD66b+ (2). Eosinophils are then distinguished as CD49d^{high}CD16^{low} (3) and a second overlapping exclusion gate captures the remaining cells (4). Non-eosinophils (4) are then gated for CD16+ neutrophils (5).

Increased gastrointestinal neutrophil frequencies and reduced neutrophil active caspase-3 expression in treated, chronic HIV infection

Using the described gating strategy, we assessed neutrophil frequencies in colorectal biopsies from HIV-infected and uninfected individuals. It is now recommended that patients begin ART immediately upon HIV diagnosis regardless of CD4 count, and a recent report by the Global Burden of Disease Study 2015 indicates that roughly 50% of infected individuals worldwide are accessing ART with current efforts aimed at continuing to increase that number⁴. Therefore, in this study we assessed individuals undergoing treatment to obtain data

generalizable to a significant proportion of the current and future infected population, and any data from infected individuals described herein refers to individuals on ART at the time of biopsy or blood draw.

Assessing neutrophils from GI biopsies via flow cytometry, we found increased neutrophil frequencies in the GI of HIV+ individuals compared to uninfected controls (Figure 3.2A). We postulated that one potential mechanism for elevated frequencies of neutrophils in the colon in HIV is prolonged neutrophil survival. Neutrophil clearance is an important mechanism for tissue homeostasis, and neutrophils are generally short-lived, with findings from studies investigating neutrophil lifespan *in vivo* ranging from 8 hours to 5 days⁵⁰. The least inflammatory mechanism of neutrophil clearance from tissues is caspase-mediated apoptosis followed by engulfment by macrophages, and caspase-3 mediates the final steps of apoptosis once activated^{65, 189, 190}. We found that the frequency of GI neutrophils expressing active caspase-3 was significantly decreased in biopsies from HIV+ individuals compared to uninfected controls (Figure 3.2B). Neutrophils undergoing apoptosis often demonstrate reduced surface CD16 expression, and we confirmed that a large proportion of cells expressing active caspase-3 were CD16low (Figure 3.2C)¹¹⁸. Importantly, in a joint regression analysis accounting for potential covariates, HIV status remained significantly associated with gut neutrophil frequency ($p=0.019$) and the percentage of active Caspase-3 neutrophils ($p=0.002$) while no association was observed with age, sex, or ethnicity. In infected and uninfected individuals, we observed a significant negative correlation between total neutrophil frequencies and the frequency neutrophils expressing active caspase-3 (Figure 3.2D). This suggests that individuals with higher neutrophil frequencies have reduced caspase-3 expression regardless of infection status and that GI neutrophil accumulation in HIV infection may be related to a reduction in homeostatic apoptosis.

While neutrophil infiltration in inflammatory bowel disease has been attributed in part to delayed neutrophil apoptosis, these are the first data demonstrating this mechanism in intestinal mucosal dysfunction in HIV infection¹⁹¹.

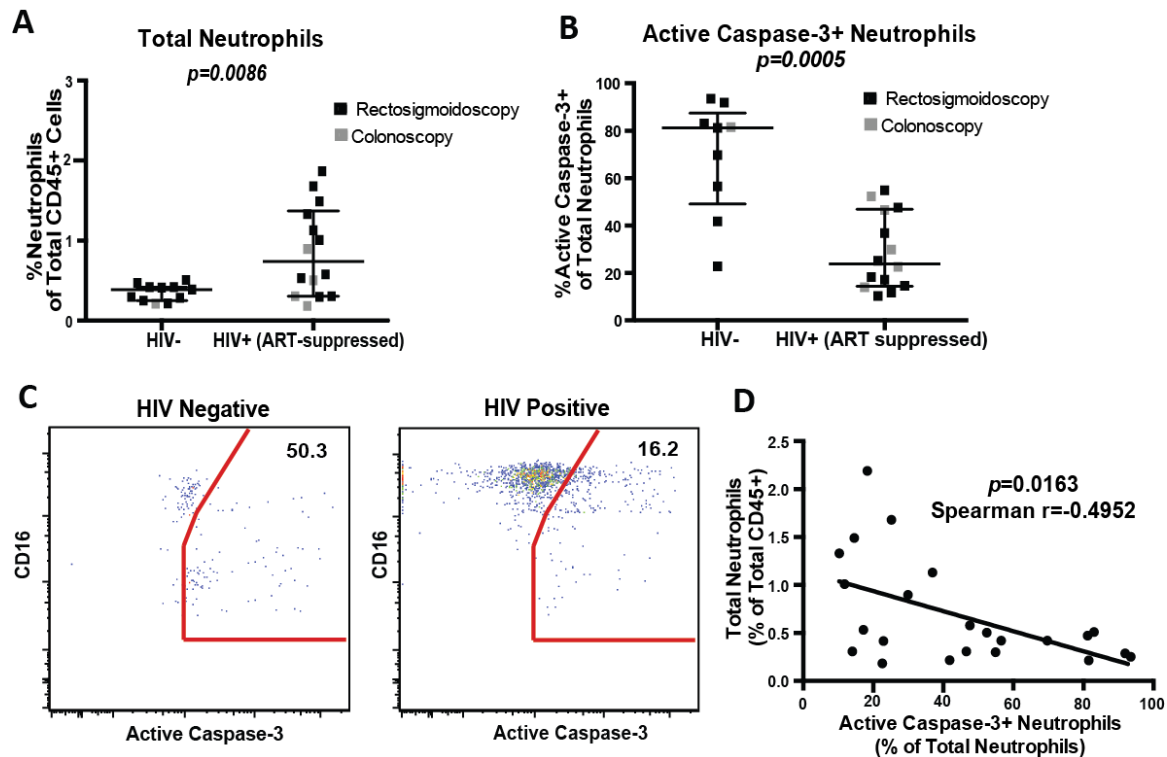


Figure 3.2. Increased gastrointestinal neutrophil frequency is associated with reduced neutrophil apoptosis in treated HIV infection. A.) The frequency of total neutrophils isolated from rectosigmoidoscopy and colonoscopy biopsies as measured by flow cytometry. B.) The frequency of total neutrophils expressing active caspase-3 in rectosigmoidoscopy and colonoscopy biopsies as measured by flow cytometry. C.) Example gating of active caspase-3 on rectosigmoid neutrophils. D.) Association of total neutrophil frequencies and active caspase-3 expression in rectosigmoidoscopy and colonoscopy biopsies as determined by a Spearman correlation analysis. Statistical differences in neutrophil frequencies and active caspase-3 expression between infected and uninfected individuals (A and B) were determined by Mann-Whitney test.

TLR-2 and TLR-4 stimulation decrease neutrophil apoptosis

There is much evidence that microbes impact neutrophil survival through both direct interactions and by influencing cytokine release by other immune cells. First, TLR-4 activation by bacterial LPS has been shown to increase neutrophil survival both through direct interaction on neutrophils and through the release of TNF- α and IL-1 β by monocytes¹¹⁵. In addition, incubation with the gram-positive bacteria *Streptococcus haemoliticus* also increased neutrophil survival *in vitro*¹¹⁶. In accordance with these previous studies, we observed a decreased frequency of active caspase-3+ neutrophils in whole blood after a 20-hour incubation with TLR-4 and TLR-2 agonists compared to unstimulated controls (Figure 3.3A). Specifically, neutrophils in media alone (unstimulated control) exhibited a range of 20.2-60.8% of total cells undergoing homeostatic apoptosis after 20 hours, while blood stimulated with different TLR agonists including LPS, FSL-1, and Pam3CSK4 only demonstrated 1.6-28.3% of neutrophils undergoing apoptosis across all conditions. Isolated neutrophils incubated with TLR-4 and TLR-2 agonists also demonstrated a decreased frequency of active caspase-3+ neutrophils after a 20-hour incubation but to a lesser extent that that observed in whole blood (Figure 3.3B), suggesting that both direct interactions with neutrophils and soluble factors released by other leukocytes contribute to neutrophil apoptosis alterations in the presence of microbial antigens.

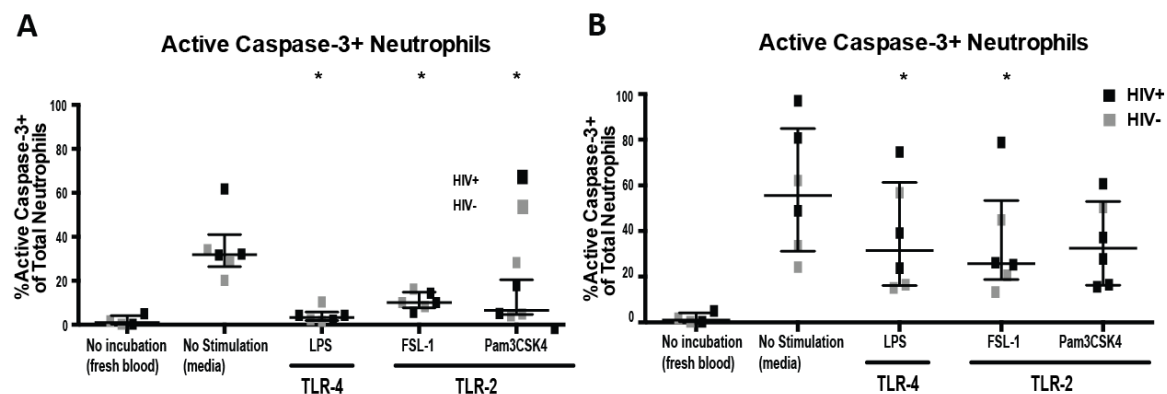


Figure 3.3. TLR-4 and TLR-2 stimulation decrease neutrophil apoptosis. A.) Active Caspase-3 expression on neutrophils in whole blood incubated with TLR-4 and TLR-2 agonists as measured by flow cytometry. B.) Active Caspase-3 expression measured by flow cytometry on neutrophils isolated using CD15+ magnetic beads and incubated with TLR-4 and TLR-2 agonists. Statistical significance was determined using a paired one-way ANOVA followed by a Dunnett's post-hoc analysis for multiple comparisons comparing each group to the media control. Asterisks (*) indicate significance by adjusted p -value from the post-hoc analysis (* P <0.005, ** P <0.01, *** P <0.001).

Lactobacillus uniquely increases neutrophil apoptosis compared to other HIV-altered mucosal bacteria and can override survival signals from TLR stimulation

Given the studies demonstrating that TLR activation alters homeostatic neutrophil apoptosis and our observed effect on neutrophil active caspase-3 expression in stimulations, we hypothesized that dysbiotic GI bacteria may play a role in GI neutrophil survival and accumulation in HIV infection. Using a panel of intestinal bacterial species from genera known to be altered in HIV infection (HIV-altered mucosal bacteria; HAMBs) we observed differential effects on neutrophil apoptosis (Figure 3.4). All bacterial species significantly decreased homeostatic neutrophil apoptosis in whole blood with the exception of the *Lactobacillus* species. Importantly, two different *Lactobacillus* species tested, *Lactobacillus plantarum* and *Lactobacillus rhamnosus*, uniquely increased neutrophil apoptosis as measured by the frequency of active caspase-3+ neutrophils (Figure 3.4A and Figure 3.4B). Additionally, to ensure that the HAMBs that reduced apoptosis were not causing a different form of cell death, such as necrosis, we assessed total neutrophil frequencies. We found that both *Lactobacillus* species uniquely

decreased total neutrophil frequencies compared to other HAMBs, confirming that the reduction in neutrophil apoptosis observed upon stimulation by non-*Lactobacillus* HAMBs resulted in sustained neutrophil frequencies (Figure 3.4C).

Finally, in order to determine if the effects of *Lactobacillus* could counteract the reduced apoptosis observed after TLR stimulation and incubation with other HAMBs, we co-stimulated whole blood with LPS and *Lactobacillus plantarum*. Importantly, we observed that *L. plantarum* incubated with whole blood in the presence of LPS induced increased apoptosis of neutrophils similar to that of *L. plantarum* alone (Figure 3.5D). This observation has important implications for the potential use of *Lactobacillus* to therapeutically target neutrophil apoptosis because it suggests that it can override survival signals induced by other microbes.

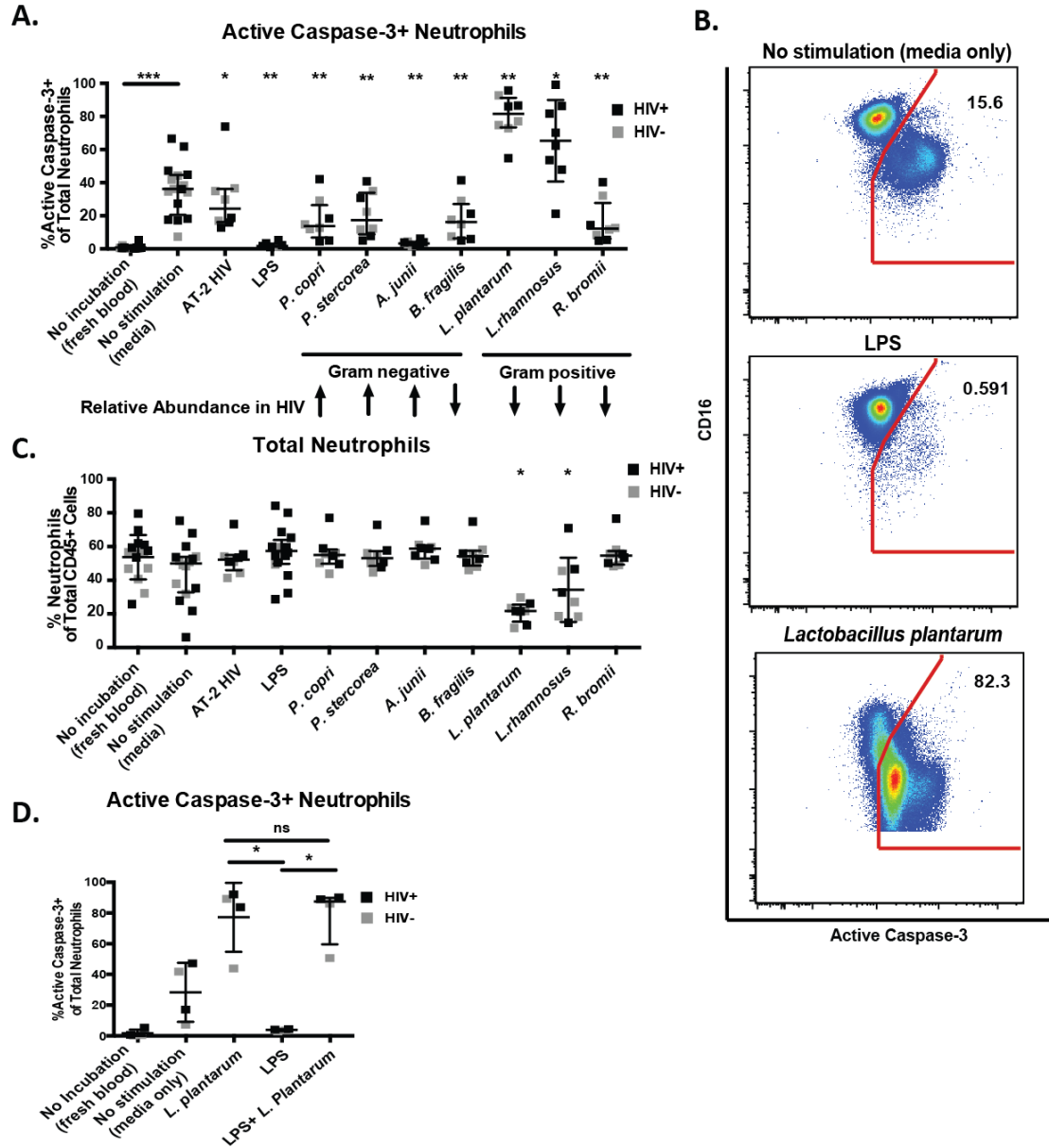


Figure 3.4. HIV altered mucosal bacteria differentially affect neutrophil apoptosis. A.)

Active caspase-3 expression on neutrophils in whole blood incubated with different HIV altered mucosal bacteria as measured by flow cytometry. B.) Example staining of active caspase-3 on neutrophils after stimulation with LPS and *Lactobacillus plantarum*. C.) Total neutrophil frequencies as a percentage of total CD45+ leukocytes in whole blood incubated with different HIV altered mucosal bacteria. D.) Active caspase-3 expression on neutrophils in whole blood

incubated with LPS alone, *Lactobacillus plantarum* alone, and LPS with *L. plantarum* as measured by flow cytometry. Statistical significance was determined using a paired one-way ANOVA followed by a Dunnett's post-hoc analysis for multiple comparisons comparing each group to the media control. Asterisks (*) indicate significance by adjusted p -value from post-hoc analysis (* P <0.005, ** P <0.01, *** P <0.001). The statistical difference between fresh blood and blood incubated in media alone was assessed using a Wilcoxon signed-rank test (* P <0.005, ** P <0.01, *** P <0.001).

We additionally assessed the effect of different HAMBs on neutrophil apoptosis in isolated neutrophils, and observed a much larger percentage of isolated neutrophils compared to those in whole blood became apoptotic after the 20 hour incubation in media alone (Figure 3.5). Due to this increase in apoptosis without stimulation, minimal effects of bacterial stimulation were observed. This suggests that the isolation procedure itself activated apoptosis pathways that could not be overridden or reversed by subsequent stimulation. We therefore cannot conclude to what extent the bacteria may alter homeostatic apoptosis of isolated neutrophils without this confounding activation upon isolation. Further studies will be necessary to better determine the effects of other leukocytes in the alteration of neutrophil survival by different bacteria.

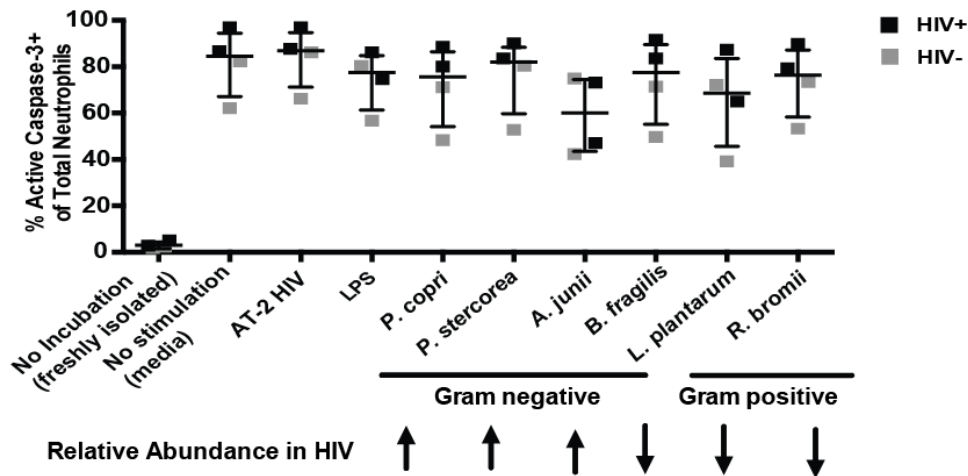


Figure 3.5. Reduced effect of HIV altered mucosal bacteria on apoptosis of isolated neutrophils. Active caspase-3 expression measured by flow cytometry on neutrophils isolated using CD15+ magnetic bead labeling and incubated with different HIV altered mucosal bacteria. Statistical significance was determined using a paired one-way ANOVA followed by a Dunnett's post-hoc analysis for multiple comparisons comparing each group to the media control. Asterisks (*) indicate significance by adjusted *p*-value from post-hoc analysis (**P*<0.005, ***P*<0.01, ****P*<0.001).

Discussion

Increased neutrophils in the gastrointestinal tract in HIV infection can contribute to epithelial tissue damage, yet the mechanisms driving neutrophil accumulation in the GI have not been elucidated. Increased microbial translocation and dysbiosis may result in both increased neutrophil recruitment and increased neutrophil lifespan. Indeed, one study demonstrated that IL-8 expression in the colorectal mucosa of HIV infected individuals was similar to that of individuals with ulcerative colitis and significantly higher relative to uninfected controls¹⁹². Importantly, in addition to being a potent chemokine involved in neutrophil extravasation into tissues, IL-8 mediates neutrophil survival in an autocrine manner upon TNF- α stimulation, and

delayed neutrophil apoptosis in inflammatory bowel disease has been attributed in part to cytokines such as IL-8^{193, 194}. Studies investigating neutrophil lifespan have demonstrated that neutrophil apoptosis is regulated by both cytokines released by monocytes and by direct interaction in response to TLR stimulation¹¹⁵. While numerous studies have linked reduced neutrophil apoptosis to disease severity in IBD, these are the first data suggesting that increased neutrophil lifespan contributes to GI neutrophil accumulation in HIV Infection. Further, we report that various HIV altered mucosal bacteria have differential effects on neutrophil apoptosis *in vitro*, suggesting that an altered microbiome may contribute to neutrophil accumulation in the GI through effects on neutrophil apoptosis.

One of the defining characteristics of microbial dysbiosis in HIV-infected individuals is a reduction in *Lactobacillus* species, which are known to maintain gut health and immune function^{124, 195}. Importantly, we observed the unique ability of *Lactobacillus* species to increase neutrophil apoptosis, which has not been previously reported and has important implications for potential therapeutic intervention in HIV and other diseases of intestinal inflammation. Anti-inflammatory effects of various *Lactobacillus* species are well described, and have been attributed to several factors: 1) the ability of superoxide dismutase secreted by *Lactobacillus* to neutralize reactive oxygen species; 2) the inhibition of the NF- κ B pathway leading to a reduction in pro-inflammatory cytokines and chemokines; and 3) the expansion of regulatory T cells^{196, 197, 198}. The observed increased neutrophil apoptosis we report in the presence of *Lactobacillus* may be caused by NF- κ B inhibition, which is known to drive the production of survival factors in neutrophils and be an important regulator of apoptosis¹⁹⁹.

These are the first data suggesting that the ability of *Lactobacillus* to reduce intestinal inflammation *in vivo*, as previously reported, may be a consequence of increased neutrophil

apoptosis and reduced neutrophil accumulation^{200, 201, 202}. Importantly, neutrophil apoptosis has emerged as a therapeutic target for the resolution of acute and chronic inflammation in the lungs, intestines, and arthritis but no strategies are approved for use in humans to-date²⁰³. Thus, the data we provide in this study opens up a new avenue of research in this area and suggests commensal bacteria, their surface antigens, and their products play a direct role in altering immunity in the GI tract, and should be further assessed for their therapeutic ability to reduce neutrophil infiltration in inflammation.

Chapter IV

The effects of fecal microbial transplantation on microbiome and immunity in SIV-infected macaques

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Introduction

Antiretroviral (ART) drugs have substantially decreased progression to AIDS in HIV-infected individuals. However, despite the ability to suppress viremia, ART-suppressed HIV-infected individuals have increased morbidities and mortality compared to uninfected individuals²⁴. One mechanism underlying the increased mortality is dysfunction of the gastrointestinal (GI) tract, which is associated with inflammation during HIV infection. Several lines of evidence support the role of GI dysfunction in HIV-related disease, including: (i.) consistent associations between mortality in HIV and markers of microbial translocation and gut epithelial dysfunction^{147, 204, 205}; (ii.) association of microbial products that translocate during HIV or SIV infection and inflammation^{20, 30, 155}; and (iii.) dysbiosis of the gut microbiome during HIV infection, which results in inflammation^{121, 122, 206}.

The recent awareness of the importance of the gut microbiome in human health has greatly improved our understanding of the interactions between GI bacteria and the immune system, as well as the importance of maintaining healthy microbial communities at mucosal surfaces. During HIV infection, the delicate balance of commensal bacterial communities is perturbed, resulting in microbial dysbiosis. This is typically characterized by an overall loss of diversity, with alterations to the phyla of Bacteroidetes, Firmicutes, and Proteobacteria¹²⁵. Specifically, loss of beneficial bacteria genera such as *Bacteroides*, *Lactobacillus* and

Bifidobacterium has been observed and associated with pathogenesis^{121, 122, 123, 124}. Furthermore, several pathogenic Proteobacteria have been observed to increase during HIV infection, including within the genera *Campylobacter*, *Escherichia*, *Acinetobacter*, *Desulfovibrio*, and *Pseudomonas*, along with increased *Prevotella* species^{121, 122, 125}. Indeed, these bacteria have been found to be more adherent to epithelium, prone to translocate, and drivers of inflammation in the context of HIV and SIV infections^{121, 122, 206, 207}. In an effort to reverse the deleterious effects of gut dysbiosis, studies have been performed using probiotic microbes to improve health in HIV-infected individuals and using the SIV macaque model. In pathogenic SIV infection, ART-suppressed animals have enhanced immunity and CD4+ T cell restoration in the GI tract after probiotic therapy or probiotics supplemented with IL-21^{208, 209}. In HIV-infected individuals, probiotics have been shown to decrease inflammation and microbial translocation^{210, 211}. Thus, replenishing the gut microbiome with beneficial commensals may improve immunity, decrease inflammation, and improve health in HIV infection.

Fecal microbiota transplantation (FMT), wherein donor fecal material is transplanted into the intestine of a recipient, has been demonstrated to robustly alter microbial communities²¹². FMT has been used to treat bacterial diseases such as *Clostridium difficile* and vancomycin-resistant *Enterococcus* (VRE) infections, which occur when there is dysbiosis (typically after antibiotic use), and these pathogenic bacteria overtake the microbiome and cause diarrheal disease, and are typically resistant to treatment^{213, 214, 215}. Unlike antibiotic therapy, FMT has an efficacy of nearly 90% at restoring a diverse microbiota and decreasing GI symptoms^{213, 216, 217}. Clinical trials evaluating the benefits of FMT in inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), and other gastrointestinal diseases are currently underway^{212, 218}.

Given that microbial dysbiosis occurs during HIV infection and is associated with pathogenesis, we sought to determine whether FMT would be a safe and efficacious method of modifying the bacterial community and improving health in HIV infected individuals. Due to immunodeficiency and chronic inflammation that occurs in HIV infection, determining the safety of FMT is crucial to prevent deleterious consequences if used as a future treatment. Here we used the macaque model of HIV infection and performed FMT on six chronically SIV-infected rhesus macaques on ART.

Methods

Study Animals

Animals were housed and cared for in Association for the Assessment and Accreditation of Laboratory Animal Care international (AAALACi) accredited facilities, and all animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) of University of Washington. Six male rhesus macaques (*Macaca mulatta*) were infected intrarectally with SIV_{MAC239x} and started on ART 91 days after infection, which consisted of subcutaneous tenofovir (PMPA; 20mg/kg) and emtricitabine (FTC; 30mg/kg) and oral raltegravir (50mg b.i.d). For longitudinal assessment of the effects of FMT, procedures and sampling were performed in two groups, with the study schedule varying by one day between groups. Sampling time points from the two groups are referred to collectively as weeks post-FMT (overview in Figure 4.1). Specifically, blood and biopsies of the colon, rectum, and jejunum were taken pre-antibiotic (pre-ABX: 3 weeks prior to FMT), and after 2 weeks on antibiotics, which was 1 week prior to FMT. Post-FMT samples were taken 2 and 6 weeks, after

transplant. In addition, we collected plasma and PBMCs pre-SIV (-47 weeks) prior to FMT and pre-ART (-33 weeks) prior to FMT.

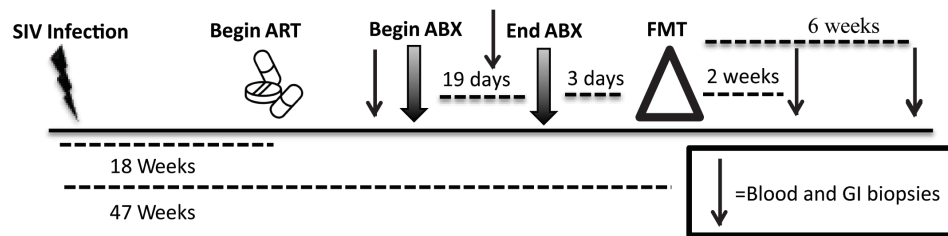


Figure 4.1. Sampling timeline showing blood and GI biopsies obtained from 6 SIV-infected rhesus macaques before and after antibiotics treatment and fecal microbial transplantation.

Antibiotic treatment and fecal microbial transplantation

First, animals were placed on an antibiotic regimen (ABX) for 19 total days consisting of oral vancomycin (15mg/kg orally b.i.d.) for 7 days followed by 12 additional days of combination vancomycin and enrofloxacin (10mg/kg orally b.i.d.). This ABX regimen was chosen to target both Gram-positive and Gram-negative intestinal bacteria. Stool from healthy (SIV-) rhesus macaque donors also housed at the WaNPRC was processed for transplantation as previously described in human protocols²¹⁹. In brief, stool was homogenized in PBS and fibrous material was filtered out using a 70- μ m filter. The solution was centrifuged and the pellet was resuspended in PBS with 10% glycerol and frozen at -80°C until use. For transplant, stool mixtures containing 50g of fecal donor material were thawed for each recipient animal. The mixtures were centrifuged and the pellet was resuspended in 80 ml PBS, and 40ml was gavaged into the duodenum and 40ml into upper colon via endoscope. The transplant procedure was performed 3/4 days after the end of ABX treatment depending on the sampling group (see Figure 4.1).

Blood and tissue processing

Plasma and PBMCs were separated by density centrifugation and cryopreserved for later analyses. Gut biopsies were processed immediately as follows: 1-2 biopsies/tissue were frozen in RNAlater (QIAGEN, Valencia, CA) for subsequent DNA extraction and 16S sequencing. The remaining biopsies were enzymatically digested with R10 media supplemented with Liberase (40 µg/ml, Sigma-Aldrich, St. Louis, MO) and DNase (4 µg/ml, Sigma-Aldrich) for 1 hour at 37°C with vigorous stirring, then ground through a 70-µm cell strainer into a single cell suspension, then analyzed by flow cytometry. Complete blood counts with differential were measured on a Beckman Coulter AC*T 5diff CP hematology analyzer. Viral loads were determined by real-time reverse transcription (RT)-PCR using primers specific for SIVgag as previously detailed¹⁵⁴.

Genomic DNA extraction and sequencing

Genomic DNA was extracted from colon tissue biopsies using the Omni Bead Ruptor with 2.8-mm ceramic beads and homogenized in RLT buffer. Genomic DNA was extracted via RNA/DNA All-Prep kit (QIAGEN, Valencia, CA) and 5 ng of genomic DNA/biopsy was used to generate a 460 base pair amplicon by targeting the V3 and V4 variable regions of the 16S ribosomal RNA (rRNA) gene. All genomic DNA underwent 20 cycles of PCR and 16S amplicons were cleaned using 0.8X AMPure XP beads (Beckman Coulter, Brea, CA). Nextera XT dual index adaptors (Illumina Inc., San Diego, CA) were incorporated by performing 12 PCR cycles using a FailSafe PCR System (Epicentre, Madison, WI), cleaned using 1.1X AMPure XP beads (Beckman Coulter, Brea, CA), quantified using a DNA High Sensitivity Qubit (Life Technologies, Carlsbad, CA), and multiplexed using equal molar ratio of DNA for each sample. Final 16S libraries were loaded on a 600-cycle MiSeq Kit at 2pM with 5% PhiX control and sequenced using Nextera sequencing read and index primers (all from Illumina, San Diego, CA).

Analysis of 16S rRNA gene sequencing data

We first combined paired-end reads using the PANDAseq assembler²²⁰ and then analyzed 16S rRNA gene sequence data using the QIIME software package²²¹. We initially clustered sequences into operational taxonomic units (OTUs) at 97% similarity and assigned taxonomy to each OTU using the Greengenes release v. 13.5²²². We performed principal coordinate analysis (PCoA) using tools within the QIIME software.

Antibodies and flow cytometry

Surface staining:

Single cell isolations from biopsies were stained immediately after isolation using the following surface antigen antibodies with clone denoted in (), from BD biosciences unless otherwise stated: CD45 PerCP (D058-1283), CD3 PE CF594 (SP34-2), CD4 Brilliant Violet 605 (Biolegend, OKT4), CD8 Brilliant Violet 570 (Biolegend, RPA-T8), CD14 Brilliant Violet 786 (Biolegend, M5E2), and HLA-DR Brilliant Violet 711 (G46-6).

Intracellular cytokine staining:

Cyropreserved PBMCs were thawed using R10 media with 5 µg/ml DNase. Thawed PBMCs were rested for 4 hours and then stimulated for 14 hours at 37°C with 10 ng/ml PMA (Sigma-Aldrich) and 1 µg/ml Ionomycin (Life Technologies) in R10 media with 1 mg/ml of Brefeldin A (Sigma-Aldrich). Stimulated cells were then stained with the following surface antigen antibodies from BD biosciences unless otherwise stated: CD45 PerCP (D058-1283), CD3 PE CF594 (SP34-2), CD4 Brilliant Violet 605 (Biolegend, OKT4), CD8 APC-H7 (SK1), CD14 Brilliant Violet 786 (Biolegend, M5E2). Following surface staining, stimulated cells were permeabilized using Cytofix/Cytoperm (BD biosciences), and stained using the following

intracellular antigen antibodies from eBiosciences: IL-17 PE (ebio64CAP17) and IL-22 PerCP-eFluor710 (IL022JOP). Stained samples were fixed in 1% paraformaldehyde and collected on an LSR II (BD Biosciences, La Jolla, California). Analysis was performed in FlowJo (version 9.7.6, Treestar Inc., Ashland, Oregon).

Assessment of plasma cytokines and soluble factors

Cytokine and chemokine levels in plasma were analyzed using a MILLIPLEX MAP Non-human Primate Cytokine Magnetic Bead Panel Premixed 23-plex kit (Millipore, Darmstadt, Germany). Levels of analytes were assessed on a Bio-plex 200 System (Biorad, Hercules, CA). Other soluble factors were assessed via ELISA using pre-prepared kits as follows: sCD14 (Quantikine ELISA Human sCD14 Immunoassay, R&D Systems Inc., Minneapolis, MN), LPS Binding Protein (Biometric, Brixen, Italy), C-Reactive Protein Monkey ELISA, (Life Diagnostics, West Chester, PA), and IL-6R Rhesus Macaque ELISA, (RayBiotech, Inc., Norcross, GA). ELISAs were read using an iMark Microplate Reader (Biorad, Hercules, CA).

Statistical analyses

Statistical analysis was performed using GraphPad Prism statistical software (Version 6, GraphPad Software, San Diego, CA). Post-ABX (week -1) and post-FMT timepoints (weeks 2 and 6) were compared to pre-ABX (week -3) as baseline, and significance was evaluated using a paired t-test. Statistical significance between pre-SIV and all post-SIV timepoints was also evaluated using a paired t-test when pre-SIV cryopreserved samples were available for comparison. We generated correlation matrices using the R statistical software package corrplot.

Results

Microbiome composition changes induced by antibiotic treatment and fecal microbial transplantation

The use of antibiotics to reduce or eliminate pathogenic bacteria, such as *C. difficile*, is a standard prerequisite for patients receiving FMT. The first goal of our study was to determine the impact of vancomycin/enrofloxacin antibiotic treatment and FMT on the colonic microbiome. By comparing the relative abundances of bacterial genera across all timepoints, we found that the microbiota composition post-ABX treatment clustered separately from pre-ABX and post-FMT timepoints, with 60.4% of the variation explained by the PC1 axis (Figure 4.2A). Specifically, ABX treatment significantly reduced the relative abundance of gram-positive bacteria, except lactobacillus, and the majority of aerobic gram-negative bacteria. Antibiotic treatment had the largest impact on the abundance levels of the most prevalent bacterial genera, *Helicobacter* and *Flexispira*, while enriching for anaerobic gram-negative microbiota compositions with greater diversity (Figure 4.2B). The microbiome composition quickly reverted by 2 weeks post-FMT to be *Helicobacter/Flexispira* dominant, similar to pre-transplant. However, differences in abundances of the minor populations remained with two of the six animals (animals 4 and 6) retaining increased microbial diversity.

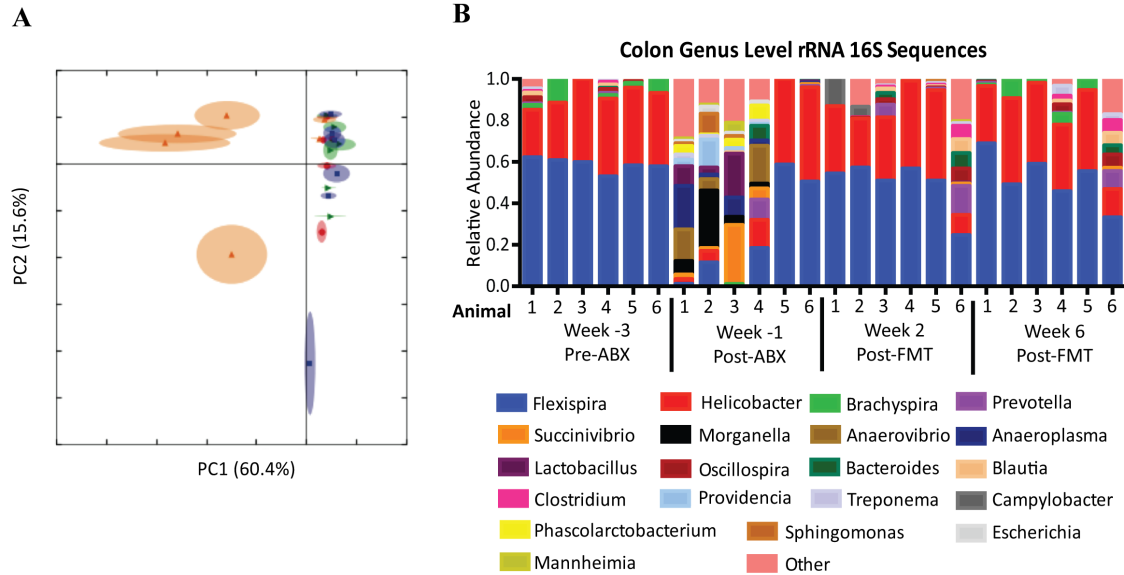


Figure 4.2. ABX and FMT treatment resulted in microbiome composition changes. A.)

Principal components analysis using all relative abundances of all genera from colon biopsies across all timepoints. Green=pre-ABX (week -3); Orange=post-ABX (week -1), Blue= 2 weeks post-FMT (week 2); Red= 6 weeks post-FMT (week 6). B.) Relative abundance of bacterial genera from colon biopsies pre- and post-ABX and FMT. Top 20 most abundant genera are shown. Less abundant genera were grouped into other the category labeled as “Other” (pink).

No negative side effects of fecal microbiota transplantation

Due to the immunocompromised nature of HIV patients, it will be critical to monitor the effects of microbial therapeutics on the overall health of the HIV-infected recipient. It will be particularly important to ensure that introduction of outside bacteria does not affect recipient welfare or cause increased inflammation and immune activation or bacteremia. In this study, FMT in SIV-infected rhesus macaques was well tolerated with no change in animal behavior and only a minor reduction in mean animal weight 2 weeks post-FMT (Table 4.1). There were no

significant changes in leukocytes measured by CBC, with the exception of monocytes, which were reduced 6 weeks post-FMT to a level more closely resembling pre-SIV levels (Table 4.1 and data not shown). Although all animals were on ART, they maintained low but measureable plasma virus levels pre-transplant, ranging from 36-208 copies/ml (Table 4.1). There was a slight yet non-significant increase in plasma virus in some animals post-ABX, which returned to pre-ABX levels by 6 weeks post-FMT.

Table 4.1. Clinical Effects of Fecal Microbial Transplantation in SIV-infected Rhesus Macaques

	Week -3 (Pre-ABX)		Week -1 (Post-ABX)		Week 2 (Post-FMT)		Week 6 (Post-FMT)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Animal Weights (kg)	10.12	1.51	9.87	1.53	9.54*	1.43	9.75	1.41
CBC Neutrophils (1000 cells/ μ l)	2.67	0.9	2.85	0.98	4.16	1.93	4.14	0.89
CBC Monocytes (1000 cells/ μ l)	0.66	0.26	0.65	0.31	0.62	0.25	0.53*	0.17
CBC Lymphocytes (1000 cells/ μ l)	2.16	0.91	1.87	0.73	2.03	0.8	1.9	1.01
CBC Basophils (1000 cells/ μ l)	0.05	0.02	0.04	0.03	0.05	0.03	0.07	0.03
CBC Eosinophiles (1000 cells/ μ l)	0.11	0.05	0.12	0.06	0.09	0.02	0.10	0.04
Viral Load (copies/ml)	123.2	64.5	319.2	376.5	315.5	286.8	109.8	68.2

* $P < 0.05$ by paired t-test, compared with pre-ABX as baseline

Additionally, we measured cytokines and soluble markers of microbial translocation and inflammation in the plasma post-treatment to ensure bacteremia or inflammation did not occur. ABX treatment resulted in increases in the cytokines IL-2 and IL-15, but both returned to pre-ABX levels by 2 weeks post-FMT (Figure 4.3). Interestingly, vascular endothelial growth factor (VEGF), a cytokine increased in HIV associated CNS diseases²²³, transiently decreased 2 weeks post-FMT. We observed no significant changes in other cytokines measured. Soluble CD14 (sCD14), a marker of microbial translocation, began increasing after ABX treatment and

remained significantly elevated 2 and 6 weeks post-FMT. Contrarily, we observed no changes in LPS binding protein (LBP), a marker of LPS stimulation, or the systemic inflammation markers C reactive protein (CRP) or soluble IL-6 receptor. Taken together, these data suggest that ABX treatment may increase inflammation and microbial translocation, but ABX and FMT treatments did not have substantial or lasting effects on overall animal health and welfare and did not result in overtly increased systemic immune activation.

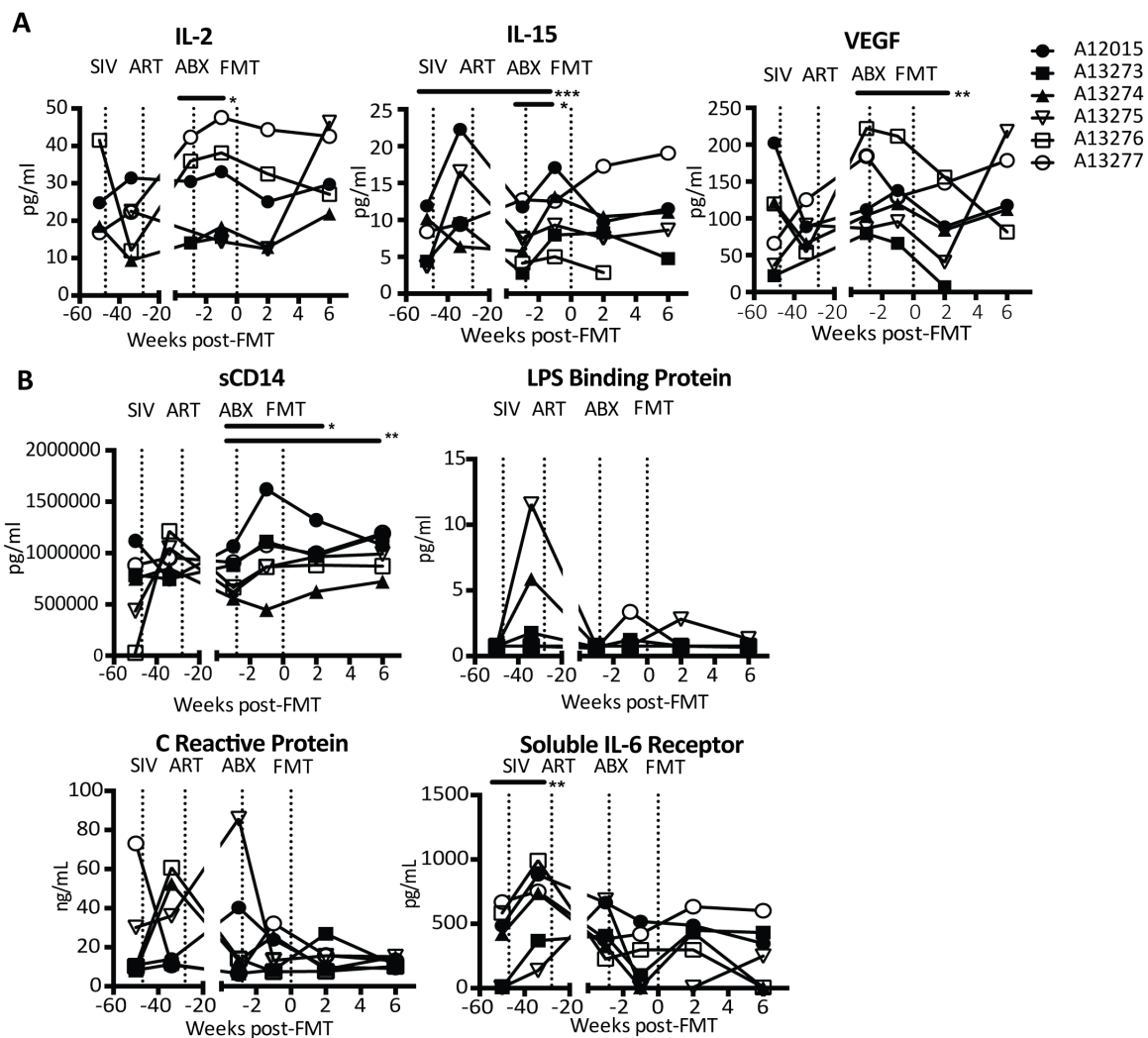


Figure 4.3. Cytokines and soluble markers of inflammation and microbial translocation after antibiotics treatment and fecal microbial transplantation in SIV infected rhesus

macaques. A.) Plasma cytokines measured by luminex. B.) Markers of microbial translocation and inflammation measured in plasma by ELISAs. Asterisks indicate significant difference by paired t test (* $P < 0.005$, ** $P < 0.01$, *** $P < 0.001$).

Increased peripheral Th17 and Th22 frequencies and decreased gut CD4+ T cell activation after fecal microbial transplantation

Th17 and Th22 cells, important components of mucosal immunity, are significantly depleted in HIV and SIV infection and this depletion is associated with reduced barrier integrity, microbial translocation, and systemic immune activation^{19, 112, 173}. Previous studies have demonstrated a connection between commensal bacteria and the ability of probiotic therapy to enhance intestinal Th17 frequencies^{110, 111, 208}. Thus, we hypothesized that alteration of the microbiome through FMT might restore Th17 and Th22 frequencies. Indeed, while we observed no significant effects on total peripheral CD4+ and CD8+ T cell frequencies, peripheral Th17 and Th22 frequencies increased significantly 6 weeks post-FMT compared to pre-ABX (Figure 3). Although the Th17 and Th22 frequencies remained significantly lower than pre-SIV levels, the increases observed suggest FMT has promising potential as a therapy for altered T cell subset homeostasis in HIV infection.

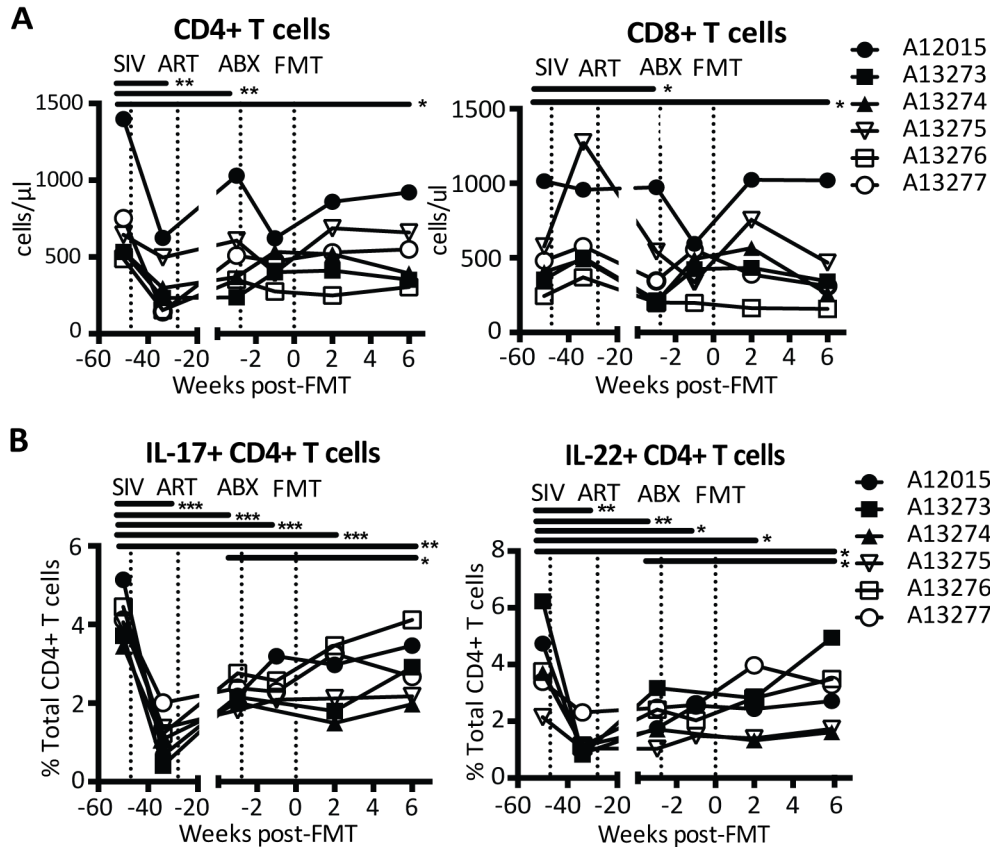


Figure 4.4. Increased peripheral Th17 and Th22 cells after fecal microbial transplantation.

A.) Absolute peripheral CD4+ and CD8+ T cells as calculated using percentage of total CD45+ cells by flow cytometry and the total leukocyte count by complete blood count analysis. B.) IL-22 and IL-17 producing CD4+ T cells in PBMCs measured via flow cytometry after stimulation with PMA/Ionomycin. CD4+ T cells were identified as CD45+CD3+CD4+CD8- cells. Asterisks indicate significant difference by paired t test (* $P < 0.005$, ** $P < 0.01$, *** $P < 0.001$).

T cell activation is associated with CD4+ T cell loss and disease progression in HIV infection^{156, 224}. We measured the effect of FMT on intestinal T cell activation, and found a decrease in activation, as measured by HLA-DR expression, of jejunum, colon, and rectum CD4+ T cells but not CD8+ T cells 6 weeks post-FMT, although the decrease in the colon was not statistically significant (Figure 4.5). These data suggest that FMT might have a beneficial

effect on intestinal T cell activation.

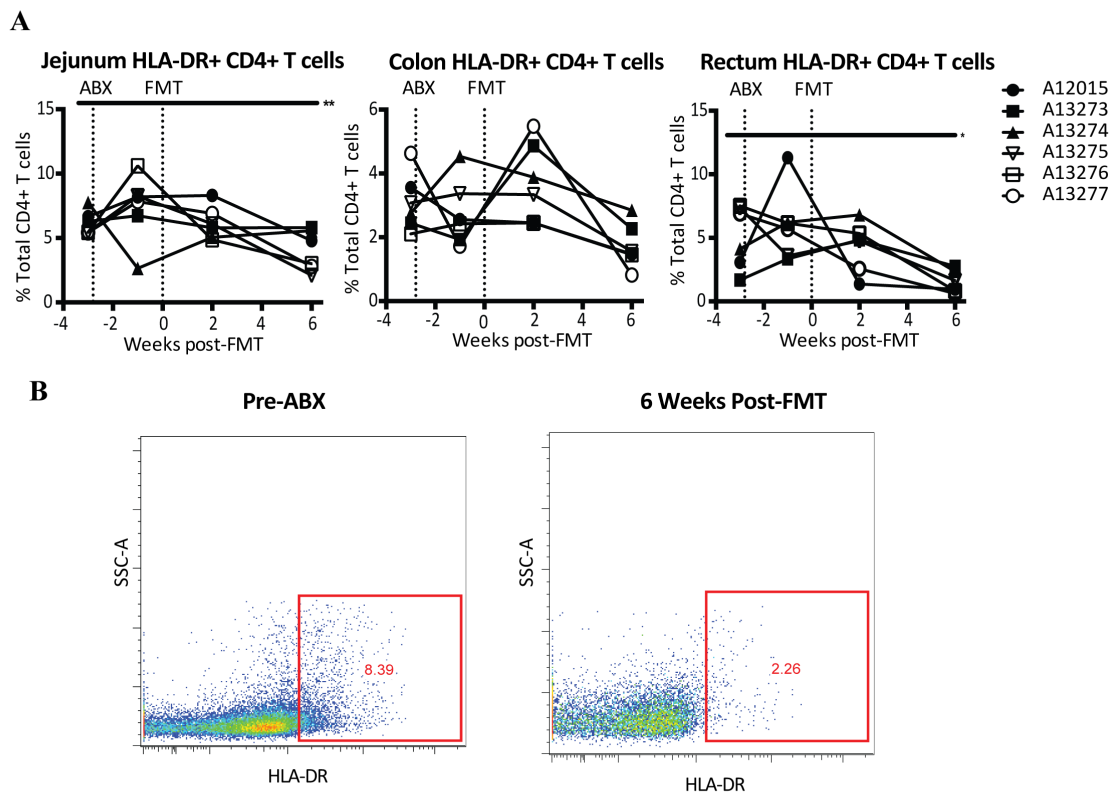


Figure 4.5. Reduced CD4+ T cell activation after fecal microbial transplantation. A.) HLA-DR+ CD4+ T cells in colon, jejunum, and rectum biopsies. CD4+ T cells were identified as CD45+CD3+CD4+CD8- cells. B.) Representative flow plots showing HLA-DR expression on CD4+ T cells from jejunum of an SIV infected rhesus macaque before ABX treatment (left) and 6 weeks post-FMT (right). Asterisks indicate significant difference by paired t test ($*P<0.05$, $**P<0.01$).

Altered immune parameters are associated with altered microbial frequencies

Finally, to investigate if specific alterations to the microbiome were associated with changes in immunological and physiological parameters, we performed correlations across all

identified genera and the immunological parameters significantly altered by FMT. Using these data, we created a correlation matrix illustrating the genera that correlated most strongly with other measured parameters in our study (Figure 4.6). Several strong correlations were observed, particularly among lower abundance genera. Specifically, animal weight was positively associated with the *Helicobacteraceae* genera but negatively associated with *Anaeroplasmataceae*, *Enterobacteriaceae*, *Mitsuokella*, and *Pasteurellaceae*. HLA-DR expression on CD4+ T cells was positively associated *Fusobacterium*, *Neisseriaceae*, and *Sphingomonadaceae*, and negatively associated with *Lachnospira*, *Lachnospiraceae*, *Roseburia*, and *Ruminococcaceae*. We observed no strong correlations between any one specific bacterial genus and peripheral Th17 or Th22 frequencies. Although none of these associations remained significant after correcting for multiple comparisons, these data suggest that microbiome alterations, particularly in the low abundance genera, are potential contributors to the physiological and immunological changes observed in response to ABX and FMT treatments.

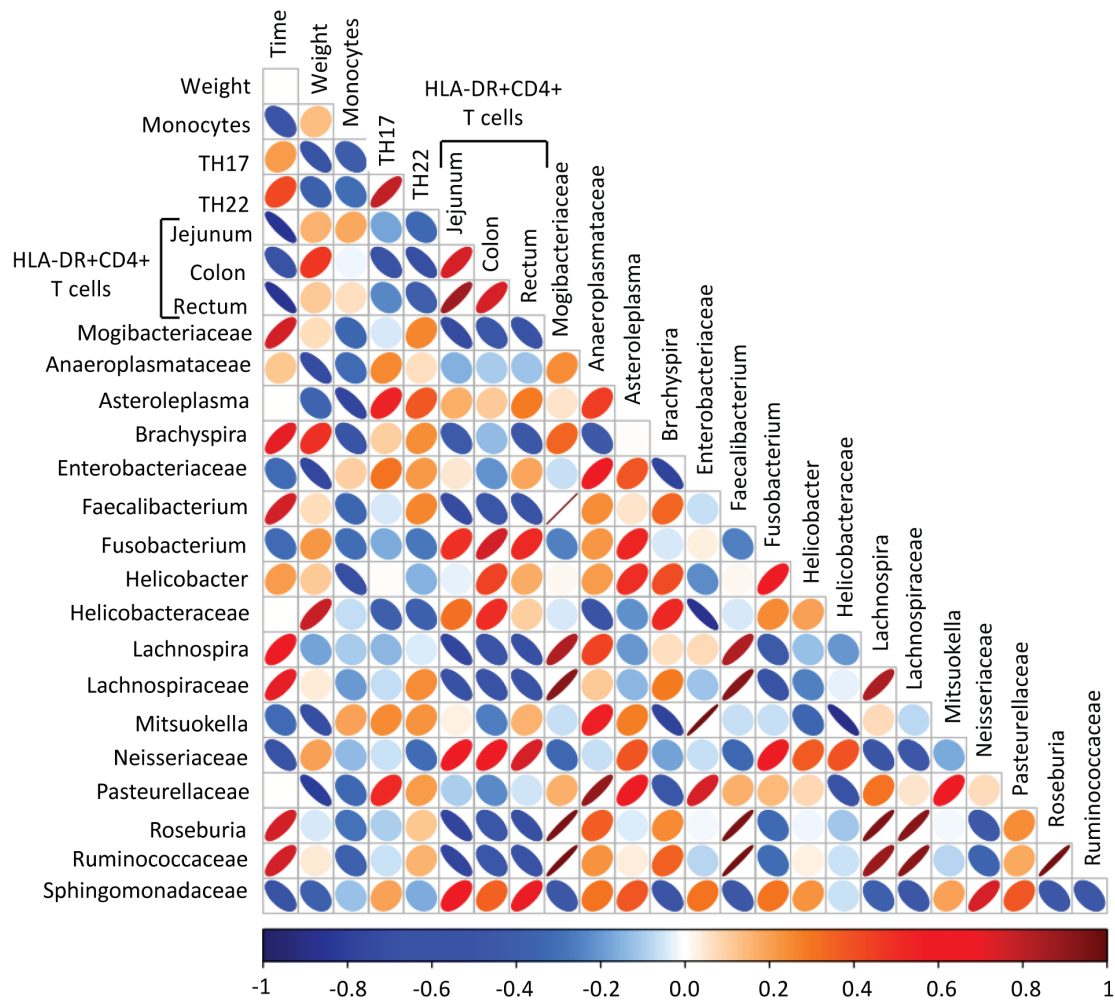


Figure 4.6. Correlation matrix of genera that most strongly correlated with immunological or physiological parameters. Colors indicate Spearman correlation coefficient value ranging from -1 (dark blue) to 1 (dark red). Ellipse shape indicates relative spread of data points and slope of correlation.

Discussion

In chronic HIV infection, an altered gut microbiome is associated with mucosal dysfunction, systemic inflammation, and disease progression^{121, 122, 123, 225}. FMT is emerging as an effective treatment option for antibiotic-refractory intestinal infections, such as *C. difficile*, as well as for chronic inflammatory conditions of the gut that are known to be associated with dysbiosis^{217, 218, 226}. ABX treatment is often used prior to transplantation, although the overall impact of this strategy on therapy efficacy is largely unknown²²⁷. Here we reported the effects of ABX treatment and FMT in 6 SIV-infected rhesus macaques on microbiome, health, inflammation, and immunity. Overall, we find that FMT is safe and well tolerated, and may have beneficial effects on immunity.

Interestingly, after FMT, the microbiome community structure reverted to a state that was strikingly similar to pre-FMT. This likely indicates that the transplant donors had similar microbiota despite being healthy, SIV-uninfected animals. This may be due to all animals being housed at WaNPRC and fed the same diet, which may outweigh the effects of SIV in these colonies. Another alternative is that the FMT did not colonize, and the microbiota rapidly reverted to pre-FMT communities. However given the extensive selection for a diverse community of anaerobic gram negative bacteria induced by antibiotics, this is unlikely, and furthermore, previous studies have demonstrated that post-FMT, recipient microbiome resembles donor microbiota²²⁸.

Despite the reversion to a microbiome similar to that of pre-FMT, we observed enhanced Th17 and Th22 frequencies post-FMT treatment. Induction of Th17 and Th22 cells is important given that these cells are essential in responding to bacterial and fungal pathogens, and critical in maintaining homeostasis of epithelial barriers^{229, 230, 231}. Additionally, during HIV and SIV

infection, IL-17 and IL-22 producing cells are lost, and this is associated with damage to epithelial barrier integrity, inflammation, and pathogenic effects^{19, 112, 173, 232}. Although we observed no correlations between Th17 and Th22 frequencies and any specific bacterial population, the alterations observed may have been induced by minor populations or combinations of populations not represented in the overall microbiome composition. Additionally, we observed decreased intestinal CD4+ T cell activation post-FMT treatment. Importantly, HLA-DR expression on CD4+ T cells of the gut correlated strongly with several lower abundance bacterial genera. The associations between bacterial genera and activated CD4+ T cells were similar between cells isolated from different GI tissue, suggesting that either FMT altered the microbiome at other intestinal sites in a similar manner as in the colon, or that changes in colonic microbial communities have widespread effects on gastrointestinal immunity. Further studies on microbiome alterations at different gastrointestinal sites after FMT should be performed to address this, however data suggest that FMT may provide a novel therapeutic intervention to enhance gastrointestinal integrity through enhanced T cell immunity.

Of interest, while the FMT tended to improve health in the animals, antibiotic treatment appeared to have an inflammatory effect. The increase in proinflammatory adaptive immune related cytokines indicates a potential T or NK cell response to altered microbiota induced by antibiotics^{233, 234}. In addition, sCD14, a marker of microbial translocation, increased in 5 of the 6 animals post-ABX treatment in our study and remained elevated through 6 weeks post-FMT. Although we cannot conclude these changes were solely induced by ABX treatment due to the lack of an ABX only control group, a recently published study in chronically-infected pigtail macaques similarly observed an increase in sCD14 in 2 of the 3 animals after treatment with the antibiotic Rifaximin and the anti-inflammatory agent Sulfasalazine²³⁵. To our knowledge, there

have been no other studies assessing inflammatory cytokines after ABX treatment in infected macaques. The drastic shift in the microbiota from an aerobic to an anaerobic dominant community observed in the gut of post-ABX treatment could potentially influence the systemic inflammatory response. The overall change in the intestine to an anaerobic environment would directly impact oxygen levels, the gut pH, the microbial biofilm structure, and the microbial byproducts produced stimulating an immune response. Importantly, ABX treatment differentially affected the animals in our study, with only minor microbiome changes observed in 2 of the animals while the other 4 animals had dramatic microbiome shifts. This demonstrates that different individuals can respond differently to microbiome-altering therapies. Further studies to assess how commonly used antibiotics may affect inflammation and immunity and the long-term consequences on microbiome are warranted.

Finally, our study provides evidence that alterations to the gastrointestinal microbiome associate with changes in immunological parameters. Microbial diversity has previously been demonstrated as an important factor in protection from intestinal pathogens and inflammatory disorders. Additionally, previous studies of dysbiosis in IBD that have shown associations between some low abundance genera, such as *Roseburia* and *Faecalibacterium*, and intestinal inflammation^{236, 237}. Indeed, in our study, physiological and immune parameters altered by ABX and/or FMT treatments correlated most strongly across all sampling timepoints with lower abundance genera in the colon. Interestingly, we also observed a negative correlation between *Roseburia* and CD4+ T cell activation in gut, further suggesting that this bacterial community may have beneficial anti-inflammatory properties. While this was a small study with few animals, these data warrant further investigation into how overall changes in the microbiome, particularly in terms of diversity and changes in minor populations, affect immunity.

Overall, this pilot study provides evidence that FMT would be a safe treatment for microbial dysbiosis in HIV infection and potentially have beneficial effects on immune dysfunction associated with chronic infection. One caveat of this small pilot study was our inability to include ABX only, FMT only, and no treatment control groups. Without these control groups we are unable to assess the relative contribution of ABX treatment and FMT treatment to the reported observations. Although changes observed after ABX treatment but before FMT are logically attributable to ABX treatment, a larger follow-up study with these control groups should be performed to confirm these findings. This will be particularly important to further understand how ABX and FMT treatments may have differentially contributed to the transient weight loss and increase in sCD14 observed in this study. Future FMT studies in macaques could also consider pre-screening potential macaque donors to identify donor feces with high diversity or enriched for beneficial bacterial communities, combining probiotics with fecal material, or using healthy human fecal material to better represent human microbiota. Additionally, a recent report demonstrated that altering the microbiome with antibiotic treatment with a luminal antibiotic (Rifaximin) together with an anti-inflammatory drug (Sulfasalazine) during acute SIV infection resulted in beneficial reductions in immune activation and microbial translocation, while treating during chronic infection had no discernible effect²³⁵. This suggests that future studies assessing the benefits of FMT in lentiviral infection may result in increased benefits if administered during the acute phase of infection. Finally, while future pre-clinical studies are of interest to determine mechanisms underlying decreased inflammation and enhanced cellular effects of FMT, ultimately clinical trials in HIV-infected individuals would be most informative as to beneficial effects of FMT to treat HIV-associated microbial dysbiosis. Importantly, the pilot study here provides evidence that FMT in immune deficient primates infected with lentivirus did

not have negative side effects, but had overall positive effects, suggesting that clinical trials may be safe and efficacious.

Chapter V

Discussion, Conclusions, and Future Directions

Summary of Major Findings

The broad purpose of this thesis was to address several unanswered questions relating to mechanisms and potential therapies for gastrointestinal mucosal dysfunction in HIV infection, summarized in Chapter I. In Chapter II, we performed an intrarectal SIV infection of rhesus macaques to assess the relative kinetics of mucosal dysfunction and neutrophil infiltration in the intestines during acute infection. In a comprehensive longitudinal examination of peripheral and mucosal tissues early after infection, we sought to answer several specific questions: 1.) When does mucosal damage and microbial translocation begin in acute infection? 2.) When are mucosal and peripheral Th17 cells depleted in acute infection? 3.) Are neutrophils recruited to the rectum in response to intrarectal SIV infection? 4.) Do neutrophils accumulate in the GI during acute infection? 5.) Do Neutrophils retain antimicrobial function in acute infection?

We hypothesized that an early loss of Th17 cells would preclude a neutrophil response to the virus in the rectum but that neutrophils would be recruited later in early infection after mucosal damage and microbial translocation were established. We found that mucosal and peripheral Th17 cells are maintained through early acute infection and begin to decrease in late acute infection (14-28 days post infection) and that neutrophils increased in the rectum, but not the colon, 3 days post-infection. These data suggest recruitment in response to the virus and are the first data demonstrating that neutrophils are in fact recruited to the site of lentivirus infection and may participate in the antiviral immune response. Additionally, we demonstrated that neutrophils exhibit increased phagocytic function in acute infection, which is in contrast to the well-documented impairment of antimicrobial functions of neutrophils in chronic infection. Finally, while we observed increased markers of microbial translocation, we found no prolonged

neutrophil accumulation in the GI mucosa, providing evidence that neutrophil accumulation in chronic infection is likely the result of prolonged mucosal damage, microbial translocation, and immune dysfunction rather than an initial contributing mechanism to mucosal dysfunction. These findings have important implications for future research into potential therapeutic strategies because they provide evidence that neutrophils may not be an appropriate target to prevent of the initiation of mucosal dysfunction in acute HIV infection.

While our data presented in Chapter II suggest that neutrophils are an unlikely contributor to initial mucosal damage, neutrophil accumulation into the gastrointestinal tract during chronic HIV infection has the propensity to exacerbate mucosal tissue damage and dysfunction. While previous studies have reported increased MPO staining in the colorectal mucosa in chronic HIV infection, no previous studies have assessed neutrophil frequencies in the GI in relation to other leukocytes. Additionally, neutrophils exhibit reduced homeostatic apoptosis and prolonged lifespan in several inflammatory conditions including IBD, yet no prior studies had examined neutrophil apoptosis as a potential mechanism for neutrophil accumulation in the GI in HIV infection or the potential connection between neutrophil lifespan and HIV altered mucosal bacteria. In Chapter III, we addressed these questions by examining neutrophils in the colorectal mucosa of HIV infected individuals on ART. Specifically, we sought to answer the following questions: 1.) Are neutrophil frequencies increased relative to other leukocytes in the GI in treated HIV infection? 2.) Is GI neutrophil apoptosis altered in treated HIV infection? 3.) Do HIV altered mucosal bacteria affect neutrophil apoptosis?

We found that neutrophil frequencies are significantly increased in the GI in treated HIV infection. Additionally, we report the first data demonstrating that GI neutrophil apoptosis is reduced in treated HIV infection. Our observed negative correlation between active Caspase-3

expression and total neutrophil frequency suggests that a reduction in homeostatic apoptosis contributes to neutrophil accumulation in the GI in treated HIV infection. These findings provide evidence that neutrophil apoptosis may be a new target for reducing GI inflammation in treated HIV infection, which is major focus in IBD research but has yet to be proposed in the HIV field²⁰³. We also demonstrated that HIV altered mucosal bacteria differentially affect neutrophil apoptosis. In contrast to other bacteria we tested, *Lactobacillus* species uniquely increased neutrophil apoptosis and decreased neutrophil frequencies in *in vitro* stimulation experiments. These data provide a novel mechanism for the ability of *Lactobacillus* to reduce inflammation in previously published *in vivo* studies and demonstrate a never before described anti-inflammatory property of *Lactobacillus*. These data have important implications beyond HIV pathogenesis, which will be discussed in more detail later in this chapter.

Given that microbial dysbiosis in HIV infection is associated with systemic immune activation and increased comorbidities, therapies aimed at restoring a healthy microbiome may provide benefits for infected individuals. In chapter IV, we sought to examine the safety and efficacy of fecal microbial transplantation (FMT) in treated lentiviral infection. Using the nonhuman primate model of ART-treated SIV-infected rhesus macaques, we asked: 1.) Are adverse outcomes associated with FMT in the immunocompromised host? 2.) Does FMT reduce immune activation or increase beneficial Th17 frequencies in treated SIV infection? 3.) How do changes in microbial communities relate to changes in measured immune parameters after FMT? Our findings demonstrated that FMT was well tolerated in SIV-infected macaques on ART. Importantly, the animals showed no signs of an inflammatory response to the donor stool and no signs of bacteremia post-FMT. Additionally, we demonstrated positive effects on immunity, including decreased mucosal T cell activation and increased Th17 cells. Finally, the study

provided important insights into the relationship between minor microbial populations and immune parameters *in vivo*. Overall, this study provided evidence that FMT would be a safe and potentially beneficial therapy to further pursue in HIV-infected individuals and should be considered for future assessment in clinical trials.

Taken together, the data presented in this thesis improves our understanding of the mechanisms contributing to mucosal dysfunction and potential therapies that can be further investigated to improve overall health in HIV infected individuals. Figure 5.1 proposes a model for the timeline and relationships between the events of Th17 depletion, neutrophil infiltration, and dysbiosis in lentiviral infection based on the data presented here and previously published studies.

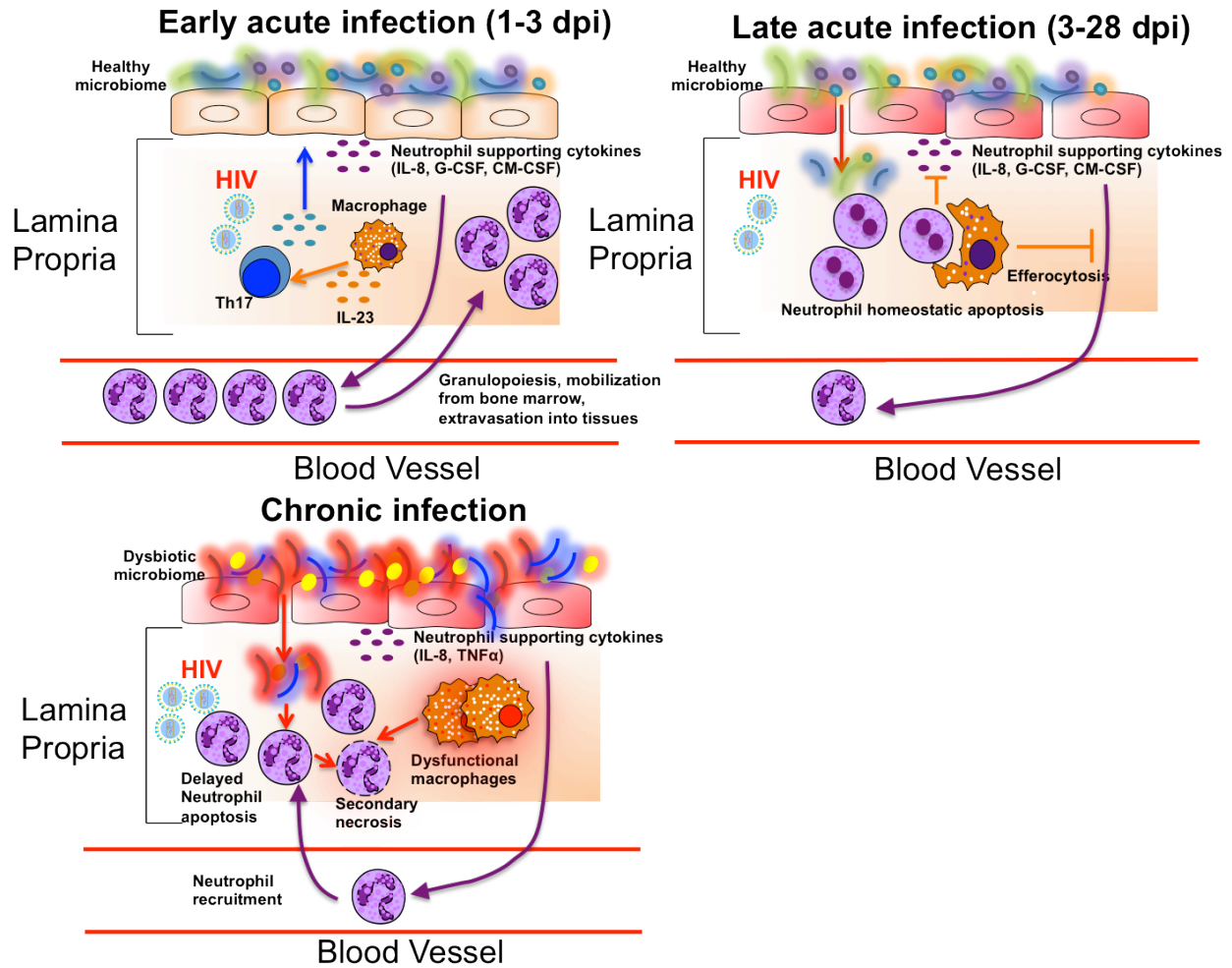


Figure 5.1. Proposed model of the relationship between gastrointestinal Th17 cells, neutrophils, and dysbiosis throughout HIV/SIV infection. Early acute infection: IL-17 is released from Th17 cells and innate lymphoid cells in response to the virus. IL-17 causes neutrophil supporting cytokines to be released from epithelial cells and macrophages that increase granulopoiesis, mobilization of neutrophils in the bone marrow, and recruitment to the site of infection. Late acute infection: Between 3 and 14 days post-SIV infection epithelial barrier breakdown begins which results in microbial translocation of healthy commensal bacteria into the lamina propria. However, neutrophils retain their ability to undergo preprogrammed homeostatic apoptosis and are engulfed by macrophages (efferocytosis). Upon efferocytosis,

functional macrophages promote the resolution of inflammation and the decrease of neutrophil recruitment, partially through reduced IL-23 production and through the release of anti-inflammatory cytokines such as IL-10 and TGF β . Additionally, Th17 cells are depleted 14-28 days post-infection, which may further contribute to resolution of neutrophil infiltration. The decrease in neutrophil supporting cytokines results in reduced neutrophil production and recruitment to the tissue. Chronic infection: Ongoing inflammation and immune dysfunction result in dysfunctional macrophages that are unable to perform efferocytosis of dying neutrophils. Additionally, an increase in IL-8 and TNF α in the tissues results in continued neutrophil recruitment and promotes prolonged neutrophil lifespan. Finally, dysbiotic bacteria interact with neutrophils and other immune cells to further promote delayed neutrophil apoptosis and increased neutrophil lifespan. Together these events result in neutrophil accumulation in the tissues despite the continued depletion of Th17 cells.

Future Directions and Ongoing Studies

Several important unanswered questions remain that should be investigated in future studies. First, the capacity in which neutrophils contribute to the antiviral response to HIV and SIV remains unclear. While several studies have linked low peripheral neutrophil frequencies to HIV acquisition risk and SIV disease progression, no studies have fully assessed the necessity of neutrophils in a protective response. Additionally, while the propensity for neutrophils to damage mucosal tissue is apparent, the extent to which they contribute to mucosal dysfunction in HIV remains unknown. Future studies could target neutrophils in the macaque model prior to low dose mucosal challenges in order to better assess their contribution to a protective antiviral response or during chronic infection to better elucidate their role in tissue damage. While

depletion of macaque neutrophils or human neutrophils would be difficult due to the lack of a specific neutrophil marker, several different approaches could be used to target neutrophil chemotaxis, lifespan, or antimicrobial functions in nonhuman primate or humanized mouse studies to better elucidate the role of neutrophils in protection and their contribution to mucosal damage (Table 5.1).

Table 5.1. Potential therapeutic targeting of neutrophils in HIV/SIV infection studies

Approach	Target	Molecule	Mechanism	Rationale	Reference(s)
Decrease Neutrophil Infiltration	CXCR2	SB-656933	Blocks IL-8 binding	Effective in murine models of neutrophil inflammation in the gut and lung. Was well-tolerated and reduced ozone-induced neutrophilia in the lung in humans.	Lazaar et al., Br. J. Clin. Pharmacol. 2011 ²³⁸
Increase Neutrophil Apoptosis	Cyclin Dependent Kinases	R-roscovitine	Accelerates the rate of apoptosis through a caspase-dependent mechanism	In murine models of inflammation, including passively induced arthritis, bleomycin-induced lung injury and carrageenan-elicited acute pleurisy, the CDKi R-roscovitine significantly improved resolution of inflammation that was associated with an increase in neutrophil apoptosis.	Fox et al., J Innate Immun., 2010 ²³⁹
Increase Neutrophil Apoptosis	MPO	Resolvin (aspirin-triggered 15-epi-lipoxin A4)	Increases neutrophil apoptosis by suppressing MPO-induced survival mechanisms	Resolvin molecules improved resolution of inflammation in lung-injury models in mice.	Fox et al., J Innate Immun., 2010 ²³⁹
Increase Neutrophil Apoptosis	Unknown; hypothesized to be NFκB	<i>Lactobacillus</i> species	Unknown	<i>Lactobacillus</i> species increased active Caspase-3 expression in neutrophils and decreased neutrophil frequencies <i>in vitro</i>	Hensley-McBain et al., (See Chapter III)

Block Neutrophil PD-L1	PD-L1	anti-PD-L1	Reduced T cell suppression and exhaustion	Demonstrated beneficial effects of PD-1 pathway blockade on T cell functionality in SIV infection, but little is known about its effects on mucosal dysfunction. Blocking of the PD-1 pathway in clinical trials in patients with cancer was well tolerated.	Mehraj et al, Open AIDS J., 2014 ²⁴⁰ ; Porichis, Current HIV/AIDS, 2012 ²⁴¹
Increase Neutrophil Antimicrobial Functions	Neutrophils	G-CSF	Restores potential defects in granulopoiesis and neutrophil priming.	G-CSF treatment increased antimicrobial function of neutrophils, reduced bacteremia, and increased survival in HIV-infected patients.	Mayer et al, Clinical Infect. Diseases, 2001 ²⁴²

Finally, GI neutrophil functionality in HIV infection has yet to be assessed, which would have important implications for their ability to contribute to containment of translocated bacteria in the GI. In an ongoing study, we are currently assessing the expression of neutrophil functional markers (Fc receptors and PD-L1) and GI neutrophil phagocytic function in the colorectal mucosa of chronically infected individuals on ART. At the conclusion of the study, we will also investigate associations between GI neutrophil frequency and functionality and markers of microbial translocation, immune activation, and inflammation to better elucidate the contribution of neutrophils to mucosal dysfunction.

Beyond HIV Pathogenesis: Broader Implications for HIV Prevention and Neutrophil-Driven Inflammation

While the work presented in this thesis has important implications for HIV pathogenesis and mucosal dysfunction, these data can also be applied to investigate new HIV prevention strategies. Importantly, studies have implicated neutrophil-associated factors in the vagina with increased risk of HIV acquisition. Specifically, cervicovaginal lavages from South African women that acquired HIV infection had increased levels of the potent neutrophil chemokine IL-8

compared to those from women who remained uninfected in a recent microbicide trial²⁴³.

Additionally, further study of genital samples from the same trial associated increased neutrophil proteases with increased inflammatory cytokines, an altered cytoskeleton, and increased CD4+ T cells²⁴⁴. The authors also demonstrated that neutrophil proteases correlated with IL-17 expression, which could contribute to increased target cells for HIV acquisition and replication as Th17 cells have recently been demonstrated to be preferentially infected in the female genital tract^{245, 246}. Taken together, these data suggest that a sustained accumulation of neutrophils in the female genital tract may be contributing to HIV acquisition in the vagina by reducing epithelial barrier integrity through the release of proteases and by promoting Th17 differentiation and recruitment.

Further studies are necessary to fully elucidate the dichotomy of ongoing neutrophil accumulation and the association with HIV risk and a transient neutrophil recruitment to the vaginal mucosa that may contribute to a protective antiviral response. However, reducing neutrophil accumulation and unresolved vaginal inflammation in high risk women has emerged as a promising HIV prevention strategy, and a potential factor contributing to increased neutrophils in the vagina may be altered microbial composition. Indeed, studies have revealed that bacterial vaginosis is linked to a 60% increase in HIV acquisition risk²⁴⁷. Bacterial vaginosis is characterized by a reduction in *Lactobacillus* species and an increase *Gardnerella vaginalis*²⁴⁸. The data presented in this thesis suggest that a reduction in *Lactobacillus* may be linked to increased neutrophil lifespan, which could drive neutrophil accumulation and HIV acquisition risk in the female reproductive tract. Preliminary data support this hypothesis, with vaginal *Lactobacillus* species increasing neutrophil apoptosis *in vitro* in a fashion similar to what we demonstrated for gut *Lactobacillus* species (data not shown). Importantly, these data suggest that

a vaginal *Lactobacillus* probiotic may reduce neutrophils in the vagina, thereby decreasing vaginal barrier damage and Th17 target cells, and ultimately reducing HIV acquisition risk.

The ability of *Lactobacillus* to drive neutrophil apoptosis also has broad implications for inflammatory conditions beyond HIV infection. The most obvious translation of these data beyond HIV would be IBD, which is characterized by dysbiosis and excessive GI inflammation that parallels the pathological presentations of HIV-induced mucosal dysfunction. Probiotics including different *Lactobacillus* species have been assessed in several previous studies for their ability to reduce IBD disease severity with variable results^{249, 250}. Our data suggest that probiotics containing *Lactobacillus* may be more effective at treating IBD in individuals where high neutrophil accumulation presents as a contributing factor. This may explain why *in vivo* studies demonstrate reduced severity of ulcerative colitis but not Chron's disease after administration of *Lactobacillus*-containing probiotics, as neutrophil activation plays a central role in ulcerative colitis but their contribution to Chron's disease is less clear¹⁸⁶.

Importantly, neutrophil apoptosis is a major therapeutic target in not only IBD but other inflammatory diseases including lung inflammation, pneumonia, arthritis, peritonitis, and meningitis²⁰³. The data we present in this thesis suggests that *Lactobacillus* or a specific component of *Lactobacillus* could be exploited to reduce excessive neutrophils, which perpetuate nonresolving inflammation and complicate many disease states²⁵¹. Future studies determining the mechanisms involved in the ability of *Lactobacillus* to increase neutrophil apoptosis will be important to better elucidate how *Lactobacillus* or a *Lactobacillus*-derived molecule may be used as therapy for neutrophil-driven inflammation.

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