

**CONTRIBUTIONS OF MACROPHAGES IN LYMPH NODES AND GUT MUCOSA TO  
SIV DISEASE CONTROL AND PROGRESSION**

by

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**ABSTRACT**

The lymph nodes and gastrointestinal tract are principal sites of inflammation and T cell dysfunction in progressive human immunodeficiency virus type 1 (HIV) infection, and a leading hypothesis is that aberrant responses by innate immune cells, such as macrophages, are actively involved in this process. Macrophages promote and resolve tissue inflammation in health and in response to pathogen insult through cytokine release, phagocytosis, and wound repair, but currently their role in HIV/AIDS pathogenesis remains unclear. Using the pathogenic simian immunodeficiency virus (SIV)-rhesus macaque model, I investigated the macrophage response at different stages of SIV infection and AIDS, as well as longitudinally in macaques anticipated to control or progress to disease based on set-point viral load. I **hypothesized** that macrophages promote a protective response during acute SIV infection that becomes inadequate and/or detrimental to the host in chronic infection and associates with disease progression. I found that macrophage density uniformly increased in lymph nodes and gut mucosa in acute SIV infection and then declined at the onset of chronic infection except for in gut mucosa of SIV progressors, where macrophage numbers remained elevated and were increased above pre-infection in AIDS. Lymph node macrophages were activated, apoptotic, and inflammatory, spontaneously secreting TNF- $\alpha$ , IL-6, and IFN- $\alpha$  in the acute and chronic stages of infection with minor differences

between groups. In contrast, macrophages in small intestine were functionally non-inflammatory, except in AIDS, and macrophage phagocytic capacity in SIV progressors was markedly impaired, inversely correlating with gut macrophage abundance, enteropathy, and plasma but not tissue viral burden. Collectively, these data suggest an inflammatory function for macrophages in lymph nodes that participates in SIV-associated inflammation and T cell loss but does not contribute to disease outcome. At the same time, macrophages in gut mucosa appear to resolve rather than promote inflammation through phagocytosis, and their impairment is associated with hallmarks of progressive SIV infection and eventual harmful activity in AIDS. Defining macrophage involvement in HIV/AIDS pathogenesis could elucidate novel pathways for adjunctive therapeutic intervention through direct or indirect augmentation of macrophages and therefore has considerable public health significance.

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## 1.0 INTRODUCTION

### 1.1 THE HIV/AIDS PANDEMIC

Human immunodeficiency virus type 1 (HIV) is one of the deadliest pathogens of the modern era. Since its isolation and discovery in 1983, over 35 million people have died of acquired immunodeficiency syndrome (AIDS)-related complications, the disease caused by HIV [1, 2]. An estimated 36.7 million people live with HIV today, and in 2015 there were a total 2.1 million new infections, a 6% decline from 2010 [3]. HIV is a lentivirus in the family *Retroviridae* that propagates by integrating viral DNA derived from its reversed transcribed positive sense single-stranded RNA genome into host DNA and co-opting host cellular machinery [4]. The virus preferentially infects memory CD4+ T-helper (Th) cells found in blood and tissues due to their expression of viral receptor and coreceptor CD4 and CCR5 [5, 6]. Transmission of HIV can occur through sexual contact, exposure to contaminated blood (e.g., via needles or syringes), or from mother-to-child during pregnancy [7]. New infections are disproportionately concentrated in developing nations, most notably Sub-Saharan Africa, and predominantly occur among women, the homosexual community, and ethnic minorities [8]. The advent of antiretroviral therapy (ART) that suppresses viral replication has increased the average life-expectancy of HIV-infected individuals dramatically; however access to therapeutics is often limited in nations with the highest prevalence of infection [9]. A small subset of HIV+ individuals (0.15%-1.5%)

referred to as elite controllers or long-term nonprogressors naturally maintains low or undetectable levels of virus replication and has delayed onset of AIDS in the absence of ART [10-12]. For elite controllers and the roughly 17 million individuals currently accessing ART, new health complications become a reality due to chronic virus exposure and/or long-term ART use [13, 14]. Today, non-infectious comorbidities, including neurological disorders and cardiovascular disease, are some of the leading causes of mortality in viral suppressed HIV-infected individuals, who have over twice the risk of developing these diseases compared to age-matched non-infected individuals [15, 16]. A growing body of literature suggests a driving factor of non-infectious comorbidities and AIDS progression is aberrant immune cell activity and the sustained exposure of host tissue to inflammatory mediators [17].

## **1.2 SIV-NONHUMAN PRIMATE MODEL**

Our current wealth of knowledge and understanding of HIV infection is in large part due to studies conducted using simian immunodeficiency virus (SIV)-infected nonhuman primates. Experiments that would otherwise involve invasive procedures in humans, such as research into the gastrointestinal tract, or challenging longitudinal protocols, such as investigating the early events of transmission, can be carried out humanely and with precision in nonhuman primates. Studies using SIV and nonhuman primates have been employed to test preclinical vaccines, discover novel therapeutics, and expand our understanding of mechanisms underlying HIV disease control and progression [18]. Currently, there are 2 primary SIV/nonhuman primate models that dominate the HIV/AIDS scientific landscape: endemically or experimentally infected natural hosts of SIV and non-natural SIV hosts that require experimental inoculation

[19]. African-origin nonhuman primates, for example the African green monkey (*Chlorocebus sabaenus*), co-evolved for thousands of years with SIV, resulting in adaptations that confer a nonpathogenic infection despite maintaining moderate levels of virus replication in blood and tissues [20, 21]. Conversely, experimental infection of Asian-origin macaques recapitulates hallmarks of pathogenic HIV infection in humans, such as systemic and mucosal immune pathologies, viral load dynamics, clinical symptoms, and eventual progression to AIDS, albeit at an accelerated rate [22, 23]. The most widely adopted model for studying progressive infection is the Indian-origin rhesus macaque (*Macaca mulatta*) infected with either pathogenic SIVmac251 swarm or SIVmac239 clone [18]. Studies investigating correlates of immune protection and disease control for therapeutic purposes and academic advancement have classically compared outcomes in natural SIV hosts to those in non-natural hosts, simulating comparisons between elite controllers and normal progressors in humans [19]. These comparisons have led to many seminal discoveries in the field, such as the observation that plasma viral burden is not the only determinant of disease progression and that gastroenteropathy and chronic immune activation also play a key role (to be discussed in detail in section 1.3) [18]. One caveat to comparing outcomes in natural and non-natural SIV models (i.e., African green monkeys vs. rhesus macaques) for pathogenesis studies is the inability to control for species-specific variance between animals. The natural SIV hosts African green monkeys, sooty mangabeys, and mandrills, for example, have distinct immunologic imprints compared to pathogenic models that bestow a protective advantage, such as a lower frequency of CD4+ T cells in circulation and mucosal tissues and lower CCR5 expression on CD4+ T cells [24, 25]. Moreover, the use of distinct species of nonhuman primates often requires comparing infection with dissimilar strains of virus. An underused approach to control for these potentially

confounding variables is to exploit certain protective major histocompatibility class (MHC) I alleles that are associated with long-term virus control (to be discussed in detail in section 1.3). By taking advantage of nonhuman primate analogs to the human protective alleles *HLA-B\*27* and *HLA-B\*57*, comparisons between controlled and progressive disease outcomes in identical species of animal infected with the same strain of virus can be made [26]. For the purposes of this dissertation, from here on unless otherwise stated, all discussion of nonhuman primates will refer to SIV infection in Indian-origin rhesus macaques.

### **1.3 HIV/SIV INFECTION DYNAMICS AND DISEASE PATHOGENESIS**

Infection of target CD4<sup>+</sup> T cells by HIV/SIV initiates a cascade of events within the host that can be divided into three discrete stages: 1) the acute or primary stage of infection, 2) the chronic, asymptomatic period of infection, and 3) AIDS. Clinical symptoms consistent with acute stage infection manifest in humans and nonhuman primates between one to four weeks after viral transmission [7, 27, 28]. Regardless of transmission route, acute infection is associated with a burst of virus replication in the plasma, which peaks on average between  $10^5$ - $10^7$  viral RNA copies/mL, and corresponds with a decline of circulating naive CD4<sup>+</sup> T cells from blood [29]. Serial necropsy studies in rhesus macaques inoculated vaginally with SIVmac251 reveal that viral RNA is detectable as early as 24-hours post-infection in the gastrointestinal tract, tonsil, spleen, lymph nodes, and female reproductive tract. In the same study, by day 10, virus could be found in lungs, liver, thymus, and spleen, but at the highest concentration in gastrointestinal and lymphoid tissues [30]. The resolution of peak viremia during the acute phase signals the onset of asymptomatic (or chronic) infection and coincides with an expansion of HIV/SIV-specific

adaptive immune responses. Here, antigen-specific CD8<sup>+</sup> T cells, and to a minor extent plasma cells, respectively combine to kill HIV/SIV-infected CD4<sup>+</sup> T cells and neutralize free virions in circulation, ultimately reducing virus burden in the plasma [31, 32]. Studies have shown that HIV-specific CD8<sup>+</sup> T-lymphocytes are detectable in tissues before peak viremia, however the magnitude and timing of their response is insufficient to overcome the rate of viral expansion [30, 33]. The extent with which virus replication in plasma is controlled and subsequently maintained throughout the asymptotic phase of infection is referred to as set-point. Set-point establishes chronic phase virus load until the emergence of AIDS and is used as a reliable measure of the severity of infection, is associated with time to progression to disease, is clinically employed as a guide to determine an ART regimen, and is used to assess ART efficacy [34]. Definitions vary worldwide but in general elite controllers of HIV maintain median set-point plasma virus RNA concentrations between 50-1000 copies/mL, whereas risk of rapid AIDS-onset is significantly increased when virus loads exceed 10<sup>4</sup> copies/mL [34, 35]. Furthermore, plasma viral burden inversely correlates with CD4<sup>+</sup> T cell levels in blood; therefore, individuals with the highest set-point viral loads tend to become immunocompromised more rapidly [36]. A diagnosis of AIDS is reached when circulating CD4<sup>+</sup> T cells fall below 200 cells/mm<sup>3</sup>, accompanied by a rise in opportunistic infections, such as *Pneumocystis pneumonia*, and in the non-infectious disorders described above.

### **1.3.1 Genetic determinants of disease outcome**

Certain host genetic factors can play an important role in determining disease prognosis because of their influence on set-point viral load. Two of the most well-studied genetic determinants associated with slow progression to disease are the CCR5-Δ32 allele and protective MHC class I

alleles [37, 38]. Tropism of HIV/SIV is nearly always dictated by the co-expression of cellular proteins CD4 and CCR5, which the viral envelope proteins gp120 and gp41 attach to for viral entry [4]. For a small subset of individuals, viral entry is impaired due to a 32 base-pair mutation in their CCR5 gene that produces a nonfunctional CCR5 receptor on the surface of their CD4+ T cells [37]. Heterozygous carriers of this allele have reduced susceptibility to infection and often maintain elite controller status, whereas homozygous expression confers complete resistance to viral entry [39, 40]. Interestingly, genetic polymorphisms that result in the increased circulation of the CCR5-ligands RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$  are also associated with enhanced protection [41]. Despite optimism for the development of a CCR5-receptor antagonist, so far only one drug, maraviroc, has reached the market. Maraviroc is detectable at high concentrations in blood, seminal plasma, and rectum in treated patients, and provides at minimum a 1.5% log reduction in plasma viral RNA compared to control groups [42, 43].

Expression of specific MHC class I alleles in humans (referred to as HLA molecules) and in rhesus macaques (Mamu molecules for *Macaca mulatta*) can also affect the rate of progression to disease by promoting cytotoxic T-lymphocyte-mediated containment of viral expansion. Most notably, expression of alleles *HLA-B\*14*, *-B\*27*, and *-B\*57* in humans and *Mamu-A\*001*, *-B\*017*, and *-B\*008* in macaques are highly correlated with low chronic phase viremia and increased survival time [44, 45]. Identification of these alleles has led to extensive research into the role of CD8+ T cell responses in virus control as well as innate immune cells that orchestrate effector CD8+ T cell specificity, such as myeloid dendritic cells (DCs) [46]. Immunization and viral sequencing studies in humans and nonhuman primates demonstrate that blood CD8+ T cells generated by protective HLA/Mamu allele-restricted epitopes suppress viral replication by targeting specific regions of the virus [47, 48]. For example, in one study,

differences in *Mamu-B\*008*-expressing SIV controllers (chronic phase viral loads  $<10^4$  gag copies/mL) compared to SIV progressors (viral loads  $>10^5$  copies/mL) were found to be a result of viral escape mutations in *Mamu-B\*008* restricted-epitopes and not a function of divergent CD8<sup>+</sup> T cell activity or frequency in blood [48, 49]. While some studies find associations between enhanced CD8<sup>+</sup> T cell responses and elite virus control, with or without expression of protective HLA class I alleles, there is still no clear consensus [50-54]. The fact that many natural controllers of HIV do not express protective class I alleles, are not carriers of the CCR5- $\Delta 32$  polymorphism, and/or have measurable CD8<sup>+</sup> T cell dysfunction underscores the complexities of immune-mediated viral suppression among HIV-infected individuals [51, 55, 56].

### **1.3.2 Pathobiology of infection in lymph nodes and gastrointestinal tract**

An overwhelming majority of research investigating HIV/SIV pathogenesis extrapolate findings from the blood as a window into the state of infection and disease despite blood containing between 0.3% and 0.5% of the total CD4<sup>+</sup> T cells in the body [57]. Instead, secondary lymphoid organs and the gastrointestinal tract are the body's largest reservoirs of CCR5-high CD4<sup>+</sup> T cells in health and the most significant sites of virus replication and latency during infection [58]. Lymphoid compartments function as infrastructure to introduce antigen-bound innate immune cells circulating in the periphery to naïve immature lymphocytes, an interaction essential for the induction of antigen-specific cellular and humoral immune responses and the development of immunologic memory [59]. Lymph nodes are stationed almost everywhere in the body but are most concentrated in and around mucosal surfaces. In fact, roughly 85% of the body's lymphoid tissue and 90% of its lymphocytes are located within the gastrointestinal tract [60-62]. Situated

at the interface of the host and external environment, the gastrointestinal tract maintains physiologic and immunologic equilibrium through elaborate symbiotic interactions with intestinal microbiota [63]. In addition to shaping the development of antigen-specific immune responses, these interactions play vital roles in nutrient absorption, in preventing non-specific immune responses towards dietary antigens and commensal bacteria, and in combating the expansion of pathogenic organisms [64].

Among the earliest sites infected by HIV or SIV is the gastrointestinal tract [65]. The magnitude of CD4<sup>+</sup> T cell depletion in gut mucosa is significantly more rapid and profound than in any other tissue in the body, occurring days before CD4<sup>+</sup> T cells decline in blood and lymph nodes [66, 67]. Studies using the rhesus macaque SIV model demonstrate that over 90% of gut CD4<sup>+</sup> T cells are depleted 2 weeks post-infection [66]. This is in part a function of the high density of effector memory CD4<sup>+</sup> T cells that occupy the gut, the most readily infected of the CD4<sup>+</sup> T-helper cell populations due to their high CCR5 expression and activated state [6, 66, 68, 69]. Unlike in the blood, however, the reservoir of gut CD4<sup>+</sup> T cells remains considerably depleted and dysfunctional in the chronic stages of infection and is only partially restored by ART medication [70]. Paradoxically, experimentally-infected natural hosts of SIV also experience an acute loss of CD4<sup>+</sup> T cells from the gut, suggesting CD4<sup>+</sup> T cell depletion from gastrointestinal tract is not an indicator or driver of disease progression [71]. Lymph node CCR5<sup>+</sup> CD4<sup>+</sup> T cells are also progressively depleted during HIV/SIV infection and depletion is strongly correlated with the degree of lymph node hyperplasia and fibrosis [72, 73]. As a consequence, the timing of drug commencement is inversely proportional to the extent of CD4<sup>+</sup> T cell recovery in HIV-infected lymph nodes [74, 75].

Pathologic changes to lymph nodes and the gut during HIV/SIV infection are profound and play a significant role in influencing the course of disease. In lymph nodes, changes associated with progressive infection include extensive collagen deposition and fibrosis, architectural damage, and an influx of activated, apoptotic, and dysfunctional innate and adaptive immune cells [74, 76-84]. Compared with SIV infection in rhesus macaques, lymph nodes from viremic natural SIV hosts have relatively little detectable virus, preserved tissue integrity, and no collagen deposition [85]. Likewise, the gastrointestinal tract is subject to a massive influx of activated leukocytes, alterations in gut microbiota, and extensive enteropathy [86-89]. Breach in the intestinal lining results in leakage of commensal microbiota from the gut luminal space into the host cavity which contributes to a persistent state of cellular activation throughout the body, a phenomenon referred to as chronic immune activation [90, 91]. In SIV-infected macaques, *Escherichia coli* (*E.coli*) and associated microbial products are detectable in lamina propria of colon as early as 2 weeks post-infection and found at high levels in colon and distal lymph nodes in SIV/AIDS [90]. One mechanism for intestinal breach is the depletion of the Th17 T-cell subset from gut lamina propria [92]. Th17 cells promote mucosal epithelial integrity by secreting IL-17 and IL-22, which maintain epithelial cell tight junctions, promote enterocyte proliferation and remodeling, and support innate defense against microbiota [93]. In contrast to pathogenic infection, gut Th17 cell frequencies remain stable in natural SIV hosts, despite ongoing virus replication in intestine, and in HIV-infected individuals that initiate ART early [21, 85, 94, 95]. The cumulative and sustained exposure of host cells to persistent virus replication and translocated bacteria, among other factors, perpetuates an environment of aberrant immune activity and unchecked inflammation that is highly correlated with disease

outcome, and the mechanisms underlying these processes are the focus of intense investigation [91, 96].

### **1.3.3 HIV/SIV infection and innate immunity**

Mononuclear cells of the innate immune system and innate-associated responses are often implicated in contributing to tissue damage and immune dysfunction in HIV/AIDS. The mononuclear phagocytes DCs and macrophages are hematopoietic cells that are central to bridging the gap between innate and adaptive immune responses [97]. There are two primary DC subsets found in humans and nonhuman primates: myeloid DCs and plasmacytoid DCs. Myeloid DCs are dedicated antigen-presenting cells that can be productively infected by HIV and have been shown to trans-infect CD4<sup>+</sup> T cells through virological synapses and endosome-associated vesicles via tunneling nanotubes [98, 99]. Myeloid DCs accumulate in lymph nodes during acute SIV infection to generate antigen-specific adaptive immunity; however, infection is associated with their impaired maturation, resulting in reduced expression of MHC class II, CD40, and CCR7 and an inability to stimulate naïve CD4<sup>+</sup> T cells, possibly a function of increased apoptosis [84, 100]. At the same time, plasmacytoid DCs, the foremost type I interferon (IFN)-producing cells in the body, accumulate in lymph nodes and gut mucosa, and their accumulation coincides with chronic IFN-stimulated gene expression and immune activation associated with pathogenic SIV infection [83, 88]. While plasmacytoid DCs are reported activated in acutely SIV-infected lymph nodes, they are also highly apoptotic, hyporesponsive to *ex vivo* viral stimulation, and the blockade of plasmacytoid DC IFN- $\alpha$  release has no effect on SIV-associated immune activation, suggesting other cell types participate in this response [83, 101, 102]. In AIDS, both DC subsets are lost in secondary lymphoid tissues,

suggesting a role for DCs in disease pathogenesis and highlighting the importance of defining mechanisms of innate immune pathology associated with divergent disease outcomes [103]. While a lot is known about the role of DCs in HIV/AIDS, relatively little is understood about macrophages and their contributions to HIV pathogenesis.

## 1.4 MACROPHAGES

Macrophages are tissue-resident effector cells of the innate immune system that mediate homeostasis through their diverse phenotypic signatures, strong phagocytic capacity, and the ability to present antigen on MHC class II molecules. They originate in tissues during embryonic development and can be derived and differentiated from monocytes in circulation during inflammation [104, 105]. Macrophages exhibit a high degree of heterogeneity and plasticity from tissue to tissue conferring the ability to respond to tissue-specific needs in health and adapt accordingly to different infections and diseases [106]. Specialized macrophages resident in tissues include Kupffer cells (liver), microglia (brain), dermal macrophages (skin), and alveolar macrophages (lung) [106]. The range of functions macrophages carry out include tissue maintenance and repair, the capacity to initiate and resolve inflammatory responses, immune surveillance and pathogen control, augmentation of adaptive immunity, and iron metabolism [106]. Macrophages were first discovered in 1893 by Elie Metchnikoff while observing large nucleated cells ingest material in the context of pathogen-induced inflammation, a process termed phagocytosis [107]. Phagocytosis is a type of endocytosis by which macrophages internalize and degrade large molecules ( $\sim 0.75\mu\text{m}$  in diameter) for the purpose of tissue maintenance, such as clearing cellular waste or pathogenic debris, and for influencing

adaptive immune responses through antigen-presentation [108]. Macrophages facilitate receptor-mediated phagocytosis by tagging cellular debris and pathogens with secreted opsonins, including mannose-binding lectin, fibronectin, and complement proteins (e.g., C3b) [108]. The capacity to present antigen on MHC class II molecules to lymphocytes and enhance their activation distinguishes macrophages from other phagocytic cells, such as neutrophils which lack surface MHC class II expression [108]. Macrophages differentiate benign host material from inflammatory cellular debris and microorganisms through a variety of internally and externally expressed pattern recognition receptors, such as Toll-like receptors (TLRs), NOD-like receptors, C-type lectin receptors, and scavenger receptors [109-112]. Macrophage expression of pattern recognition receptors varies across tissues in health reflecting their adaptation to different compartmentalized microenvironments [113]. As a consequence, resident macrophages have the capacity to contribute a spectrum of responses throughout the host that are amenable to maintaining homeostasis within each tissue [114]. Unlike macrophages in lymph nodes, for example, macrophages in the intestine constitutively downmodulate expression of lipopolysaccharide (LPS) co-receptor CD14, co-stimulatory molecules CD40, CD80, and CD86, and innate response pathway adaptor proteins MyD88 and TRIF to avoid inappropriate responses towards commensal enteric microbiota and food antigens [115-118].

#### **1.4.1 M1-M2 macrophage paradigm**

The M1-M2 classification system was designed to simplify macrophage diversity based on their responses to specific TLR-ligands, cytokine signals, and their induction of either Th1-cytotoxic or Th2-regulatory adaptive immune responses [119, 120]. Labeling of macrophages as M1- or M2-like represents extremes in macrophage behavior on a continuum of activity, and it is likely

most macrophages exist in the center of this spectrum [121]. Traditionally, tissue macrophages have been delineated by their expression of various surface and intracellular antigens such as HAM56, CD68, CD163, CD14, CD206, CD11b, CD169, and MAC387 [113, 122, 123]. However, the promiscuity of many of these antigens on different cell-types and between M1 and M2 cell populations has underscored the importance of pairing surface antigen expression with certain gene transcriptional signatures and/or functional attributes [114]. M1 or classically activated macrophages are typified by their heightened antigen-presentation ability and disposition for cytolytic and microbicidal responses in the context of infection and disease [124]. Polarization of M1 macrophages is driven by exposure to LPS, IFN- $\gamma$ , and GM-CSF which induce an enhanced capacity for the production of pro-inflammatory mediators IL-6, TNF- $\alpha$ , IL-1 $\beta$ , and iNOS [125, 126]. Due to their direct-killing function, M1 macrophages are found activated and in abundance in tissues infected with many intracellular bacterial pathogens such as *Salmonella typhi*, *Mycobacterium tuberculosis*, and *Listeria monocytogenes* [127-129]. For example, granuloma lesions in *M. tuberculosis* infection of cynomolgus macaques and humans are characterized by clusters of M1 macrophages within the inner regions that may function to restrict bacteria growth [130]. M1 macrophages also exhibit antitumor potential [124]. Studies in lung cancer models have uncovered benefits of M1 macrophage cytolytic activity in suppressing tumor cell growth and metastasis by decreasing tumor cell viability [131]. Irregularity of M1 macrophage activity, however, can be a source of tissue pathology in chronic infections and non-infectious diseases. Crohn's disease is a chronic intestinal disorder associated with a disrupted intestinal barrier and an infiltration of TNF- $\alpha$ - and iNOS-expressing macrophages [132]. The FDA approved biologics Natalizumab (an anti- $\alpha$ 4 integrin antibody) and Adalimumab (an anti-TNF- $\alpha$  antibody) are currently marketed for treatment of Crohn's

disease-associated inflammation by inhibiting M1 macrophage recruitment to and related-responses within the gut mucosa [133, 134].

M2 or alternatively activated macrophages sit at the opposite end of the spectrum from M1 macrophages. Activation and accumulation of M2 macrophages are correlated with tissue repair and remodeling as well as the resolution of inflammation, and they are found in the presence of fungal, helminth, bacterial, and some viral infections [124]. For example, lack of an early M2 response in respiratory syncytial virus-infected mice results in protracted proinflammatory activity by recruited immune cells within the lung and is correlated with increased alveolar pathology [135]. Gene expression associated M2 polarization, such as *Arg1*, *Fn1*, and *Egr2* are increased in the presence of IL-4, IL-10, IL-13, and M-CSF, and downregulated in response to LPS and IFN- $\gamma$  [126, 136]. M2 macrophages can be loosely phenotyped by their expression of arginase-1, CD206, and CD163, however in some instances these markers have been found on M1-like cells [113]. For example, tissues from patients with Crohn's disease, infectious mononucleosis, and Hodgkin's lymphoma, all conditions associated with a Th1-predominant phenotype, have an abundance of CD163-positive macrophages expressing signature M1-like genes [137, 138]. While not as efficient at presenting antigen as M1 cells, M2 macrophages excel at phagocytosis and have high expression of scavenger receptors that facilitate endocytic clearance of inflammatory cellular byproducts, bacterial endotoxins, and IgG-opsonized pathogens [113]. The scavenger receptor CD163, for instance, has a strong binding affinity for hemoglobin-haptoglobin complexes [139]. A byproduct of hemolysis, hemoglobin can induce inflammatory IL-8 and TNF- $\alpha$  responses from leukocytes [140, 141]. CD163-expressing macrophages actively patrol tissues and scavenge for hemoglobin to prevent hemoglobin-induced cytotoxicity [142]. M2 macrophages also mediate wound repair

and angiogenesis by production of growth factors such as VEGF-A, IGF-1, and HGF, and studies in Crohn's disease patients have shown inadequate HGF secretion is associated with reduced intestinal healing [143, 144]. Similarly, the production of TGF- $\beta$  by M2 macrophages also encourages tissue healing by promoting fibroblast growth, which has been found to decrease plaque rupture in atherosclerosis by increasing plaque stability [145]. Chronic production of growth factors can conversely fuel tumor cell expansion, and studies have shown that tumor microenvironments actively produce signals to recruit and polarize macrophages towards an M2 phenotype, making them an enticing target for anti-cancer therapeutics [146, 147]. In this document, I use the marker CD163 to define macrophages independent of M1/M2 because of its strict expression by cells of the monocyte/macrophage lineage.

#### **1.4.2 Dynamics of macrophages during HIV/SIV infection**

Infection with HIV in humans and SIV in rhesus macaques is associated with a marked increase in macrophage abundance in the lymph nodes, spleen, lung, heart, brain, small intestine, and colon, suggesting an important role for macrophages in HIV/SIV pathogenesis [89, 148-154]. Increased macrophage numbers in the lung, duodenum, spinal cord, and brain have been found to associate with pronounced monocyte activation, turnover, and trafficking, implicating monocytes as a potential source of newly recruited macrophages in HIV/SIV infection [89, 149, 155, 156]. Under homeostatic conditions, monocytes exist as three discreet subsets in blood, identified as either CD14<sup>+</sup>CD16<sup>-</sup> (classical), CD14<sup>+</sup>CD16<sup>+</sup> (intermediate), or CD14<sup>-</sup>CD16<sup>+</sup> (nonclassical), the former constituting roughly 80% of all monocytes [157]. Increasing CD16 expression on monocytes is reflective of monocyte maturation, and HIV infection is associated with a preferential expansion of CD16-high monocytes in circulation [158-161]. In ART-treated

macaques with acute SIV infection, macrophage abundance is significantly diminished compared to ART-naïve animals and coincides with decreased monocyte BrdU incorporation, a measure of recent cell division, suggesting a relationship between macrophage recruitment to tissues and plasma viral burden [101]. Furthermore, inhibition of monocyte trafficking correlates with reduced macrophage burden in heart and dorsal root ganglia, providing convincing evidence for monocytes as a mechanism of macrophage accumulation in HIV/SIV-infected tissues [155, 162].

## **1.5 PUTATIVE CONTRIBUTIONS OF MACROPHAGES TO HIV/SIV PATHOGENESIS**

### **1.5.1 Macrophages as targets of HIV/SIV infection**

One means by which macrophages might contribute to HIV/SIV pathogenesis is by serving as targets of viral infection. Macrophages are among the first cells infected in tissues after viral transmission, along with other myeloid cell types and Th17 cells, and it is posited the main mechanism by which they acquire virus is through phagocytosis of infected CD4<sup>+</sup> T cells [163-165]. Some studies have demonstrated that macrophages can also transmit virus and establish *de novo* infection of CD4<sup>+</sup> T cells through virological synapses and the secretion of virus-containing exosomes or trans-infection [166-170]. Although the number of virus infected macrophages is low relative to CD4<sup>+</sup> T cells, macrophages do represent an important longstanding source of replication competent virus due in part to inefficient suppression of viral replication by cytotoxic CD8<sup>+</sup> T cells [171-174]. Viral RNA and DNA have been detected in macrophages located within lymph nodes, lung, heart, liver, spleen, central nervous system,

brain, and regions of the gastrointestinal tract, and infection has been implicated in driving aberrant macrophage activity that may promote focal inflammation and immune dysfunction [175-181]. Monocytes also represent a pathway to infection as macrophage precursors that cross the blood-tissue barrier and differentiate into newly immigrated macrophages [182, 183]. CD16-expressing monocytes are preferentially infected by HIV due to their expression of CCR5 compared to other monocyte subsets and are found significantly diminished in frequency in ART-treated individuals and elite controllers [184-187]. It is currently unclear how ART-mediated suppression of virus effects the ratio of viral positive to viral negative macrophages in tissues. Despite ART-treatment, HIV DNA and p24 are detectable in macrophages from brain, duodenum, and lung from HIV-infected individuals, and therefore macrophages can function as persistent sources of new virion production in the presence or absence drugs [181, 188, 189].

### **1.5.2 Alterations in macrophage function during HIV/SIV infection**

In addition to being reservoirs for viral antigen, abnormalities in macrophage functional behavior are implicated in both directly and indirectly contributing to HIV/AIDS pathogenesis. To date, a preponderance of evidence supports a pathologic or detrimental role for macrophages in HIV/SIV-infected tissues. In SIV-infected lymph nodes, lung, and heart, increased macrophage numbers correspond with increased lymphoid fibrosis, pulmonary tissue destruction, and cardiac pathology, respectively [149, 190]. In addition, macrophage and monocyte activation as measured by plasma soluble CD163 (sCD163) levels is strongly correlated with measures of systemic T cell activation, plasma viral burden, neurocognitive impairment, and atherosclerotic plaque buildup [191-193]. The level of monocyte turnover from the bone marrow is significantly correlated with plasma sCD163 levels and has been shown to be a strong predictor

of progression to AIDS [194, 195]. Moreover, recent studies demonstrate that antibody-mediated suppression of monocyte migration to heart and brain decreases focal inflammation and tissue pathology and is associated with reduced tissue macrophage density [155, 162]. In HIV-infected colon, macrophage recruitment is positively correlated with the level of LPS in plasma, associated with *in situ* expression of TNF- $\alpha$  and IL-1 $\beta$ , and they display increased responsiveness to *ex vivo* LPS stimulation compared to uninfected controls [196].

Alternatively, evidence also exists that suggests macrophage dysfunction might indirectly contribute to disease pathogenesis due to an inadequate or weakened immune response during infection. Reported macrophage and monocyte phagocytic defects coincide with the emergence of several opportunistic pathogens in HIV/AIDS [197-199]. During the chronic stages of infection, in SIV-infected colon and HIV-infected duodenum, macrophage phagocytic capacity is significantly impaired and associated with increased *E.coli* products in plasma and tissues [89, 90, 200]. Moreover, some *in vitro* and *in vivo* assessments reveal widespread deficiencies in macrophages' ability to respond to HIV antigens and in HIV/SIV infection [101, 201, 202]. For example, while *in situ* stained macrophages in acute SIV-infected lymph nodes co-express TNF- $\alpha$  and IFN- $\alpha$ , lymph node macrophages stimulated *ex vivo* with SIV *env* nucleotides produce significantly less TNF- $\alpha$  and IFN- $\alpha$  compared to in health [101, 102]. In lung, alveolar macrophages ability to convey an effective cytokine response towards bacterial pneumonia is impaired in the context of HIV infection [203]. Furthermore, impaired macrophage cytokine responses are also implicated in contributing to inadequate CD4<sup>+</sup> T cell proliferation and IFN- $\gamma$  production in SIV-infected lymph nodes [100].

Lastly, some findings are consistent with a beneficial or nonpathologic role for macrophages in HIV/SIV infection. *In vitro* HIV infection of monocyte-derived macrophages

does not induce expression of Nf- $\kappa$ B, IRF3, and other inflammatory-related transcription factors, suggesting inflammatory M1-type macrophages do not arise from virus exposure [204]. Additionally, some studies report enhanced production of anti-inflammatory IL-10 from macrophages after exposure to HIV proteins gp120 and Tat, which in turn might inhibit inflammatory IL-12 [205-207]. CD163 is upregulated by microglia in encephalitic brain during chronic SIV infection and found co-localizing with haptoglobin, an indicator of a breach in the blood-brain barrier, suggesting macrophage-mediated clearance and degradation [208]. On the other hand, increased macrophage density in the brain may be a double-edge sword, complicating interpretations of macrophage mobilization to brain and perhaps other tissues. Several studies have shown an aberrant monocyte/macrophage response may underlie HIV-associated dementia, encephalitis, and neurocognitive degeneration, with the level of monocyte/macrophage accumulation associated with increased macrophage-derived proinflammatory mediators and increased disease severity [156, 194, 209, 210].

Despite an abundance of literature regarding phenotypic and functional alterations in macrophages associated with progressive HIV/SIV infection, to date no studies have addressed the macrophage response in the context of stable or controlled infection. Furthermore, whether infection-mediated changes in macrophages within peripheral tissues parallel changes in mucosal sites is currently unclear, as few studies have addressed the anatomical relationship of the macrophage response in the context of divergent disease outcomes. This represents a major barrier to progress in the field and complicates our understanding of how macrophages contribute to HIV/AIDS pathogenesis.

## 2.0 HYPOTHESIS AND SPECIFIC AIMS

Macrophages accumulate in tissues including the principal viral reservoirs lymph nodes and gut mucosa during HIV and pathogenic SIV infections, and measures of macrophage activation are strongly correlated with progression to AIDS. Nevertheless, much of what we know about macrophage involvement in HIV/SIV infection is based on conclusions drawn from findings in a single tissue compartment and in the context of a single disease state. Whether these findings suggest that differences in the macrophage response to infection are desirable or detrimental is currently unclear and is a major impediment to our understanding of HIV/AIDS pathogenesis. In the present study, I will investigate macrophages in lymph nodes and gut mucosa across different stages of SIV infection and prospectively in animals that develop diverging disease courses. This multifaceted approach will allow for the analysis of anatomical differences in macrophage function as it relates to infection stage and disease status, as well as the analysis of events before and after SIV control is achieved and how those events differ in animals predicted to progress to AIDS. I **hypothesize** that macrophages promote a protective response during acute SIV infection that becomes inadequate and/or detrimental to the host in chronic infection and associates with disease progression. To address this hypothesis, I propose three specific aims:

**Aim 1: Assess how different stages of SIV infection and AIDS impact macrophage frequency and turnover in lymph nodes and small intestine.** Using a cross-sectional

approach, I will evaluate changes in macrophage kinetics with archived samples of lymph node and gut mucosa taken from uninfected macaques and SIV-infected macaques with acute and chronic SIV infection and AIDS. Cross-sectional comparisons between animals with chronic infection that lack disease and tissues taken from animals with AIDS will allow for a preliminary observation of differences in macrophages associated with disease status.

**Aim 2: Determine whether prospective changes in gut and gut-draining lymph node macrophages differ in SIV-infected rhesus macaques that go on to develop progressive or controlled disease.** To expand on the cross-sectional study, I will design a longitudinal model of diverging disease courses based on viral set-point using differential expression of SIV controlling allele *Mamu-B\*008*. Here, small intestine and gut-draining mesenteric lymph nodes will be harvested prior to infection and then at weeks 2, 12, and 20 post infection for temporal assessment of changes in macrophage numbers, activation, death, and function with infection. Longitudinal assessment of macrophages in macaques that go on to develop controlled and progressive SIV infection will allow for determination of macrophage involvement in disease pathogenesis as well as whether changes in macrophages are either causally related to or are a consequence of disease outcome.

**Aim 3: Determine whether the macrophage response in peripheral lymph nodes differs significantly from macrophages in mucosal tissues in controlled and progressive SIV infection.** To provide clarity on whether there are anatomical differences in macrophages role in HIV/AIDS pathogenesis, I will also investigate the macrophage response to infection in peripheral lymph nodes as it relates to changes in mucosal tissues using the model described in

Aim 2. If we are to effectively intervene or augment macrophage activity for therapeutic purposes, it is imperative to understand whether changes in macrophages in response to SIV infection are uniform or differ across tissues.

**3.0 CHAPTER ONE: MACROPHAGE ACCUMULATION IN GUT MUCOSA  
DIFFERENTIATES AIDS FROM CHRONIC SIV INFECTION IN RHESUS  
MACAQUES**

**3.1 PREFACE**

This chapter is adapted from a published study (Zachary D. Swan<sup>1,3</sup>, Elizabeth R. Wonderlich<sup>1,3</sup>, and Simon M. Barratt-Boyes<sup>1,2,3</sup>. 2016. *European Journal of Immunology* 46(2):446-54).

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Work described in this chapter is in fulfillment of specific aim 1.

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### 3.2 ABSTRACT

The relationship between recruitment of mononuclear phagocytes to lymphoid and gut tissues and disease in HIV and SIV infection remains unclear. To address this question, we conducted cross-sectional analyses of dendritic cell (DC) subsets and CD163+ macrophages in lymph nodes (LNs) and ileum of rhesus macaques with acute and chronic SIV infection and AIDS. In LNs significant differences were only evident when comparing uninfected and AIDS groups, with loss of myeloid DCs and CD103+ DCs from peripheral and mesenteric LNs. In contrast, there were fourfold more macrophages in ileum lamina propria in macaques with AIDS compared with chronic infection, and this increased to 40-fold in Peyer's patches. Gut macrophages exceeded plasmacytoid DCs and CD103+ DCs by ten- to 17-fold in monkeys with AIDS but were at similar low frequencies as DCs in chronic infection. Gut macrophages in macaques with AIDS expressed IFN- $\alpha$  and TNF- $\alpha$  consistent with cell activation. CD163+ macrophages also accumulated in gut mucosa in acute infection but lacked expression of IFN- $\alpha$  and TNF- $\alpha$ . These data reveal a relationship between inflammatory macrophage accumulation in gut mucosa and disease and suggest a role for macrophages in AIDS pathogenesis.

### 3.3 INTRODUCTION

Dendritic cells (DCs) and macrophages are mononuclear phagocytes that bridge innate and adaptive immunities while also contributing to pathogen control and inflammation. In HIV infection of humans and SIV infection of Asian macaques, a loss of the two major DC subsets, myeloid DCs (mDCs) and plasmacytoid DCs (pDCs), from blood is negatively correlated with viral load and disease outcome [211-219]. This loss is driven by apoptosis and recruitment of DCs to lymph nodes (LNs) and gut mucosa, coincident with altered function and production of inflammatory cytokines [83, 84, 88, 101, 103, 219-223]. Chronic SIV infection of rhesus macaques is also associated with loss from intestinal mucosa of CD103<sup>+</sup> DCs [92], an important DC subset that traffics from gut to mesenteric LNs and imprints gut homing on lymphocytes [224-226]. Loss of CD103<sup>+</sup> DCs is thought to contribute to the loss of IL-17-producing lymphocytes from gut and damage to gastrointestinal mucosal integrity [92], factors that lead to microbial translocation [91, 227]. Collectively, these findings have led to a focus on DCs as potential contributors of immune activation that is a hallmark of AIDS [228-230].

Much less is understood about the mobilization and trafficking of macrophages in HIV and SIV infection and the potential contribution of macrophages to disease control or progression [231, 232]. Macrophages accumulate in inguinal and axillary LNs during acute SIV infection of rhesus macaques [101, 154], and increased monocyte turnover is correlated with progression to AIDS in this model [194, 195]. Macrophages are most abundant in health in intestinal tissues [233], and further accumulation of macrophages in the intestine is seen in untreated HIV infection of humans and SIV infection of rhesus macaques [89, 153]. Evidence also exists for intestinal production of macrophage-associated proinflammatory molecules and altered macrophage phagocytic function and responsiveness to LPS in HIV and SIV infection

[89, 153]. However, whether alteration in macrophage dynamics in SIV or HIV infection is beneficial or detrimental remains an open question, as there have been no studies to our knowledge evaluating macrophage dynamics across different stages of infection and disease.

To address these gaps in our understanding, in this study we determined the frequency and turnover of macrophages and DC subsets in LNs and gut mucosa of rhesus macaques with acute and chronic SIV infection and AIDS. Our data reveal relatively modest differences between cell types in LNs only in macaques with AIDS when compared with uninfected animals. In contrast, up to 40-fold more macrophages were present in intestinal mucosa in macaques with acute SIV infection and AIDS relative to macaques with chronic SIV infection lacking disease. Gut macrophages from animals with AIDS but not acute infection have evidence of an inflammatory function, consistent with a role in disease progression.

## **3.4 RESULTS**

### **3.4.1 mDC loss and marked increases in cell turnover in peripheral LNs in macaques with AIDS**

To evaluate the impact of pathogenic SIV infection on macrophages and DC subsets in lymphoid and mucosal tissues, we conducted cross-sectional analyses using specimens from rhesus macaques infected with the biologic isolate SIVmac251 as part of previous studies [83, 102, 219]. Tissues were harvested either at the acute stage of infection (week 2, n = 6), at the chronic stage of infection but without disease (range week 66 to 78, median week 71, n = 6), or from animals with rapid progression to AIDS (range week 11 to 43, median week 33, n = 8; Table 1).

**Table 1. Animal characteristics.**

Table 1. Animal characteristics

Animal	Time post infection (weeks)	Plasma virus load (RNA copies/mL)	Blood CD4 T cells (cells/ $\mu$ L)	Status
M182-06	2	2,340,000	272	Acute
M183-06	2	8,120,000	773	Acute
M184-06	2	4,470,000	584	Acute
M185-06	2	9,410,000	730	Acute
M186-06	2	104,000	600	Acute
M187-06	2	3,580,000	645	Acute
R479	66	81,200 <sup>a)</sup>	360	Chronic
R486	67	490 <sup>a)</sup>	924	Chronic
M135-08	67	2108	144 <sup>b)</sup>	Chronic
M138-08	75	51,928	66 <sup>c)</sup>	Chronic
M9010	75	425	294 <sup>c)</sup>	Chronic
M131-08	78	26,386	238 <sup>c)</sup>	Chronic
R189	11	44,000,000	823 <sup>d)</sup>	AIDS
R488	18	27,000,000	1249	AIDS
R482	27	1,210,000	1164	AIDS
R477	32	14,900,000	592	AIDS
R485	33	2,330,000	317	AIDS
M5206	36	8,730,000	384	AIDS
R180	43	4,460,000	255	AIDS
R183	43	151,000	361	AIDS

a) Week 58.

b) Week 62.

c) Week 70.

d) Week 8.

Samples from SIV-infected macaques were compared to healthy SIV-naïve macaques (n = 8). We first analyzed mononuclear phagocytes in cell suspensions of inguinal, axillary, or popliteal LNs, which we collectively termed peripheral LNs. To delineate mononuclear phagocytes we used established flow cytometry methods [101] gating on CD3/CD20(lineage)-MHC-II+CD163-CD11c+CD123- cells for mDCs, lineage-MHC-II+CD163-CD11c-CD123+ cells for pDCs, and lineage-MHC-II+CD163+ cells for macrophages (Fig. 1A). To determine changes in individual subsets independently, the frequency of DCs and macrophages were calculated as a proportion of all live cells, which was based on lack of fluorescence after exposure to an amine-reactive dye

(Fig. 1A). Preliminary findings using peripheral LN samples from macaques with acute (week 2,  $n = 6$ ) and chronic (week 20,  $n = 11$ ) SIVmac251 infection showed that mDC, pDC, and macrophage frequencies determined in this manner are highly correlated to frequencies based on tissue weight ( $R = 0.8589$ ,  $p < 0.0001$ ; data not shown), indicating that changes in T and B lymphocytes over time do not erroneously affect DC and macrophage frequencies when based on total number of live cells. In peripheral LNs only the frequency of mDCs differed significantly across conditions, with loss of mDCs in monkeys with AIDS relative to uninfected macaques. There was a trend toward loss of pDCs in chronic infection and an increase in macrophages in acute infection (Fig. 1B). SIV infection was associated with marked differences in the proportion of each subset expressing Ki67, a marker of recent cell division [83]. The proportion of Ki67+ mDCs in peripheral LNs of monkeys with AIDS exceeded all other groups, and Ki67+ pDCs in monkeys with AIDS and acute infection were greater than in uninfected macaques. The frequency of Ki67+ macrophages in peripheral LNs in chronic infection and AIDS was greater than that in uninfected and acutely infected macaques (Fig. 1C). When comparing the frequency of each cell type at different stages of infection or disease, the proportion of macrophages exceeded pDCs only in acute infection (Fig. 1D). Flow cytometry findings were supported by immunohistochemistry analysis showing evidence of a loss of mDCs and pDCs in chronically infected relative to uninfected tissues, and a relative abundance of macrophages in acute infection (Fig. 1E).

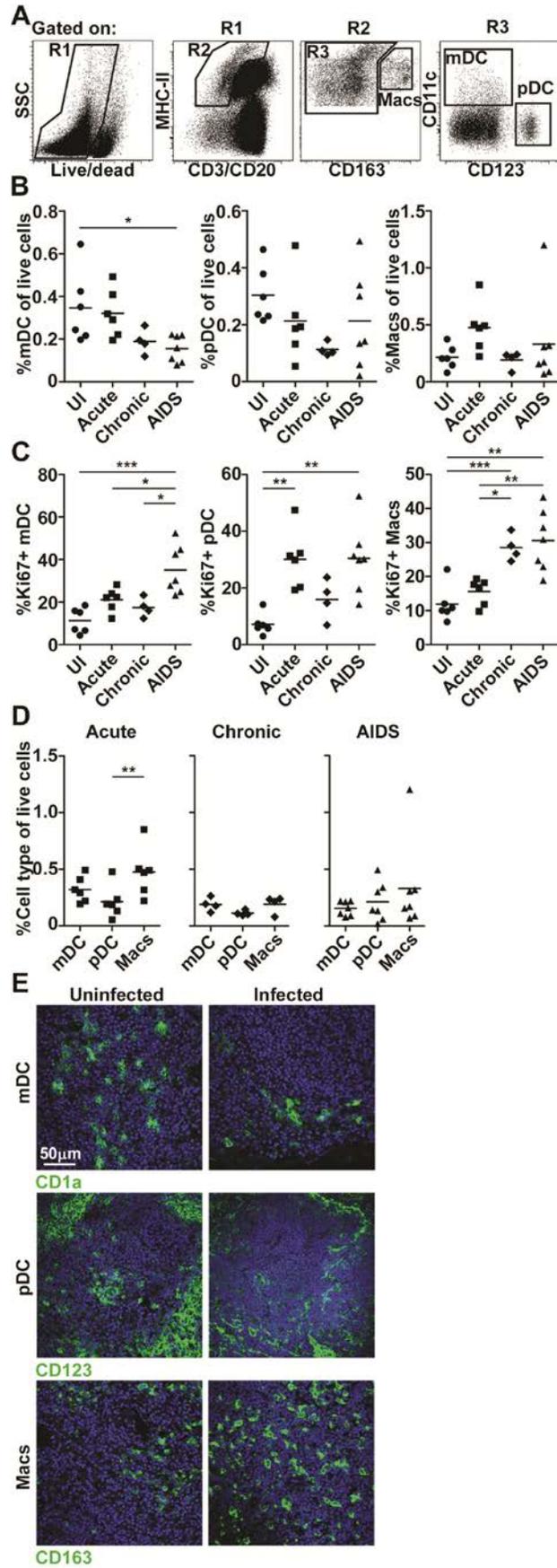


Figure 1. mDCs are lost from peripheral LNs in macaques with AIDS.

(A) Representative flow cytometric analysis of peripheral LN single-cell suspensions showing gating strategy to define macrophages (Macs), mDCs, and pDCs after exclusion of dead cells by amine reactive dye labeling. (B, C) The proportion of each mononuclear phagocyte population in the live cell fraction (B) and the proportion of each population expressing Ki67 (C) in peripheral LNs from uninfected (UI) macaques and macaques with acute and chronic SIV infection and AIDS. (D) The proportion of cells in the live cell fraction of peripheral LNs that is mDCs, pDCs, or macrophages in acute and chronic SIV infection or AIDS. (B-D) Each symbol represents an individual animal and horizontal bars represent means.  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ . Statistical comparisons were done using (B, C) ANOVA followed by Bonferroni's multiple-comparison test or (D) repeated-measures ANOVA followed by Tukey's range test. (A-D) Data shown represent 11 independent experiments. (E) Immunofluorescence of peripheral LN sections from uninfected (left) and SIV-infected (right) macaques labeled with Ab to CD1a, CD123, and CD163 to identify mDCs, pDCs, and macrophages, respectively. SIV-infected sections were taken at chronic infection for mDCs, and at acute infection for pDCs and macrophages. Blue staining in each section is nuclei labeled with Hoechst dye. Original magnification = 400x. Images shown are representative of five independent experiments.

### **3.4.2 pDCs and macrophages accumulate but CD103+ DCs are depleted in mesenteric**

#### **LNs in monkeys with AIDS**

We next investigated the influence of SIV infection on DC and macrophage dynamics in mesenteric LNs that drain the gut mucosa using similar strategies. In contrast to peripheral LNs, there was no change in the frequency of mDCs over the course of infection, whereas pDCs and macrophages both significantly increased in frequency in monkeys with AIDS relative to uninfected monkeys (Fig. 2A). The frequency of Ki67+ mDCs and pDCs increased significantly in acute infection and AIDS, whereas the frequency of Ki67+ macrophages increased in chronic infection and AIDS (Fig. 2B). We next evaluated CD103+ DCs, which were identified as a subset of CD11c+ mDCs. CD103+ DCs constituted about 30% of mDCs prior to infection in mesenteric LNs but were absent from peripheral LNs (Fig. 2C and data not shown). CD103+ DCs were lost from mesenteric LNs and the frequency of Ki67+ CD103+ DCs significantly increased in AIDS relative to uninfected animals (Fig. 2C). The frequency of macrophages exceeded mDCs (with the CD103+ subset included) in acute infection and AIDS, and exceeded

pDCs in acute infection, but similar frequencies of each cell type were present in mesenteric LNs in chronic infection (Fig. 2D). To support the flow cytometry findings, we performed immunohistochemistry of mesenteric LNs focusing on macrophages and CD103+ DCs, which were distinguished from T cells by lack of CD3 staining. As expected, *in situ* staining revealed an increase in macrophages but a marked loss of CD103+ DCs as infection progressed (Fig. 2E).

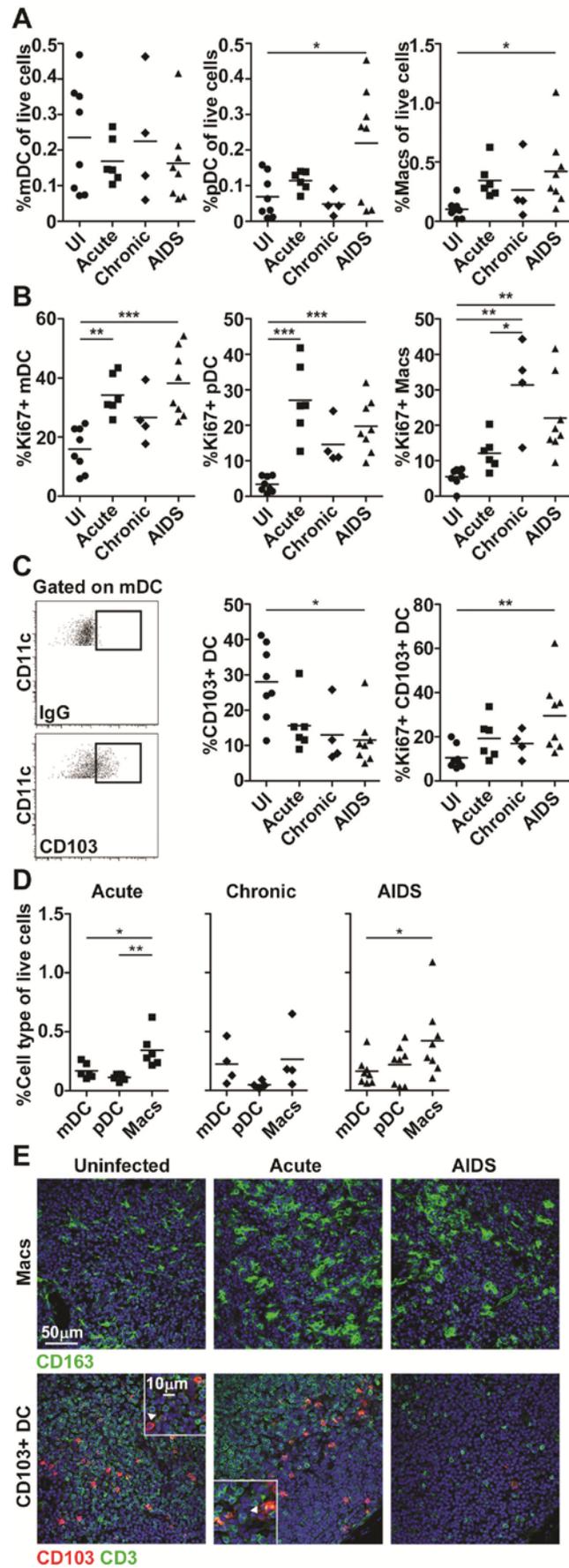


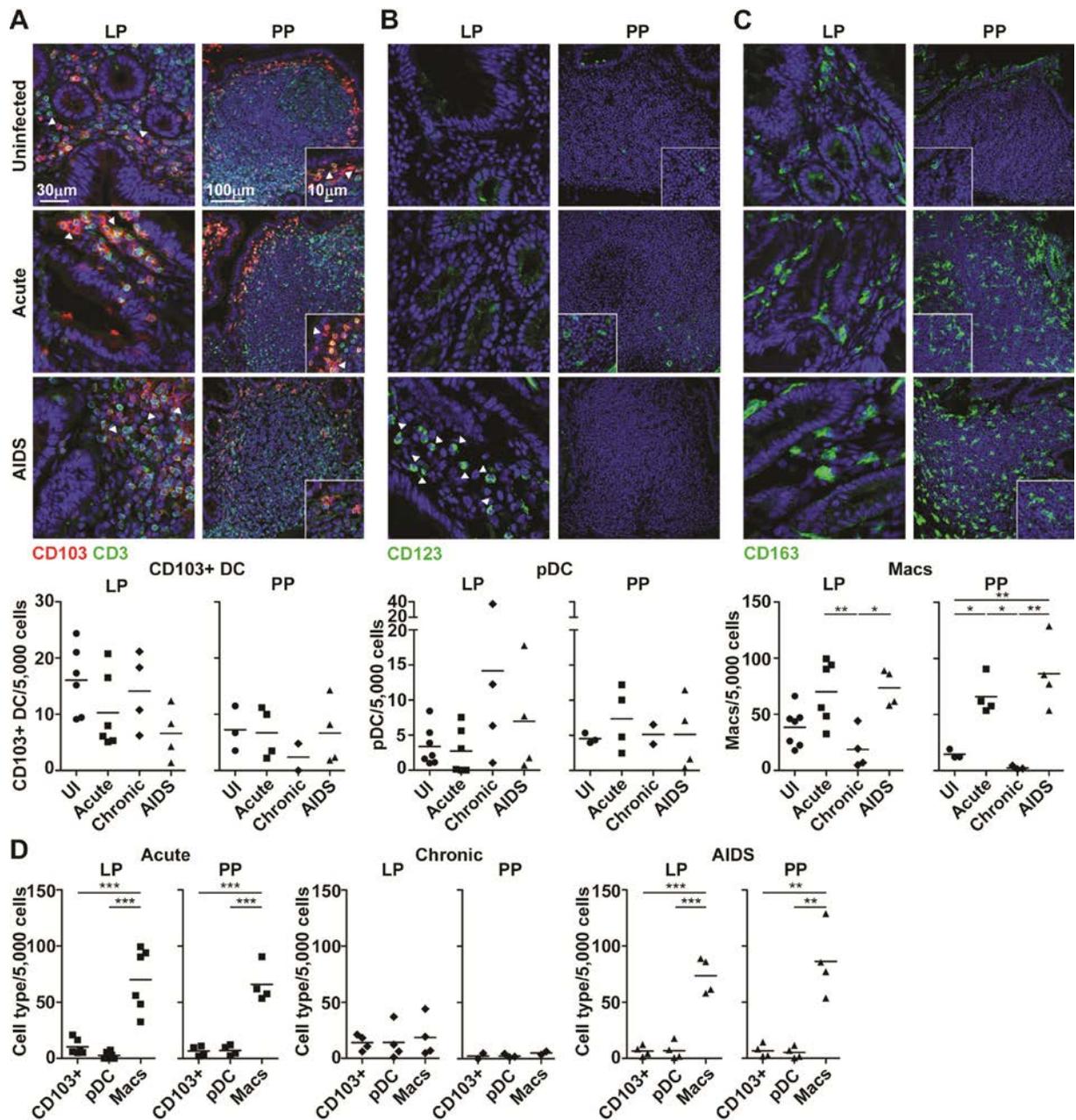
Figure 2. Loss of CD103+ DCs but accumulation of pDCs and macrophages in mesenteric LNs in macaques with AIDS.

(A, B) The proportion of each mononuclear phagocyte in the live cell fraction (A) and the proportion of each population expressing Ki67 (B) in mesenteric LNs from uninfected (UI) macaques and macaques with acute and chronic SIV infection and AIDS. (C) Left: representative flow cytometric analysis of mesenteric LN single-cell suspensions showing gating to define the CD103+ fraction of mDC based on isotype control Ab (IgG) labeling. Right: the proportion of mDCs that is CD103+ and the proportion of these cells that express Ki67 in mesenteric LNs from uninfected (UI) macaques and macaques with acute and chronic SIV infection or AIDS. (D) The proportion of cells in the live cell fraction of mesenteric LNs that is mDCs, pDCs, or macrophages (Macs) in acute and chronic SIV infection or AIDS. (A-D) Each symbol represents an individual animal and horizontal bars represent means. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . Statistical comparisons were done using (A-C) ANOVA followed by Bonferroni's multiple-comparison test or (D) repeated-measures ANOVA followed by Tukey's range test. Data shown represent 14 independent experiments. (E) Immunofluorescence of mesenteric LN sections from uninfected macaques and macaques with acute SIV infection or AIDS labeled with Ab to CD163 to identify macrophages and CD3 and CD103 to identify CD103+CD3- DC (highlighted by arrowheads). Blue staining in each section is nuclei labeled with Hoechst dye. Original magnification = 400x. Images shown are representative of five independent experiments.

### **3.4.3 Macrophages accumulate in gut mucosa in acute SIV infection and AIDS but not chronic infection**

Next we used immunofluorescence and confocal microscopy to detect mononuclear phagocytes in the ileum in a subset of macaques for which tissues were available. We chose to image and quantify cells *in situ* to provide information on the local distribution within Peyer's patches (an immune inductive site) and lamina propria (an immune effector site) and to avoid prolonged tissue processing associated with the generation of cell suspensions that may lead to unintended cell loss. For mDCs, we focused on the CD103+ subset given the profound loss from mesenteric LNs. CD103+ DCs in naïve macaques were primarily found in the subepithelial dome and T-cell-rich interfollicular zones of Peyer's patches and scattered throughout the lamina propria, and there was a trend toward loss of CD103+ DCs from lamina propria in monkeys with AIDS (Fig. 3A). CD123+ pDCs were infrequent in both lamina propria and Peyer's patches of uninfected ileum and there was a nonsignificant trend toward accumulation of pDCs in lamina propria in

chronic infection and AIDS associated with considerable animal-to-animal variation (Fig. 3B). CD163<sup>+</sup> macrophages were present at high frequencies relative to CD103<sup>+</sup> DCs and pDCs prior to infection, most notably in lamina propria (Fig. 3C). In marked contrast to DCs, the frequency of macrophages in gut mucosa differed significantly as a function of stage of infection and disease status. Monkeys with acute infection and AIDS had significantly greater frequencies of macrophages in both lamina propria and Peyer's patches than did monkeys with chronic infection lacking disease (Fig. 3C). The frequency of macrophages in these compartments was 10 to 20 times higher than CD103<sup>+</sup> DCs and pDCs in acute infection and AIDS but did not differ from the DC subsets in chronically infected tissues (Fig. 3D). The frequency of macrophages in gut mucosa did not correlate with peripheral CD4<sup>+</sup> T-cell counts or plasma virus load (data not shown).



**Figure 3. Accumulation of macrophages in gut mucosa in macaques with acute SIV infection**

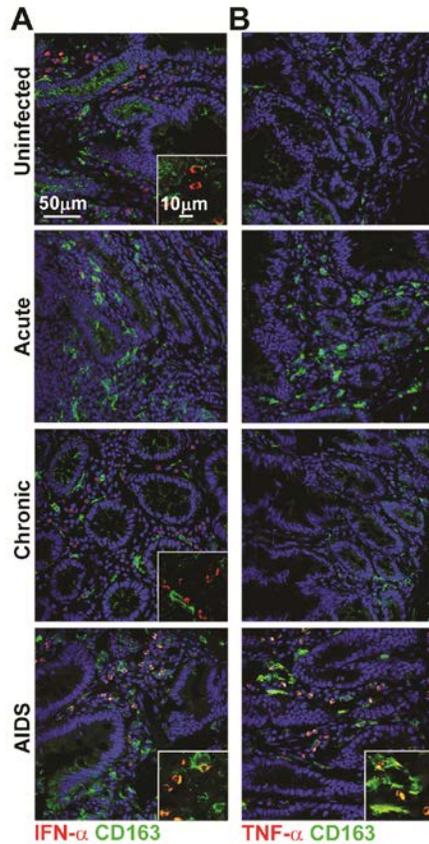
**AIDS but not chronic infection.**

(A-C) Top: immunofluorescence of ileum sections from uninfected macaques and macaques with acute SIV infection and AIDS stained with antibodies to CD103 and CD3 to identify CD103+CD3- DC (A), CD123 to identify pDC (B), and CD163 to identify macrophages (Macs, C) in lamina propria (LP) and Peyer's patches (PP). Arrowheads highlight cells of interest. Blue staining in each section is nuclei labeled with Hoechst dye. Original magnification = 200x. Images shown are representative of 12 independent experiments. (Bottom) The frequency of each cell type per 500 nucleated cells enumerated using ImageJ and MetaMorph software. (D) The proportion of cells in lamina propria and Peyer's patches that is CD103+CD3- DCs, pDCs, or macrophages in acute and chronic SIV infection or AIDS. (A-D) Each symbol represents an individual animal and horizontal bars represent means. The number of animals differs between lamina propria and Peyer's patches as in some cases Peyer's patches were

not identified.  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ . Statistical comparisons were done using (A-C) ANOVA followed by Bonferroni's multiple-comparison test or (D) repeated-measures ANOVA followed by Tukey's range test.

#### **3.4.4 Evidence for inflammatory macrophages in gut mucosa of monkeys with AIDS**

To address whether the macrophages that accumulate in gut mucosa in acute infection and AIDS may contribute to the inflammatory response, we performed *in situ* staining for CD163 along with proinflammatory cytokines IFN- $\alpha$  and TNF- $\alpha$ . In both uninfected and chronically infected tissues small numbers of cells expressing IFN- $\alpha$  but not TNF- $\alpha$  were detected, but in both cases cytokine staining did not localize with macrophages. Negligible staining for either cytokine was detected in acutely infected tissues, despite macrophage accumulation (Fig. 4). In contrast, a substantial number of macrophages present in gut mucosa of macaques with AIDS expressed IFN- $\alpha$  and TNF- $\alpha$ , consistent with an inflammatory function, and expression of these cytokines appeared to be restricted to macrophages.



**Figure 4. Gut macrophages from macaques with AIDS but not acute infection express IFN- $\alpha$  and TNF- $\alpha$ .**

(A and B) Representative immunofluorescence of ileum sections from uninfected macaques and macaques with acute and chronic SIV infection and AIDS labeled with Ab to CD163 to identify macrophages and Ab to IFN- $\alpha$  (A) and TNF- $\alpha$  (B). Blue staining in each section is nuclei labeled with Hoechst dye. Original magnification = 400x. Images shown are representative of 12 independent experiments.

### 3.5 DISCUSSION

This is the first report to our knowledge to provide a detailed comparative analysis of mononuclear phagocyte subsets in lymphoid and mucosal tissues across different stages of SIV infection and disease. Significant differences existed between DCs and macrophages in peripheral and mesenteric LNs when comparing uninfected macaques and macaques with AIDS, consistent with earlier reports [103, 234]. However, only the frequency of macrophages in gut

mucosa distinguished macaques with AIDS from those with chronic SIV infection lacking disease. Even with the relatively small number of animals studied this effect was striking, with a fourfold difference in mean macrophage frequency in lamina propria and a 40-fold difference in mean macrophage frequency in Peyer's patches of macaques with AIDS compared with chronic infection. These data indicate that accumulation of macrophages in gut mucosa correlates with disease and suggest a role for macrophages in AIDS pathogenesis.

Macrophages are abundant in intestinal mucosa in health and are important in maintaining a noninflammatory environment and normal tissue homeostasis [116, 233]. In contrast, our findings indicate that CD163+ macrophages accumulating in the gut of macaques with AIDS express TNF- $\alpha$  and IFN- $\alpha$ , consistent with an inflammatory function, and data from untreated HIV-infected patients reveal secretion of a number of macrophage-related proinflammatory molecules in gut mucosa [89]. Similarly, in inflammatory bowel disease and ulcerative colitis, activated macrophages producing proinflammatory cytokines including TNF- $\alpha$  infiltrate the bowel and contribute to an inappropriate innate response [235-237]. Macrophage production of TNF- $\alpha$  may directly affect the integrity of gut epithelium by inducing epithelial cell apoptosis and disrupting intracellular tight junctions [132, 238], and gut macrophages in HIV infected-individuals have reduced capacity to phagocytose bacteria that may transit the damaged epithelium [89]. Collectively, these findings suggest a potential contribution of macrophages to gut inflammation and microbial translocation in AIDS.

It is notable that CD163+ macrophages also accumulated in gut mucosa in acutely infected macaques but lacked production of IFN- $\alpha$  and TNF- $\alpha$ , suggesting they may be functionally distinct from gut macrophages in AIDS. A key function of macrophages is antigen presentation, and CD163+ macrophages in SIV-infected LNs lose capacity to stimulate CD4+ T

cells [100]. It will be important to assess antigen-presenting function of gut macrophages at different stages of SIV infection, particularly as they accumulate heavily in Peyer's patches, an immune inductive site.

Previous studies have revealed loss of CD103+ DCs from both gut mucosa and mesenteric LNs in rhesus macaques infected with SIV for more than 90 days, although distinction was not made between animals with and without disease [92]. In our study with similar numbers of animals, we found significant depletion of CD103+ DCs from mesenteric LNs in macaques with AIDS but not chronic SIV infection relative to uninfected macaques, suggesting that CD103+ DC depletion is a function of disease. CD103+ DCs that migrate from gut mucosa to mesenteric LNs produce retinoic acid from dietary vitamin A that in turn drive development and maintenance of IL-17- and IL-12-producing cells and Treg [226, 239, 240]. IL-17 is associated with maintenance of epithelial integrity, and loss of Th17 cells in acute pathogenic SIV infection correlates with mucosal immune dysfunction and is predictive of systemic immune activation [241, 242]. There is also evidence that Treg are depleted from intestinal lamina propria but not blood or LNs in acute SIV infection [243]. The loss of CD103+ DCs from mesenteric LNs could, therefore, indirectly impact chronic immune activation and together with macrophage accumulation in gut mucosa could promote microbial translocation through Th17-mediated loss of epithelial integrity.

Accumulation of pDCs in colorectum and ileum of macaques with chronic SIV infection has been noted previously, with frequencies roughly fourfold greater than in uninfected samples, although there was no comparison done between animals with chronic infection and AIDS [88, 222]. In our study there was a trend toward increased frequency of pDCs in lamina propria of macaques with chronic infection relative to uninfected animals, but no detectable differences in

pDC frequency when comparing chronic infection and AIDS. Moreover, macrophages were tenfold more numerous in lamina propria and 17-fold more numerous in Peyer's patches in AIDS monkeys compared with pDCs, whereas the frequency of both pDCs and macrophages was low and indistinguishable in chronic infection, reflecting a far greater accumulation of gut macrophages than pDCs in disease.

Both DCs and macrophages appear to have dysregulation of pro- and antiapoptotic molecules that favors apoptosis in SIV and HIV infection [244], and apoptotic DCs and macrophages can readily be identified in LNs of SIV-infected macaques [83, 103, 195, 220]. Despite relatively minor changes in frequency of mononuclear phagocytes in LNs over the course of infection our findings reveal an underlying strong regenerative response with marked increases in Ki67 expression, consistent with previous findings of BrdU incorporation *in vivo* [83, 101, 195]. These data suggest an increase in cell turnover within LNs, with increased death of cells being offset by increased recruitment of DCs and monocytes from blood.

Our study showed distinct production of IFN- $\alpha$  in gut mucosa in uninfected animals that may serve to protect the mucosa from viral invasion. The source of IFN- $\alpha$  remains to be determined, but constitutive expression of other type I IFN including IFN- $\epsilon$  by intestinal epithelial cells is known to occur in gut mucosa of SIV-naïve macaques [245]. Our findings provide further support for the notion that pDCs are not the exclusive producers of IFN- $\alpha$  in tissues during SIV and HIV infection, and that other cells including macrophages, lymphocytes, and mDCs can contribute significantly to this response [102, 246]. The data suggest that focus should be directed toward macrophages and their potential contribution to the inflammatory response associated with chronic immune activation during AIDS, particularly in the gut mucosal environment.

## **3.6 MATERIALS AND METHODS**

### **3.6.1 Animals and tissues**

Indian-origin rhesus macaques housed at the University of Pittsburgh (U.S. Public Health Service Assurance Number: A3187-01) were used in this study and all animal manipulations and procedures were done with appropriate institutional regulatory oversight and approval. Macaques were infected with SIVmac251 as part of previously published studies [83, 102, 219]. Tissues were harvested either at the acute stage (week 2, n = 6) or chronic stage of infection (range week 66 to 78, median week 71, n = 6), or during AIDS (range week 11 to 43, median week 33, n = 8; Table 1). Samples from eight uninfected, healthy macaques were used as controls. Not all animals were used in all analyses depending on availability of tissues. To generate LN suspensions, freshly harvested LNs were digested using 1 mg/mL collagenase D (Sigma-Aldrich) and 20 µg/mL DNase I (Roche) in RPMI 1640 medium with 2% FBS and 10 mmol HEPES and cryopreserved for later use. To generate tissue sections for immunohistochemistry, harvested tissues were fixed in 2% paraformaldehyde for 2 h at 4°C and then infused with 30% sucrose overnight. Tissues were frozen with an aerosol of chlorodifluoromethane (Histofreeze 22, Fischer Scientific).

### **3.6.2 Flow cytometry**

LN single-cell suspensions were stained with the following cross-reactive surface-labeling anti-human antibodies for mononuclear phagocyte detection (all antibodies were purchased from BD Biosciences unless otherwise noted): CD11c (clone S-HCL-3), CD123 (7G3), CD103 (2G5,

Beckman Coulter), CD163 (GH1/61, BioLegend), HLA-DR (L243), CD20 (2H7, eBioscience), and CD3 (SP34-2). Nonviable cells were excluded by staining with a fluorescent LIVE/DEAD cell stain (Invitrogen). For analysis of nuclear protein Ki67 (B56), LN cells were permeabilized with Cytfix/Cytoperm (BD Biosciences) and then stained with Ab in Perm/Wash buffer (BD Biosciences). Data were acquired using an LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo 7.6.4.

### **3.6.3 Immunofluorescence microscopy**

LN and ileum sections were hydrated in PBS on Superfrost Plus glass slides and then blocked for 1 h with 10% serum matching the species of the secondary Ab. Endogenous peroxidase was quenched using 0.15% hydrogen peroxide. Sections were incubated for 1.5 h with antibodies to CD163 (GH1/61, eBioscience), CD123 (7G3), CD1a (SK9, BioLegend), CD3 (polyclonal, DAKO), and/or CD103 (2G5.1, AbD Serotec). The remaining steps were conducted in accordance with the standard tyramide signal amplification kit protocol (Invitrogen). To detect cytokine expression, sections were incubated with Ab to IFN- $\alpha$  (MMHA2, PBL Interferon Source) or TNF- $\alpha$  (Mab11) overnight and developed using goat-anti-mouse-HRP AlexaFluor 546 with tyramide amplification. Sections were costained using biotinylated mouse Ab to CD163 followed by mouse HRP-streptavidin AlexaFluor 488. All staining included an appropriately concentrated isotype-matched Ab. Cell nuclei were identified using membrane permeable Hoescht dye. Slides were mounted in gelvatol mounting medium and viewed on an Olympus Fluoview 1000 confocal microscope. For quantification of cells in gut mucosa, an average of seven nonoverlapping regions of lamina propria and at least three entire Peyer's patches were imaged. Cells of interest were enumerated manually using ImageJ software (U.S.

National Institutes of Health) and the total number of nuclei per image was determined digitally using MetaMorph Software (Molecular Devices). Cell frequencies were then calculated using the formula: (total number of cells of interest x 5000) / total number of nuclei.

#### **3.6.4 Statistical analysis**

Cross-sectional comparisons between uninfected and infected groups were performed using a one-way ANOVA followed by Bonferroni's multiple-comparison test. Comparisons made between cell types within the same animal were done using repeated-measures ANOVA followed by a Tukey's range test. All statistical tests were performed using GraphPad Prism version 5. A *p*-value < 0.05 was considered significant.

**4.0 CHAPTER TWO: GUT MACROPHAGE ACCUMULATION AND  
PHAGOCYTTIC DYSFUNCTION IS PREDICTIVE OF PROGRESSIVE DISEASE IN  
SIV-INFECTED RHESUS MACAQUES**

**4.1 PREFACE**

This chapter is adapted from a submitted manuscript (Zachary D. Swan<sup>1,3</sup>, Anthea L. Bower<sup>1,3</sup>, Elizabeth R. Wonderlich<sup>1,3</sup>, and Simon M. Barratt-Boyes<sup>1,2,3</sup>). Work described in this chapter is in fulfillment of specific aim 2.

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## 4.2 ABSTRACT

The gastrointestinal tract is a major site of inflammation and macrophage accumulation in HIV-infected humans and SIV-infected rhesus macaques, however macrophage contribution to disease progression remains unclear. To address this issue, we prospectively studied macrophages in gut and gut-draining mesenteric lymph nodes (LNs) from SIV-infected macaques with dichotomous expression of controlling MHC class I alleles that were predicted to be SIV controllers or progressors based on diverging set-point virus loads. Infection induced a massive accumulation of CD163<sup>+</sup> macrophages into gut mucosa in the acute phase which persisted into chronic infection in SIV progressors but was resolved in controllers. Sustained gut macrophage accumulation was associated with the level of CD163 expression on  $\alpha 4\beta 7$ +CD16<sup>+</sup> blood monocytes and was correlated with the extent of epithelial damage. Macrophages isolated from intestine of SIV progressors in chronic infection had reduced phagocytic function relative to SIV controllers and uninfected macaques, and the proportion of phagocytic macrophages in gut negatively correlated with gut macrophage density, epithelial breach, and plasma but not tissue virus burden. Macrophages in intestine produced low levels of cytokines regardless of disease course, while mesenteric LN macrophages from SIV progressors became increasingly responsive to stimulation as infection advanced. These data indicate that CD163<sup>+</sup> macrophages accumulate in gut mucosa early in progressive SIV infection in response to sustained intestinal damage but fail to adequately phagocytose debris, potentially establishing a positive feedback loop promoting recruitment. The non-inflammatory function of gut macrophages may serve to limit inflammation early in infection.

### 4.3 INTRODUCTION

Macrophages are mononuclear phagocytes that mediate innate homeostatic and inflammatory functions in tissues throughout the body. Macrophages are most abundant in the gastrointestinal tract, where they maintain a non-inflammatory environment, expressing low levels of innate response receptors and primarily scavenging debris and promoting wound repair [115, 116, 144, 247]. Under inflammatory conditions, such as with Crohn's disease and ulcerative colitis, colonic tissues have an increased frequency of activated proinflammatory macrophages that are hyper-responsive to bacterial stimuli and correlate with increased turnover of CD14-high inflammatory monocytes [132, 248-251]. These findings have implications for macrophage recruitment and accumulation as a central mechanism in the pathogenesis of inflammatory bowel disorders [252]. Macrophages also infiltrate the intestine in HIV-infected humans and SIV-infected macaques [89, 150, 196]. However, whether macrophage accumulation in intestine reflects a beneficial or detrimental role in HIV/SIV pathogenesis remains unknown.

Comparative studies between natural SIV hosts that have a nonpathogenic disease course, such as the sooty mangabey and African green monkey, and macaques that generally progress to AIDS have improved our understanding of intestinal biology in HIV/SIV pathogenesis [71], but interpretation of findings are complicated by inherent differences between the species. Similarly, comparative studies in rhesus macaques infected with pathogenic and attenuated strains of SIV are highly informative but are limited by differences in virus replication capacity and persistence [253]. In HIV infection, viral set-point is used as a predictive measure of disease prognosis and set-point plasma virus loads above  $10^4$  RNA copies/mL are statistically correlated with an increase in AIDS-related morbidity [34, 35]. The rhesus macaque MHC class I allele *Mamu-B\*008* is correlated with long-term control of SIV replication, generating CD8+ T cell responses

against comparable peptides as the human elite controlling allele *HLA-B\*27* [26, 49, 254]. Macaque controlling alleles *Mamu-A\*001* and *Mamu-B\*017* are also associated with a low set-point viral load, whereas rhesus macaques that lack expression of all 3 controlling alleles have high set-point virus loads and progress to disease in a predictable fashion [38, 255, 256]. By using SIV-infected rhesus macaques with or without expression of protective MHC class I alleles, immune responses in animals predicted to control or progress to disease based on viral set-point can be examined in the same species of monkey infected with the same strain of virus. In this study, we used differential expression of SIV controlling alleles to explore the potential contribution of gut macrophages early in infection to disease progression or control.

Our results reveal that CD163+ macrophages accumulate in gut mucosa early in progressive SIV infection in association with intestinal damage, but have reduced phagocytic capacity and fail to clear debris, providing a positive feedback for sustained macrophage recruitment. The findings also reveal that gut macrophages in both SIV progressors and controllers, in contrast to macrophages in mesenteric lymph nodes (LNs), do not spontaneously release proinflammatory cytokines in chronic infection and do not respond to viral stimuli *ex vivo*. These findings suggest that gut macrophages may serve to limit the inflammatory response early in progressive infection rather than promote it.

## 4.4 RESULTS

### 4.4.1 SIV infection induces a persistent accumulation of CD163+ macrophages in small intestine in macaques with progressive infection

To investigate the role of intestinal macrophages in SIV pathogenesis, we developed a model of diverging disease course using differential expression of protective MHC molecule *Mamu-B\*008* [26]. We elected to use *Mamu-B\*008* over other SIV controlling alleles because fewer than 50% of SIVmac-infected macaques expressing *Mamu-B\*008* develop escape mutants compared to nearly 75% of primates expressing *A\*001* or *B\*017* [26, 254, 257]. We identified 12 rhesus macaques with and 12 without expression of *Mamu-B\*008* based on a pre-study haplotype screen. Of the 12 that expressed *Mamu-B\*008*, 3 co-expressed controller allele *Mamu-A\*001* and 1 animal expressed alleles *Mamu-B\*008*, *A\*001*, and *B\*017* (Table 2). *Mamu-B\*008*-negative macaques also lacked expression for *Mamu-A\*001* and *Mamu-B\*017*. Macaques were inoculated intrarectally with a single high dose ( $10^5$  TCID<sub>50</sub>) of pathogenic isolate SIVmac251 and monitored out to week 20 post-infection. A threshold of  $10^4$  SIV RNA copies/mL plasma at virus load set-point (week 8) was used to sort animals into predicted SIV progressors or controllers based on evidence that SIVmac escape mutants occur within the first 8 weeks of infection [49]. In total, 14 macaques had set-point virus loads  $>10^4$  SIV RNA copies/mL and were defined as SIV progressors and 10 had set-point virus loads  $<10^4$  SIV RNA copies/mL and were defined as SIV controllers (Table 2). Viral load divergence in the two groups was statistically significantly by week 3 post-inoculation (Fig. 5A).

**Table 2. Animal characteristics.**

Table 2. Animal characteristics

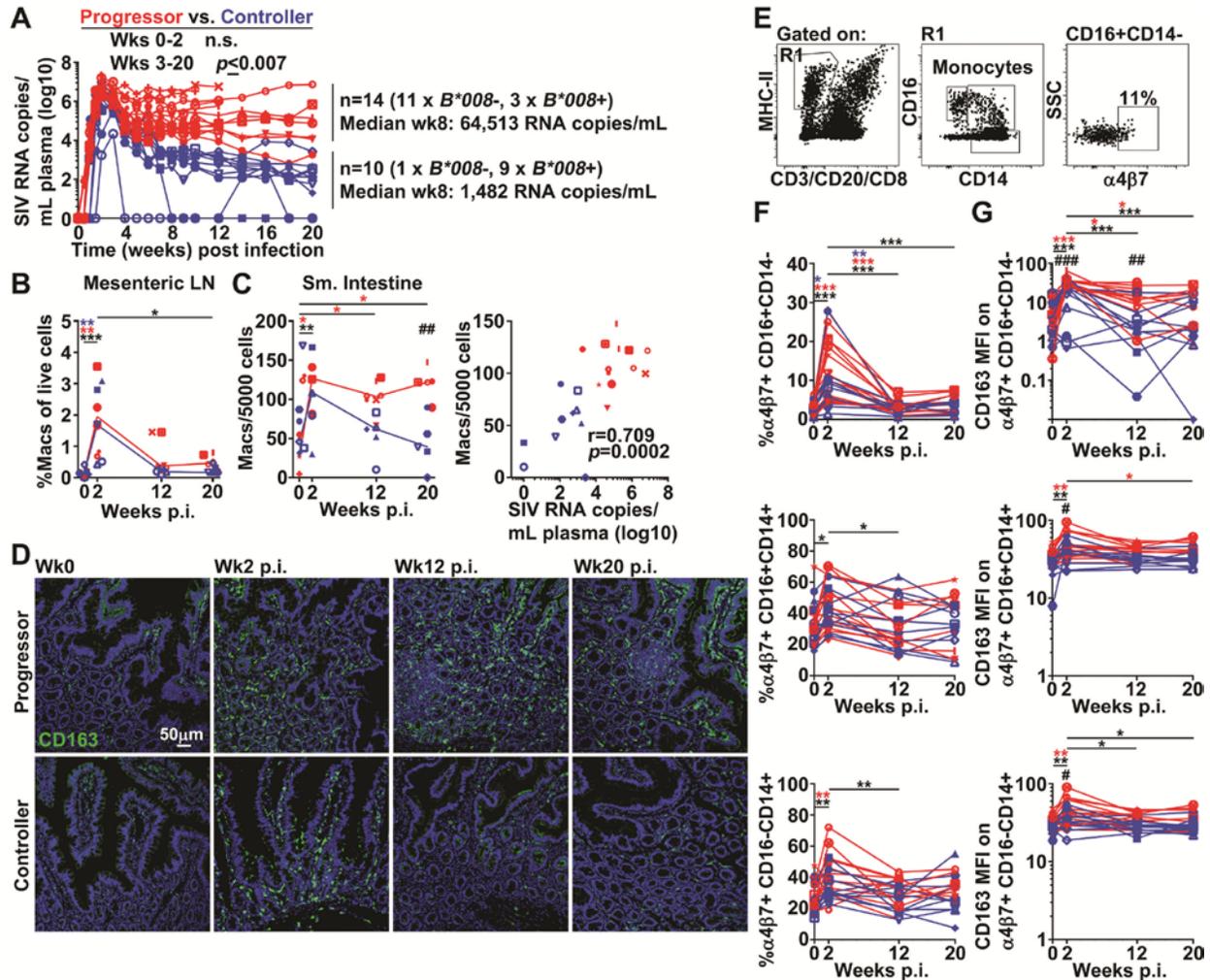
Animal ID	MHC class I haplotype			Wk8 plasma VL (RNA copies/mL)
<b>SIV controller (VL &lt;10<sup>4</sup> RNA copies/mL)</b>				
M12-13	B*008+	A*001+	B*017+	<200
M174-12	B*008+	A*001+	B*017-	2,702
M11-13	B*008+	A*001+	B*017-	1,953
M13-13	B*008+	A*001+	B*017-	1,542
M57-13	B*008+	A*001-	B*017-	435
M58-13	B*008+	A*001-	B*017-	1,632
M175-12	B*008+	A*001-	B*017-	<200
M176-12	B*008+	A*001-	B*017-	2,669
M177-12	B*008+	A*001-	B*017-	1,422
M132-14	B*008-	A*001-	B*017-	1,305
<b>Median</b>				<b>1,482</b>
<b>SIV progressor (VL &gt;10<sup>4</sup> RNA copies/mL)</b>				
M59-13	B*008+	A*001-	B*017-	21,802
M60-13	B*008+	A*001-	B*017-	22,826
M173-12	B*008+	A*001-	B*017-	168,148
M131-14	B*008-	A*001-	B*017-	636,401
M133-14	B*008-	A*001-	B*017-	79,026
M134-14	B*008-	A*001-	B*017-	48,212
M135-14	B*008-	A*001-	B*017-	15,110
M136-14	B*008-	A*001-	B*017-	289,716
M172-12	B*008-	A*001-	B*017-	4,000,000
M181-13	B*008-	A*001-	B*017-	2,400,000
M182-13	B*008-	A*001-	B*017-	410,006
M183-13	B*008-	A*001-	B*017-	44,017
M184-13	B*008-	A*001-	B*017-	38,742
M185-13	B*008-	A*001-	B*017-	50,000
<b>Median</b>				<b>64,513</b>

Using these 2 groups, we first evaluated the frequency of macrophages in intestine using established *in situ* techniques [102, 150]. To do this, sections of small intestine harvested prior to infection and at weeks 2, 12, and 20 post-infection were stained with fluorescent antibodies to

CD163 for macrophage identification and then quantified relative to the number of cell nuclei present. In parallel, we identified and analyzed macrophage frequency in mesenteric LNs using previously published flow cytometry methods, gating on CD3/CD20-MHC-II+CD163+ cells [150]. The density of CD163+ macrophages increased over 2-fold in both the mesenteric LNs and gut mucosa in acute SIV infection compared with pre-infection (Fig. 5B, C). By week 12, the proportion of macrophages in mesenteric LNs had declined to pre-infection levels, however in gut there was a stark divergence in macrophage abundance between controllers, which had declined in numbers, and progressors, which was sustained in numbers, that achieved statistical significance at week 20 (Fig. 5B, D). Spearman analysis revealed that gut macrophage frequency at weeks 12 and 20 strongly correlated with the level of chronic phase plasma viremia (Fig. 5C)

To determine if monocyte trafficking represented a potential mechanism for increased macrophages in SIV-infected intestine, we stained PBMC for CD14 and CD16 to delineate the three monocyte subsets found in blood and assessed for expression of gut-homing integrin  $\alpha 4\beta 7$  by flow cytometry (Fig. 5E). Monocytes were identified as CD16-CD14+, CD16+CD14+, or CD16+CD14- within the MHC-II+ population lacking expression for CD3, CD20, and CD8 as described previously [258]. In the absence of SIV, all monocyte subsets expressed low levels of  $\alpha 4\beta 7$ . After infection, both SIV controllers and progressors had an increased frequency of  $\alpha 4\beta 7$ + monocytes regardless of subset at week 2 post-infection that declined by chronic infection and was not significant between groups (Fig. 5F). To evaluate if gut-homing monocytes could be a source of accumulating CD163+ macrophages, we next looked at expression of CD163 on  $\alpha 4\beta 7$ + monocytes. Consistent with previous reports, the CD16+CD14- subset expressed the lowest level of CD163 in SIV-naïve blood, whereas expression of CD163 on CD16+CD14+ and

CD16-CD14+ monocytes was nearly 5-fold higher by comparison [259]. Notably, CD163 expression was selectively elevated on all  $\alpha 4\beta 7+$  monocytes in macaques with progressive SIV infection during the acute phase compared to controllers and remained elevated at week 12 only on the CD16+CD14- subset (Fig. 5G).



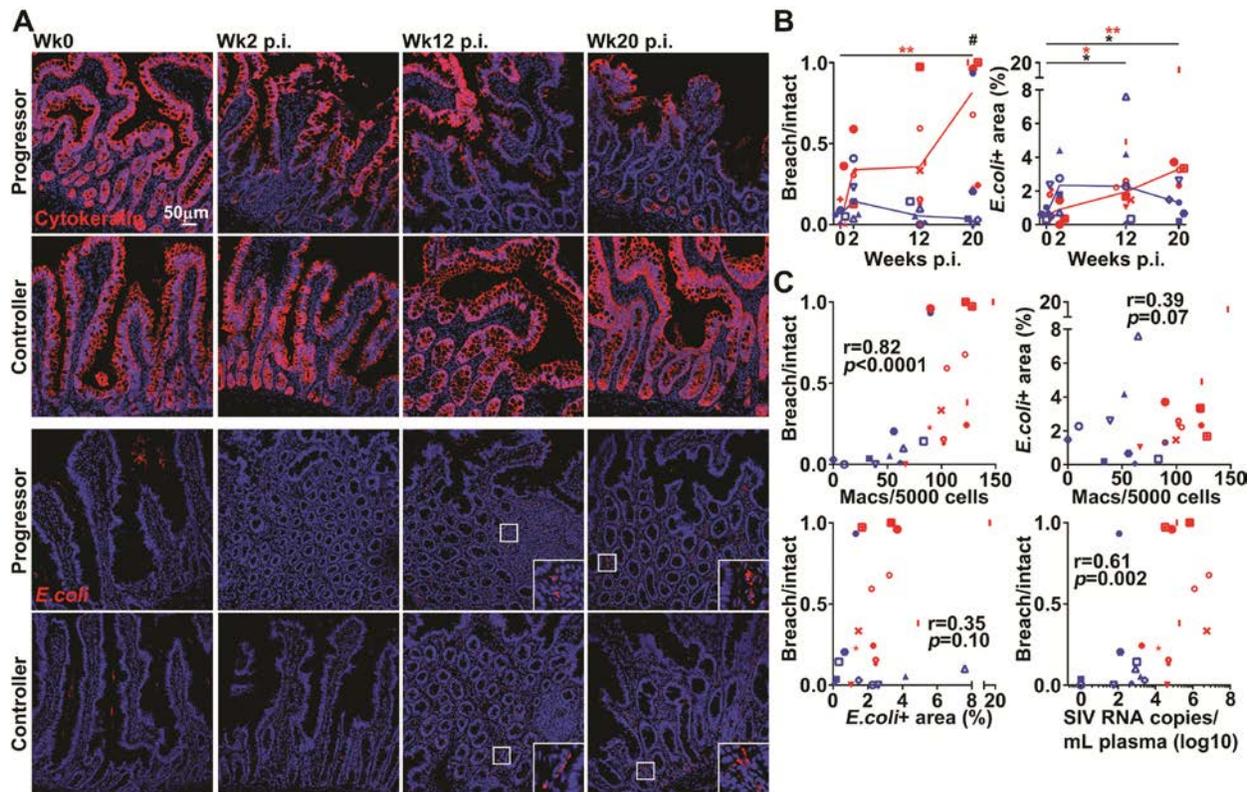
**Figure 5. Relationship between plasma viral burden and CD163+ macrophage frequency in SIV-infected small intestine.**

(A) Longitudinal viral RNA burden in plasma (n = 24). Red depicts macaques with week 8 viral loads  $>10^4$  RNA copies/mL (SIV progressors) and blue depicts macaques with week 8 viral loads  $<10^4$  RNA copies/mL (SIV controllers). (B) The proportion of CD163+ macrophages in mesenteric LNs as determined by flow cytometry. (C) (Left) The frequency of intestinal CD163+ macrophages enumerated using ImageJ and MetaMorph software. (Right) Correlation between the number of intestinal CD163+ macrophages and plasma viral loads at weeks 12 and 20. (D) Representative immunofluorescence of small intestine sections from macaques prior to infection and at weeks 2, 12, and 20 post-infection stained with antibodies to CD163 to identify macrophages. Original magnification = 200x. (E) Representative flow cytometric analysis of PBMC showing gating to define

CD16+CD14-, CD16+CD14+, and CD16-CD14+ monocytes and monocytes expressing  $\alpha 4\beta 7$ . (F) The proportion of each monocyte subset expressing  $\alpha 4\beta 7$ . (G) Median fluorescence intensity of CD163 on each subset of  $\alpha 4\beta 7$ + monocytes. Horizontal lines in (B) and (C) represent medians. # signifies cross-sectional differences between progressors and controllers and \* signifies longitudinal differences within the same groups. For (A) statistical comparisons were done using a two-tailed nonparametric Mann-Whitney U test. For (B), (F), and (G) statistical comparisons were done using a regular two-way ANOVA followed by Sidak's post test (#) or nonparametric one-way ANOVA followed by Dunn's post test (\*). Correlations in (C) were determined using a two-tailed nonparametric Spearman rank test. Black asterisks reflect all animals. \*/#  $p < 0.05$ ; \*\*/##  $p < 0.01$ ; \*\*\*/###  $p < 0.001$ .

#### **4.4.2 Progressive SIV infection is associated with increased intestinal pathology and microbial translocation**

We next sought to investigate the relationship between CD163+ macrophage frequency and hallmarks of SIV pathogenesis in the gut, namely damage to the intestinal epithelial barrier and microbial translocation [90]. Sections of small intestine were stained with antibodies to cytokeratin to assess intestinal epithelial integrity and antibodies to *E.coli* to assess microbial translocation by immunohistochemistry as previously published [90]. Confocal microscopy revealed that tissues taken prior to infection displayed uniform staining for cytokeratin along the interface of the lumen and lamina propria reflecting an uncompromised barrier of columnar epithelial cells (Fig. 6A). Qualitative and quantitative analysis revealed a pronounced disruption of cytokeratin expression by weeks 12 and 20 in SIV progressors that was not apparent in macaques with controlled infection (Fig. 6A, B). Evidence of microbial translocation as measured by *E.coli* staining was sparse in the intestinal lamina propria prior to infection, with the majority of *E.coli* present on the luminal side of the gut (Fig. 6A). Infection was associated with a statistically significant increase in percent lamina propria occupied by *E.coli*, primarily among SIV progressors, however there was considerable variation between animals (Fig. 6B). Increased intestinal damage in chronic infection strongly correlated with macrophage abundance and chronic phase viremia, but not microbial translocation (Fig. 6C).



**Figure 6. Increased intestinal pathology and microbial translocation is associated with progressive SIV infection.**

(A) Representative immunofluorescence of small intestine sections from macaques taken prior to infection and at weeks 2, 12, and 20 post-infection stained with antibodies to cytokeratin (Top) or *E.coli* (Bottom) to identify epithelial integrity and extent of microbial translocation. Original magnification = 200x. (B) Quantitative image analysis showing ratio of breach to intact intestinal epithelium determined by cytokeratin+ staining (Left) and percentage of lamina propria occupied by *E.coli*+ staining (Right). Quantitative of cytokeratin+ staining and *E.coli*+ staining was carried out using NIS-Elements software. Horizontal lines in (B) represent medians. # signifies cross-sectional differences between progressors and controllers and \* signifies longitudinal differences within the same groups. Statistical comparisons were done using a regular two-way ANOVA followed by Sidak's post test (#) or nonparametric one-way ANOVA followed by Dunn's post test (\*). (C) Correlation between the ratio of breach to intact intestinal epithelium and the number of intestinal CD163+ macrophages, the percentage of lamina propria occupied by *E.coli* and the number of intestinal CD163+ macrophages, the ratio of breach to intact intestinal epithelium and the percentage of lamina propria occupied by *E.coli*, and ratio of breach to intact intestinal epithelium and plasma viral load at weeks 12 and 20. Correlations were determined using a two-tailed nonparametric Spearman rank test. Black asterisks reflect all animals. Red depicts SIV progressors and blue depicts SIV controllers. \*/#  $p < 0.05$ ; \*\*/##  $p < 0.01$ ; \*\*\*/###  $p < 0.001$ .

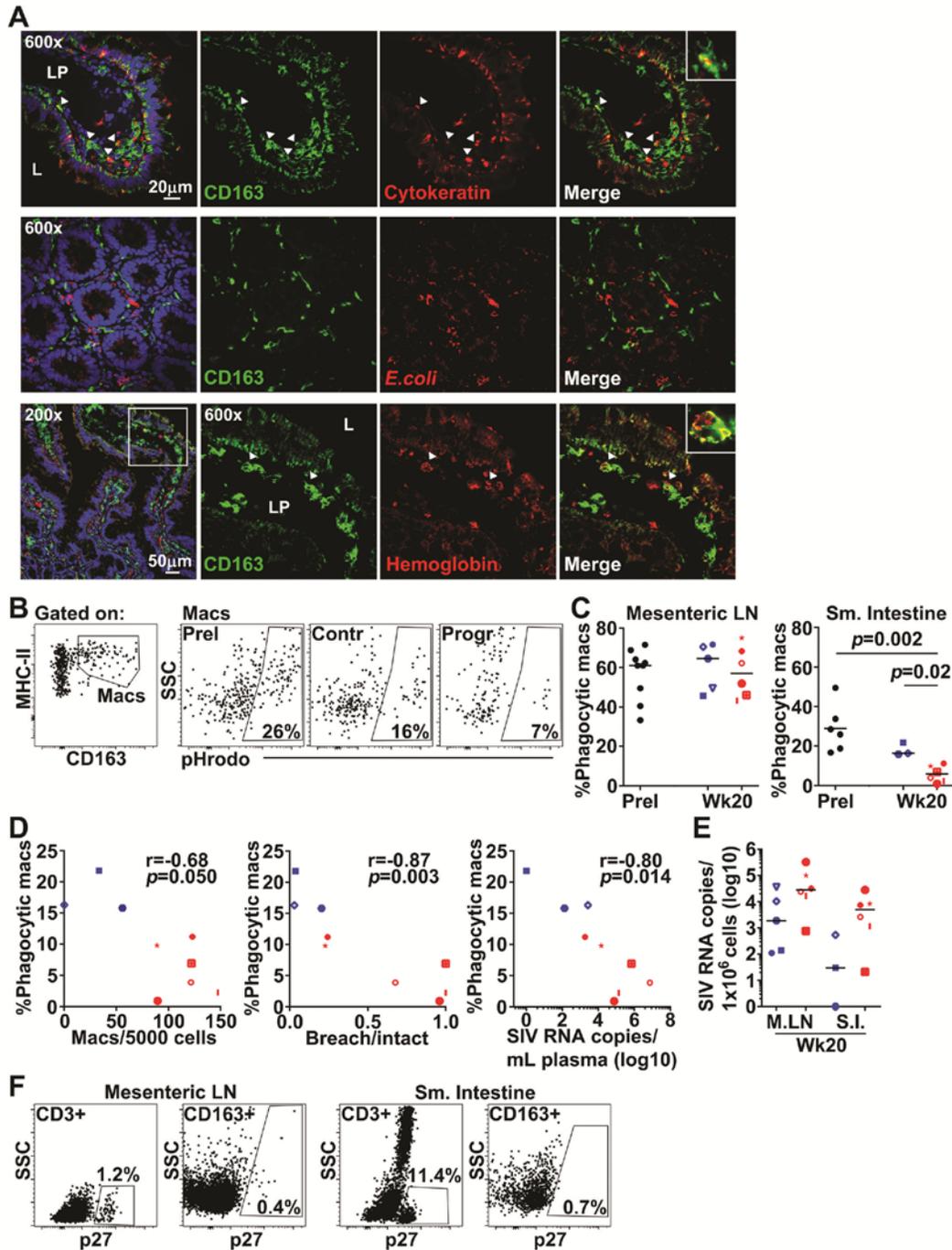
#### **4.4.3 Intestinal CD163+ macrophages have impaired phagocytic function in macaques with progressive SIV infection**

Next we used confocal microscopy to analyze *in situ* whether accumulating macrophages phagocytose cellular and bacterial debris in small intestine. Sections of intestine were stained with antibodies to CD163 and either cytokeratin, *E.coli*, or hemoglobin, the biological ligand for CD163, and then analyzed for co-expression, a measure of macrophage phagocytosis (Fig. 7A). Staining for cytokeratin in infected gut, particularly in regions with intestinal breach, revealed cytokeratin+ debris within the lamina propria where large clusters of macrophages could also be found. Co-staining for CD163 and lamina propria-associated cytokeratin could be detected in infected tissues (Fig. 7A, top). In contrast, co-localization of CD163 with *E.coli*+ staining was rare in both uninfected and infected intestine, even in tissues where microbial translocation was most abundant (Fig. 7A, middle). Hemoglobin staining was plentiful in infected intestine, suggestive of focal hemorrhage and hemolysis, and confocal imaging revealed hemoglobin deposits within CD163+ macrophages (Fig. 7A, bottom).

To quantitatively address the impact of SIV infection on macrophage phagocytic function, we next assessed the ability of isolated gut macrophages to engulf antigen *ex vivo*. Phagocytosis was determined by measuring real-time uptake of fluorescently-labeled bioparticles using a pH-sensitive assay. Intestinal suspensions from before infection and at week 20 post-infection were incubated with bioparticles and then stained with antibodies for detection of phagocytic macrophages by flow cytometry. Macrophages were delineated in suspension by gating CD45+CD3/CD20-MHC-II+CD163+ cells and phagocytic macrophages identified as pHrodo+ (Fig. 7B). We also analyzed bioparticle uptake in macrophages from mesenteric LNs for comparison. Phagocytic macrophages in gut represented a median 27% of the total CD163+

population prior to infection, and roughly twice as many macrophages were able to uptake antigen in mesenteric LNs (Fig. 7C). After infection, however, we found a dramatic decline in bioparticle uptake by intestinal macrophages at week 20 in SIV progressors compared to macrophages in SIV controllers and uninfected macaques. In contrast, we found no change in mesenteric LN macrophage's ability to phagocytose bioparticles with infection and no differences between groups (Fig. 7C). The frequency of phagocytic macrophages in gut mucosa inversely correlated with gut macrophage abundance, intestinal breach, and chronic phase viral burden (Fig. 7D).

To determine whether a decline in phagocytic function could be related to the presence of virus in tissues and/or direct viral infection, we next quantified cell-associated virus in mesenteric LNs and intestine by RT-PCR as well as determined the percentage of SIV p27 (gag)+ macrophages in suspension by flow cytometry. In both mesenteric LNs and gut, there was a nonsignificant trend towards more cell-associated virus in progressors compared to controllers at week 20 (Fig. 7E). Interestingly, virus in mesenteric LNs tended to be more prevalent per  $1 \times 10^6$  cells than in small intestine. Flow cytometric assessment of p27+ cells revealed infected T cells in mesenteric LN and intestinal suspensions, however p27+ macrophages were rare at both sites at week 20 post-infection (Fig. 7F).



**Figure 7. Intestinal CD163+ macrophages from macaques with progressive SIV infection have impaired phagocytic function.**

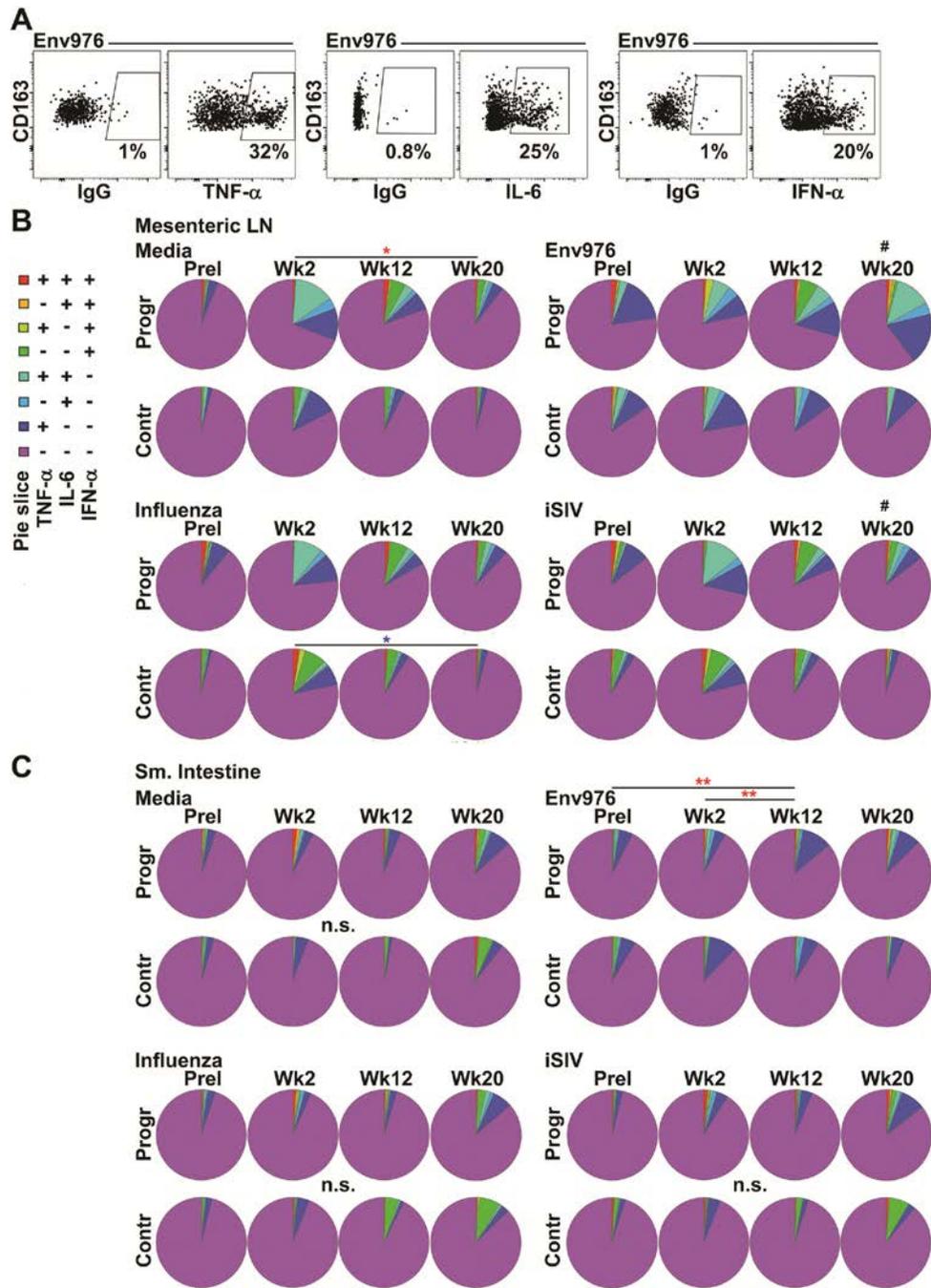
(A) Representative immunofluorescence of small intestine sections from SIV-infected macaques stained with antibodies to CD163 and either cytokeatin to identify cytokeatin+ macrophages (Top), *E.coli* to identify *E.coli*+ macrophages (Middle), or hemoglobin to identify hemoglobin+ macrophages (Bottom). LP = lamina propria and L = lumen. (B) (Left) Representative flow cytometric staining in small intestine single-cell suspensions showing detection of CD163+ macrophages. (Right) Representative flow cytometric staining for pHrodo on CD163+ macrophages in small intestine at pre-infection and at week 20 in high and low viral load samples. (C) Quantitation

of pHrodo+ macrophages in mesenteric LNs (Left) and small intestine (Right) analyzed by flow cytometry at pre-infection and at week 20 in progressors and controllers. Statistical comparisons were done using two-tailed nonparametric Mann-Whitney U test. (D) Correlations between the percentage of pHrodo+ intestinal macrophages and (Left) the number of CD163+ macrophages per nucleated cells, (Middle) the ratio of breach to intact intestinal epithelium, and (Right) the plasma viral loads at week 20. Correlations were determined using a two-tailed nonparametric Spearman rank test. (E) Quantitation of cell-associated viral RNA in mesenteric LNs and small intestine at week 20. Horizontal bars in (C) and (E) represent medians. (F) Representative flow cytometric analysis of SIV-infected mesenteric LNs (Left) and small intestine (Right) at week 20 showing staining for SIV p27 in CD3+ T cells and CD163+ macrophages. Black asterisks reflect all animals. Red depicts SIV progressors and blue depicts SIV controllers. \*/#  $p < 0.05$ ; \*\*/##  $p < 0.01$ ; \*\*\*/###  $p < 0.001$ .

#### **4.4.4 Intestinal CD163+ macrophages do not spontaneously produce inflammatory cytokines and are non-responsive to viral stimulation before and after SIV infection**

We next evaluated whether increased macrophages in SIV-infected gut mucosa may contribute to gut-associated inflammation. To address this question, we assessed the capacity of CD163+ macrophages in suspension to respond to *ex vivo* stimulation with aldrithiol-2-inactivated SIV (iSIV) that activates macrophages through TLR8, and influenza virus, which can stimulate RIG-I and TLR3, 7, and 8 [260-262]. We also stimulated macrophages with Env976, a uridine rich ssRNA oligonucleotide sequence derived from SIVmac251 *env* gene that induces macrophages from SIV-naïve peripheral LNs to produce IFN- $\alpha$  and TNF- $\alpha$  through TLR8 (Fig. 8A) [101]. In naïve mesenteric LNs, macrophages were moderately responsive to Env976 and iSIV stimulations but not influenza and produced low levels of cytokines spontaneously (Fig. 8B). After infection, there was a nonsignificant increase in the frequency of macrophages spontaneously producing cytokines that was sustained into chronic infection. Similar changes in cytokine+ macrophages were seen after *ex vivo* stimulation during the acute stage, however the proportions were comparable to the level of cytokines produced spontaneously. By week 20 post-infection, macrophages from progressors were significantly more responsive to Env976 and iSIV compared to controllers (Fig. 8B). In comparison, in gut mucosa, the percentage of

unstimulated or stimulated gut macrophages producing combinations of TNF- $\alpha$ , IFN- $\alpha$ , and/or IL-6 was below 5% in SIV-naïve macaques (Fig. 8C). SIV progressors exposed to Env976 had significantly more cytokine+ macrophages at week 12 compared to pre-infection and week 2; a finding not observed in macrophages from animals with low set-point viral loads. Nevertheless, differences in gut were minor, especially compared to mesenteric LNs, and overall very few intestinal macrophages produced any combination of TNF- $\alpha$ , IFN- $\alpha$ , or IL-6 spontaneously or in response to iSIV or influenza after infection regardless of predicted disease outcome (Fig. 8C).



**Figure 8. Intestinal CD163<sup>+</sup> macrophages are non-inflammatory and non-responsive to viral stimulation in SIV infection.**

(A) Representative flow cytometric staining for CD163<sup>+</sup> macrophages producing TNF- $\alpha$ , IL-6, and IFN- $\alpha$  in Env976 stimulated mesenteric LN single-cell suspensions. (B, C) SPICE analysis of the proportion of mesenteric LN (B) and intestinal (C) CD163<sup>+</sup> macrophages producing different permutations of TNF- $\alpha$ , IL-6, and IFN- $\alpha$  prior to infection and at weeks 2, 12, and 20 post-infection either spontaneously or after stimulation by Env976, influenza, or iSIV. Statistical comparisons were done using a permutation test performed with SPICE software. # signifies cross-sectional differences between progressors and controllers and \* signifies longitudinal differences within the

same groups. Red asterisks reflect SIV progressors and blue asterisks reflect SIV controllers. \*/#  $p < 0.05$ ; \*\*/##  $p < 0.01$ ; \*\*\*/###  $p < 0.001$ .

## 4.5 DISCUSSION

In this study, we report differences in macrophage abundance in gut mucosa of SIV-infected rhesus macaques that exist at the early stages of chronic infection and are correlated with plasma virus burden and degree of damage to gut epithelium. In contrast to macrophages that dominate colon in inflammatory bowel disease, however, we found no evidence of a corresponding proinflammatory response from infiltrating macrophages in SIV-infected animals with increased enterocyte loss [132, 248, 250, 263]. Instead, gut macrophages in SIV infection were non-responsive to viral agonists and had decreased phagocytic capacity in SIV progressors but not controllers. These results suggest that increased intestinal pathology in animals with a progressive disease course is a mechanism of recruitment for macrophages into gut, and that macrophage phagocytic impairment may lead to continued recruitment in a positive feedback loop.

It is not clear what role viral burden plays in mediating macrophage infiltration to tissues. Although there was a nonsignificant trend towards more cell-associated virus in gut in SIV progressors as compared to controllers, this could be related to the small sample size of our controller group, as others have reported a strong correlation between plasma viral load and viral replication in intestine [264]. Interestingly, CD163+ macrophages also accumulate in macaques with experimentally-induced colitis that lack SIV, suggesting factors other than virus contribute to recruitment, such as inflammation [265]. SIV-infected macaques administered an anti-TNF- $\alpha$  therapy had reduced CD163+ macrophage infiltration of peripheral LNs compared to treatment

controls, despite no change in plasma viremia and peripheral blood or lymph node T cell activation [190].

Currently the cause of intestinal macrophage dysfunction in animals with progressive SIV infection is unclear but likely to be multifactorial. Macrophages are secondary reservoirs of HIV/SIV in the gastrointestinal tract and support infection and active replication of virus in explanted vaginal mucosa [266]. In small intestine, however, macrophages lack surface receptors CD4, CCR5, and CXCR4 for viral entry and are not permissive to infection [266-268], consistent with our data. Our finding that gut macrophages from animals with controlled SIV infection have largely intact phagocytic function is consistent with normal T cell and myeloid dendritic cell responses in intestine from natural SIV hosts [20, 223]. Moreover, animals with controlled SIV infection in our study had minimal intestinal damage and microbial translocation, comparable to findings in nonpathogenic SIV models that have less systemic immune activation and tissue-associated inflammation despite high levels of virus replication in blood and gut [21, 85]. Together, these results suggest that altered macrophage phagocytic activity in SIV-infected small intestine is likely a consequence of the state of the local microenvironment and not direct viral infection.

Our results allude to a compartmentalized cytokine response by accumulating CD163+ macrophages in gut compared to gut-draining mesenteric LNs, although in neither site did spontaneous macrophage cytokine activity appear to differentiate controllers and progressors. In health, intestinal macrophages downregulate TLR-adaptor proteins MyD88 and TRIF as well as other innate signaling molecules in response to stromal-derived TGF- $\beta$  and IL-10 to avoid adverse reactions towards enteric microbiota [115, 269, 270]. Previous studies have shown that TGF- $\beta$  and IL-10 expression levels are elevated in HIV/SIV-infected gut mucosa, and therefore

these cytokines could potentially continue to act on newly recruited macrophages to promote a non-inflammatory phenotype [271-273]. Conversely, macrophages in mesenteric LNs from SIV progressors became increasingly responsive to stimulation by virus-encoded ligands, suggesting an enhanced ability to respond to mounting antigenic challenges as infection advances. It is not clear whether other subsets of macrophages might accumulate in SIV-infected mucosal tissues and contribute a harmful response. In HIV infection, CD14<sup>+</sup> macrophage numbers are increased in colon from AIDS patients and express TNF- $\alpha$  and IL-1 $\beta$ , but whether these cells also express CD163 was not addressed [196]. Alternatively, it is possible that gut macrophages in animals with a progressive disease course switch to have an inflammatory function with the onset of AIDS [150].

Preservation of intestinal integrity is a key homeostatic function of gut macrophages [144, 247]. Functionally, CD163 can mediate such protection by sensing bacterial antigen and excessive hemoglobin deposits from lysed blood cells and promoting their uptake and degradation [274-276]. Our findings of increased non-inflammatory CD163-expressing macrophages in animals with increased intestinal breach suggest a role for macrophage recruitment and phagocytosis in mitigating tissue-associated inflammation and subsequent intestinal barrier breakdown [277]. Microbial translocation is a hallmark of HIV/SIV infection and a cause of chronic immune activation associated with progression to AIDS, and studies in HIV-infected humanized mice demonstrate a link between impaired macrophage phagocytic function, plasma LPS burden, and systemic T cell activation [91, 278]. Less appreciated are the effects of macrophage functional impairment on clearing excessive hemoglobin in tissues, which could have equally important pathologic consequences due to hemoglobin's ability to induce

leukocyte production of TNF- $\alpha$ , IL-8, and IL-6 that may further trigger enterocyte apoptosis [140, 141].

Our results suggest that CD163<sup>+</sup> macrophage accumulation in intestine is derived from extravasated monocytes co-expressing CD163 and gut-homing marker  $\alpha$ 4 $\beta$ 7. Plasmacytoid dendritic cells in blood similarly upregulate  $\alpha$ 4 $\beta$ 7 during SIV infection and accumulate in rectal tissues [88, 222]. Others have reported that differentiation of sorted monocytes into M2 macrophages induces intra- and extra-cellular expression of CD163, and that the frequency of CD163<sup>+</sup>CD16<sup>+</sup> monocytes is positively correlated with viremia in HIV infection [160, 259]. Interestingly, at week 2 post-infection, all three monocyte subsets displayed evidence of increased gut homing, however only CD16<sup>+</sup> monocytes had elevated CD163 levels that reflected differences in macrophage abundance in the chronic stage. Previous studies have revealed that CD16<sup>+</sup>CD14<sup>-</sup> monocytes are preferentially expanded in acute and chronic SIV infection and share functional characteristics with intestinal CD163<sup>+</sup> macrophages in our study [161, 279]. Collectively, these data suggest that the CD16<sup>+</sup>CD14<sup>-</sup> monocyte subset and not CD14<sup>-</sup> expressing monocytes likely represent the predominant source of gut macrophages during SIV infection. Likewise, in Crohn's disease patients, classical CD14<sup>+</sup> monocytes selectively migrate to inflamed colon and phenotypically and functionally resemble newly accumulated CD14<sup>-</sup> high inflammatory macrophages [249]. A recent study found that blockade of  $\alpha$ 4 $\beta$ 7-mediated homing in combination with ART treatment promoted virological control better than ART alone [280]; our results indicate it is likely this therapeutic approach might also have considerable impact on macrophage recruitment to gut mucosal tissues.

## 4.6 MATERIALS AND METHODS

### 4.6.1 Animals and tissue preparation

Indian-origin rhesus macaques housed at the University of Pittsburgh (U.S. Public Health Service Assurance Number: A3187-01) were used in this study and all animal manipulations and procedures were done with appropriate institutional regulatory oversight and approval. Twelve macaques were selected with and 12 without expression of *Mamu-B\*008*, a macaque MHC class I allele correlated with control of chronic phase plasma viral burden [26]. Macaques were inoculated intrarectally with  $10^5$  TCID<sub>50</sub>/mL of SIVmac251 and plasma was extracted from whole blood drawn periodically to monitor viral burden and for analysis of cells in circulation. Viral RNA in plasma was determined by RT-PCR as previously described [83]. Mesenteric LNs and resections of small intestine averaging 25cm in length were taken from each animal at two of the following timepoints: pre-infection, or at weeks 2, 12, or 20 post-infection. For isolation of mononuclear cells from intestine, tissues were minced and incubated in 5mM EDTA HBSS. Intestinal slurries were subsequently digested in 15 U/mL collagenase media, mechanically disrupted, and run on a Percoll gradient for leukocyte separation. Suspensions of freshly isolated mesenteric LNs were generated as previously described [150]. Viral RNA in mesenteric LNs and intestinal suspensions was determined using RT-PCR and quantified per  $1 \times 10^6$  cells. Prior to processing, a portion of resected intestine was fixed in 2% paraformaldehyde and infused with 30% sucrose for *in situ* analysis. Tissues for IHC were frozen with an aerosol of chlorodifluoromethane and stored at  $-80^\circ\text{C}$ .

#### 4.6.2 Immunofluorescence microscopy and quantitative analysis

Six-micron sections were cut from frozen intestine blocks and mounted on Superfrost Plus slides. Sections were briefly treated with 1% sodium dodecyl sulfate for epitope retrieval, washed thoroughly, and blocked in 5% of the secondary antibody host's normal serum (goat, mouse, and/or donkey). The following primary antibodies were then diluted in 0.5% serum and applied to slides at 4°C: monoclonal mouse anti-human Cytokeratin (Clone MNF116, Dako), rabbit polyclonal anti-*E.coli* (Dako), monoclonal rabbit anti-hemoglobin (EPR3608, abcam), and monoclonal mouse anti-human CD163 Biotin (GHI/61, eBioscience). Primary antibodies raised in rabbit were detected by donkey-anti-rabbit IgG Alex Fluor 546 secondary (Thermo Scientific) and antibodies raised in mouse detected using either goat anti-mouse Alex Fluor 546 or HRP-Streptavidin Alexa Fluor 488 Tyramide Signal Amplification kits (Thermo Scientific) according to the manufacturer's instructions. Lastly, cell nuclei were identified using membrane permeable Hoescht dye and tissues mounted in gelvatol media for viewing on an Olympus Fluoview 1000 confocal microscope. All staining included an appropriately concentrated isotype-matched control antibody. Quantification of *in situ* staining was carried out as previously described [102, 150]. Briefly, macrophages were enumerated manually using ImageJ software (U.S. National Institutes of Health) and the total number of nuclei per image determined digitally using MetaMorph software (Molecular Devices). Cell frequencies were calculated using the formula:  $(\text{total number of macrophages} \times 5000) / \text{total number of nuclei}$ . To determine extent of intestinal breach, the length of the epithelial interface between the lumen and lamina propria with and without cytokeratin expression was manually determined using the length measurement tool in NIS-Elements software (Nikon). Percent breach was then ascertained using the formula:  $((\text{length of no cytokeratin expression}) / (\text{length of no cytokeratin expression} + \text{length of$

cytokeratin expression)) x 100. Percent area of *E.coli* was determined manually by tracing using the NIS-Elements polygonal ROI function. Percent *E.coli*+ area was calculated using the formula: (area of lamina propria occupied by *E.coli* staining / total area of lamina propria) x 100. All data represent averages of quantification from ten nonoverlapping regions of lamina propria.

#### **4.6.3 Flow cytometry and phagocytosis assay**

Small intestine, mesenteric LNs, and PBMC cell suspensions were stained with the following cell surface-labeling anti-human antibodies (all antibodies were purchased from BD Biosciences unless otherwise noted): HLA-DR (clone L243), CD163 (GHI/61), CD3 (SP34-2), CD4 (L200), CD20 (2H7, eBioscience), CD45 (D058-1283), CD14 (M5E2), CD16 (3G8), CD8 (RPA-T8), p27 (55-2F12, NIH AIDS Reagents), and  $\alpha 4\beta 7$  (A4B7, NHP Reagent Resource). Nonviable cells were excluded with a fluorescent LIVE/DEAD cell stain (Invitrogen). To measure responses to stimulation, freshly isolated intestinal and mesenteric LN single cell suspensions were cultured for 5 h with H7N3 influenza virus (MOI of 5), Env976 oligonucleotides (10 $\mu$ g/mL), or AT-2 iSIVmac239 (200ng capsid/mL) with or without brefeldin A (Sigma) treatment after 2 h before cell surface labeling. Cells were then fixed and permeabilized using Cytotfix/Cytoperm reagent (BD Biosciences) and subsequently stained with anti-human antibodies to TNF- $\alpha$  (Mab11), IL-6 (MQ2-6A3), and IFN- $\alpha$  (225.C, Chromaprobe). Phagocytic activity was determined using the pHrodo *Escherichia coli* BioParticles Conjugate for Phagocytosis kit (Invitrogen) according to the manufacturer's instructions with minor modifications. Briefly, a suspension of 2x10<sup>6</sup> intestinal cells were incubated at 4°C or 37°C for 1 h in a fixed volume of media before the addition of 50 $\mu$ L pHrodo for an additional 1 h. Cells

were subsequently washed and stained with surface markers for macrophage identification. All data were acquired using an LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo 7.6.4 (TreeStar).

#### **4.6.4 Statistical analysis**

Cross-sectional comparisons between SIV progressors and controllers were performed using a regular two-way ANOVA followed by Sidak's post test. Longitudinal comparisons across multiple timepoints were performed using a nonparametric one-way ANOVA followed by Dunn's post test. Comparisons between two timepoints (e.g., pre-infection vs. week 20) were performed using a two-tailed nonparametric Mann-Whitney U test. Correlations were determined using a two-tailed nonparametric Spearman rank test. All aforementioned statistical tests were carried out using GraphPad Prism version 6. Pie charts were analyzed using a permutation test performed with SPICE (version 5.22). A  $p$ -value  $< 0.05$  was considered significant.

## **5.0 CHAPTER THREE: LYMPH NODE MACROPHAGES ARE ACTIVATED AND INFLAMMATORY IN SIV-INFECTED RHESUS MACAQUES REGARDLESS OF DISEASE COURSE**

### **5.1 PREFACE**

This chapter is in fulfillment of specific aim 3 and data herein will be incorporated into a separate manuscript for future publication.

### **5.2 INTRODUCTION AND RESULTS**

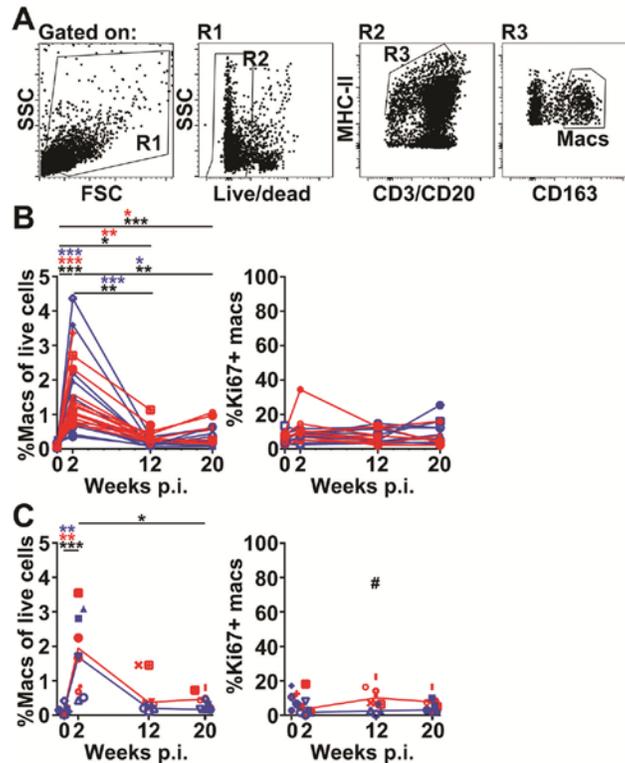
Macrophages are specialized phagocytic cells of the innate immune system that participate in host inflammatory and antiviral responses in tissues throughout the body. In lymph nodes (LNs), macrophages are situated within the subcapsular sinus and medullary regions where they uptake lymph-borne antigens and modulate adaptive immunity through antigen-presentation and cytokine production [117]. During pathogenic HIV and SIV infections, virus accumulates in lymphoid tissue, promoting progressive CD4<sup>+</sup> T cell loss and profound anatomical damage associated with an unregulated inflammatory response [78, 281]. Cross-sectional studies demonstrate that macrophages infiltrate T-cell rich zones of secondary lymphoid tissue with acute SIV infection and AIDS suggesting a role for macrophages in disease pathogenesis [150,

190]. To date, no studies have prospectively analyzed the macrophage response in LNs to SIV infection and whether that response differs in animals predicted to control or progress to disease. Here, we utilized differential expression of SIV protective allele *Mamu-B\*008* (described in extensive detail in Chapter 2) to study macrophage kinetics and response in LNs from macaques with diverging disease courses [26]. Specifically, we evaluated macrophages prior to infection and at weeks 2, 12, and 20 post infection in axillary LNs, which drain lymph vessels from peripheral sites, and mesenteric LNs, which drain the mucosa.

### **5.2.1 Acute SIV infection is associated with an accumulation of macrophages in LNs**

To monitor changes in macrophages following SIV infection, we generated single-cell suspensions of LNs using the same animals described in Chapter 2 (Table 2) and identified macrophages by flow cytometry gating on CD3/CD20-MHC-II+CD163+ cells (Fig. 9A). CD163+ macrophage frequency was dramatically increased in axillary LNs by week 2 or acute SIV infection compared to baseline percentages prior to inoculation (Fig. 9B). By week 12, macrophage frequency had declined considerably, yet remained slightly elevated above pre-infection levels at week 20. Changes in macrophage frequency with infection were not associated with differences in cell turnover based on intracellular staining for proliferation marker Ki67 (Fig. 9B). Moreover, cross-sectional analyses revealed no statistical differences in the percentage of macrophages in axillary LNs between SIV controllers and progressors. Similar observations were made in gut-draining mesenteric LNs, with macrophage frequency markedly increasing in concert with peak viremia at week 2 and then declining at the onset of chronic infection regardless of disease course (Fig. 9C). There was a minor but statistically significant

difference in Ki67+ macrophages between controllers and progressors at week 12, however in general there was no measurable change in cell turnover with infection (Fig. 9C).



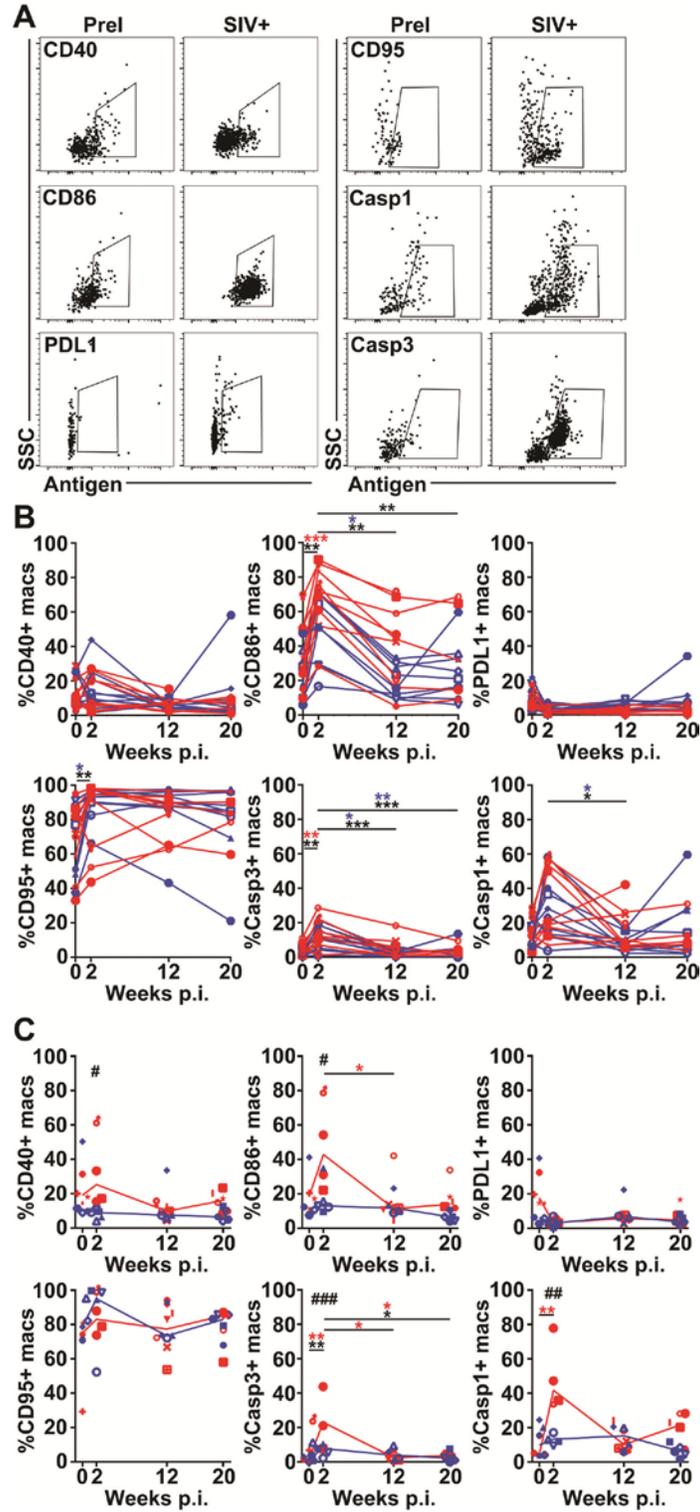
**Figure 9. Macrophages accumulate in LNs during acute SIV infection.**

(A) Representative flow cytometric analysis of peripheral LN single-cell suspensions showing gating strategy to define CD163+ macrophages. (B) The proportion of CD163+ macrophages in the live cell fraction (Left) and the proportion of macrophages expressing Ki67 (Right) in axillary LNs (B) and mesenteric LNs (C) prior to infection and at weeks 2, 12, and 20 post-infection. Red depicts macaques with set-point virus load  $>10^4$  RNA copies/mL plasma (SIV progressors), and blue depicts macaques with set-point virus load  $<10^4$  RNA copies/mL plasma (SIV controllers). Horizontal lines in (C) represent medians. # signifies cross-sectional differences between progressors and controllers and \* signifies longitudinal differences within the same groups. Statistical comparisons were done using a regular two-way ANOVA followed by Sidak's post test (#) or nonparametric one-way ANOVA followed by Dunn's post test (\*). Black asterisks reflect all animals. \*/#  $p < 0.05$ ; \*\*/###  $p < 0.01$ ; \*\*\*/####  $p < 0.001$ .

### 5.2.2 LN macrophages are differentially activated and apoptotic in acute SIV infection

We next evaluated whether differences in disease course were reflected in macrophage activation and cell death in SIV-infected macaques. We stained for co-stimulatory molecules CD40 and CD86, as well as PDL1, a molecule that inhibits T cell activity, to assess macrophage activation

(Fig. 10A). For analysis of cell death, we measured expression of Fas receptor CD95, also an indicator of cell activation, and intracellular active caspase-3 and caspase-1, which independently control induction of two mutually exclusive death pathways, apoptosis and pyroptosis, respectively [282]. Axillary LNs taken at week 2 displayed a uniform increase in CD86<sup>+</sup> and CD95<sup>+</sup> macrophages compared to pre-infection, reflecting heightened cellular activation, however infection had no impact on CD40 or PDL1 expression, and there were no statistical differences between groups (Fig. 10B). Caspase-3 expression was also increased in macrophages during acute infection and there was a trend towards an increased frequency of caspase-1<sup>+</sup> macrophages. By week 12, macrophage activation and apoptotic status had returned to pre-infection levels and remained low out to week 20. In contrast, in mesenteric LNs, macrophage activation status was significantly altered between groups but notably occurred before plasma viral load divergence. Animals that went on to progress but not control SIV infection had a significantly higher proportion of macrophages expressing CD40 and CD86 during acute infection, as well as higher active caspase-3 and caspase-1 expression (Fig. 10C). By week 12, macrophage activation and death in mesenteric LNs had reverted to pre-infection levels, similar to in peripheral LNs, reflecting the transient nature of these responses during infection.



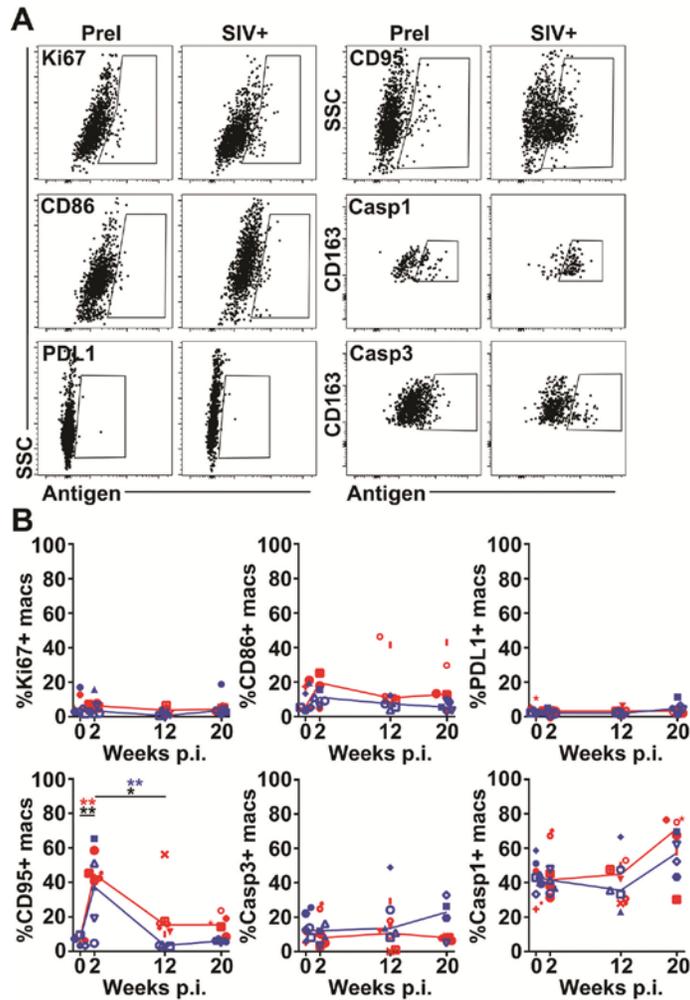
**Figure 10. Differential activation and apoptosis of LN macrophages during acute SIV infection.**

(A) Representative flow cytometric analysis of axillary LN single-cell suspensions showing CD163+ macrophages expressing CD40, CD86, PDL1, CD95, active caspase-1, or active caspase-3 pre- and post-SIV infection. (B and C) The proportion of macrophages expressing CD40, CD86, PDL1, CD95, active caspase-1, or active caspase-3 in

axillary LNs (B) and mesenteric LNs (C) prior to infection and at weeks 2, 12, and 20 post-infection. Red depicts SIV progressors and blue depicts SIV controllers. Horizontal lines in (C) represent medians. # signifies cross-sectional differences between progressors and controllers and \* signifies longitudinal differences within the same groups. Statistical comparisons were done using a regular two-way ANOVA followed by Sidak's post test (#) or nonparametric one-way ANOVA followed by Dunn's post test (\*). Black asterisks reflect all animals. \*/#  $p < 0.05$ ; \*\*/##  $p < 0.01$ ; \*\*\*/###  $p < 0.001$ .

### **5.2.3 Gut macrophages in SIV infection are not activated or apoptotic**

In our previous study, gut macrophage abundance after viral set-point was strongly correlated with plasma viral load, in contrast to macrophage numbers in LNs. We next determined whether macrophages that accumulate in gut mucosa in progressive SIV infection are differentially activated and apoptotic compared to macrophages in SIV controllers as well as assessed how changes in gut macrophage kinetics relates to changes in LN macrophages. As carried-out in LNs, gut macrophages were identified as CD163-positive by flow cytometry and evaluated for expression of Ki67, CD86, CD95, and active caspases-3 and -1 before and after infection (Fig. 11A). In contrast to axillary and mesenteric LNs, however, there was no alteration in the frequency of gut macrophages expressing CD86 or caspases-3 and -1 and no indication of increased macrophage turnover with SIV infection (Fig. 11B). Acute SIV infection was associated with a temporary increase in the percentage of CD95+ macrophages, but this subsided by week 12.

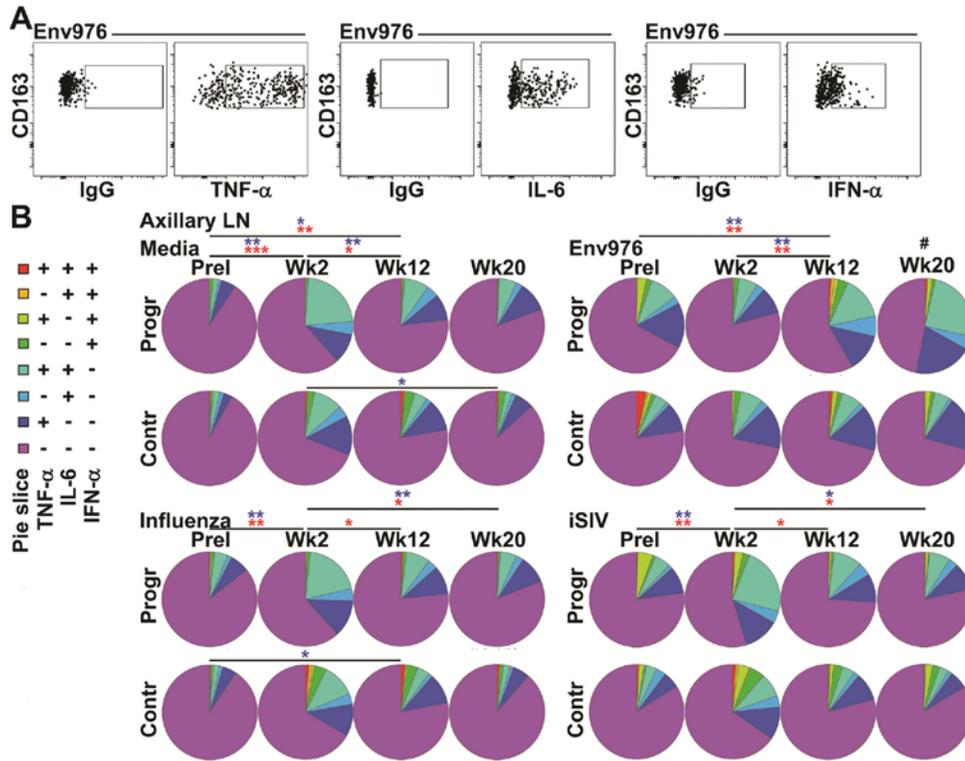


**Figure 11. SIV infection has no impact on macrophage activation or apoptosis in gut mucosa.**

(A) Representative flow cytometric analysis of small intestine single-cell suspensions showing CD163+ macrophages expressing Ki67, CD86, PDL1, CD95, active caspase-1, or active caspase-3 pre- or post-SIV infection. (B) The proportion of macrophages expressing Ki67, CD86, PDL1, CD95, active caspase-1, or active caspase-3 in small intestine. Red depicts SIV progressors and blue depicts SIV controllers. Horizontal lines in (B) represent medians. # signifies cross-sectional differences between progressors and controllers and \* signified longitudinal differences within the same groups. Statistical comparisons were done using a regular two-way ANOVA followed by Sidak's post test (#) or nonparametric one-way ANOVA followed by Dunn's post test (\*). Black asterisks reflect all animals. \*/#  $p < 0.05$ ; \*\*/##  $p < 0.01$ ; \*\*\*/###  $p < 0.001$ .

#### **5.2.4 Increased proinflammatory response by macrophages in axillary LNs during SIV infection**

To determine if controlled and progressive SIV infection is associated with differential inflammatory activity by LN macrophages, we next measured macrophage cytokine response *ex vivo* to viral agonists iSIV, influenza, and Env976 (Fig. 12A). Few LN macrophages from uninfected animals secreted combinations of TNF- $\alpha$ , IL-6, and IFN- $\alpha$  spontaneously but readily produced cytokines following stimulation with Env976 and iSIV (Fig. 12B). After SIV infection, there was a marked increase in the frequency of cytokine+ macrophages in axillary LNs, predominantly expressing TNF- $\alpha$  and IL-6, that remained elevated over pre-infection levels out to week 12. Furthermore, this contrasted with findings in mucosal tissues, where there was little change in cytokine production by gut macrophages with SIV infection and only trends in mesenteric LNs (see Fig. 8, Chapter 2). The percentage of cytokine+ macrophages in axillary LNs also increased after agonist exposure with SIV infection; however, the levels were comparable to cytokines produced spontaneously (Fig. 12B). Unexpectedly, SIV controllers had just as robust a cytokine response as animals anticipated to progress to disease. Only in LNs stimulated with Env976 did we detect differences between groups, with macrophages from SIV progressors producing significantly more cytokines than SIV controllers at week 20. Together, these data are consistent with an inflammatory function for accumulating LN macrophages in SIV infection, however there was no relationship with controlled or progressive disease outcomes.



**Figure 12. Macrophages in axillary LNs produce proinflammatory cytokines during SIV infection.**

(A) Representative flow cytometric analysis of axillary LN single-cell suspensions showing CD163+ macrophage production of TNF- $\alpha$ , IL-6, and IFN- $\alpha$  after Env976 stimulation compared to an IgG control. (B) SPICE analysis of the proportion of axillary LN CD163+ macrophages in culture producing different permutations of TNF- $\alpha$ , IL-6, and IFN- $\alpha$  prior to infection and at weeks 2, 12, and 20 post-infection either spontaneously or after stimulation by Env976, Influenza, or iSIV. Statistical comparisons were done using a permutation test performed with SPICE software. # signifies cross-sectional differences between progressors and controllers and \* signifies longitudinal differences within the same groups. Red asterisks reflect SIV progressors and blue asterisks reflect SIV controllers. \*/#  $p < 0.05$ ; \*\*/##  $p < 0.01$ ; \*\*\*/###  $p < 0.001$ .

### 5.3 DISCUSSION

In this study, we investigated the impact of controlled and progressive SIV infection on macrophage kinetics and function in secondary lymphoid tissues. Our results expand on previous reports of increased macrophage numbers in acutely SIV-infected LNs and demonstrate accumulation occurs regardless of whether macaques go on to control chronic phase viremia

[101, 190]. We also reveal LN macrophages are activated and produce inflammatory TNF- $\alpha$  and IL-6 in SIV infection, supporting the supposition that macrophage recruitment to tissues is harmful and may contribute to disease pathogenesis. Our data however provide nuance to this notion, as we uncover negligible differences in the macrophage inflammatory response between animals anticipated to control or progress to disease. Instead, we propose that while macrophages may contribute to early tissue inflammation, immune activation, and T cell loss in LNs, they do not appear to define a progressive disease outcome.

In addition to responding to TLR ligands *ex vivo* after SIV infection, LN macrophages also produced cytokines spontaneously. This suggests macrophages may be stimulated by the presence of virus in tissues, similar to myeloid DCs in HIV patients [283]. The likeness in macrophage responses between SIV controllers and progressors points towards comparable levels of viral antigen in LNs, at least until week 20. Indeed, prospective studies on viral kinetics in SIV-infected macaques have shown that virus replication is stable in LNs out to day 100 and not reflective of changes in plasma viral load [264]. Cytokines produced by macrophages in chronic infection, however, are likely elicited from the combined exposure to viral and microbial antigens, as *E.coli* products are not detectable in peripheral LNs during the acute stage [90]. Notably, Env976-activated macrophages became increasingly responsive after viral set-point in SIV progressors, achieving statistical significance compared to macrophages from controllers in the chronic stage. Although we did not examine macrophages beyond week 20, these data allude to a potential pathologic role for macrophages in advanced infection that may participate in ongoing T cell activation and associate with AIDS.

The factors driving changes in macrophage dynamics that distinguish the acute and chronic stages of SIV infection are currently unknown. Antiretroviral treatment at day 7 post-

SIV infection inhibits macrophage influx to LNs and is associated with reduced BrdU incorporation by monocytes, suggesting accumulation may be mediated by initial virus expansion and/or virus-induced inflammation [101]. Furthermore, therapy partially reverses expression of several chemotactic factors, including CCL5, which are otherwise upregulated in ART-naïve HIV-infected LNs and promote monocyte extravasation from blood [281, 284, 285]. HIV and SIV infections are associated with an expansion of CCR5-high CD16<sup>+</sup> monocytes, indicating macrophage recruitment in the acute stage might be derived from monocyte trafficking via a CCR5-CCL5 dependent pathway [161, 182, 184]. Interestingly, the percentage of macrophages in LNs declined considerably by week 12, regardless of viral set-point. Our data point towards caspase-3 and caspase-1 dependent cell death playing a role in the macrophage decline, possibly a function of direct viral infection and/or interactions between tissue-associated TNF- $\alpha$  and death-receptor CD95, a mechanism of T cell loss in HIV/SIV infection [286-288].

In our study, infection with SIV had no impact on the proportion of Ki67<sup>+</sup> macrophages in LNs or small intestine. Circulating monocytes therefore likely represent the initial and predominant source of macrophages in LNs during the early stages of infection. Conversely, others have shown that LN macrophages undergo a pronounced regenerative response in advanced chronic infection, defined as well beyond week 20 post-infection, and in AIDS, and is consistent with findings of increased perivascular macrophage proliferation in HIV-infected brain [150, 195, 289]. It is possible macrophage self-renewal may be to counteract increasing monocyte dysregulation due to monocytes becoming progressively more apoptotic with infection and being persistently recruited across multiple tissues, including lung and gut mucosa [244, 284, 290, 291].

Collectively, our data reveals significant anatomical differences in macrophage activation during SIV infection and is in agreement with a cross-sectional comparison of macrophages across multiple tissues [153]. Our data suggest that evidence of macrophage functional and phenotypic heterogeneity in health, governed largely by cytokines derived in the environment, is likewise manifested across a spectrum of responses in disease, including HIV/SIV infection [292-294]. Under homeostatic and inflammatory conditions, tissues serve unique functional roles that also shape macrophage diversity, such as immuno-priming and augmentation in LNs versus immunoregulation in gut mucosa. Accordingly, we observed enhanced co-stimulatory molecule expression by macrophages in both axillary and mesenteric LNs but minimal activation of macrophages in gut mucosa in response to SIV infection. Further comprehensive assessments of macrophages across multiple tissues and different stages of infection will be important to elucidating the contributions of macrophages to disease control and progression.

#### **5.4 MATERIALS AND METHODS**

Information regarding animals, virus infection, tissue sampling and processing, macrophage identification by flow cytometry, and *ex vivo* cell stimulations with virus carried-out in this chapter are reported in Chapter 2. Information regarding analysis of macrophage activation and death by flow cytometry is reported below:

### 5.4.1 Flow cytometry

Single-cell suspensions of LNs and small intestine were stained with the following cross-reactive surface-labeling anti-human antibodies for analysis of CD163+ macrophage activation, turnover, and death (all antibodies were purchased from BD Biosciences unless otherwise noted): CD86 (clone FUN-1), CD40 (5C3), PDL1 (29E.2A3, BioLegend), and CD95 (DX2). Nonviable cells were excluded by staining with a fluorescent LIVE/DEAD cell stain (Invitrogen). Detection of active caspase-1 was conducted using the FAM-FLICA Caspase-1 Assay Kit (ImmunoChemistry Technologies) per the manufacturer's instructions with minor modifications. Briefly, 5 $\mu$ l of 30x FLICA probe was added to 2.5x10<sup>6</sup> cells in 300 $\mu$ L of MACS buffer, incubated at 37°C for 1 h, and then washed thoroughly before surface stain. Intracellular analysis of nuclear protein Ki67 (B56) and active caspase-3 (C92-605) were conducted after permeabilization with Cytotfix/Cytoperm (BD Biosciences). Cells were exposed to 610 $\mu$ W/cm<sup>2</sup> UV-B light for 5 mins as a positive control for apoptosis and pyroptosis. Data were acquired using an LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo 7.6.4.

## **6.0 OVERALL DISCUSSION**

### **6.1 CD163 AS A MARKER OF MACROPHAGES IN SIV INFECTION**

The phenotypic characterization of cells based on surface antigen expression is complicated with respect to macrophages given their remarkable heterogeneity and plasticity across tissues. CD11b, CD68, CD14, calprotectin (MAC387), and CD206 have all been used to some extent to identify macrophages in different organs and in different models; however, antibodies to each of these molecules also overlap in specificity with fibroblasts, endothelial cells, and other leukocytes [295-298]. Alternatively, the markers HAM56, CD169 (Siglec-1), and CD163 are putatively restricted to cells of the monocyte/macrophage lineage, however these markers notably do not encompass all macrophages, and furthermore, macrophages they are expressed on may behave differently depending on the tissue they occupy [298-304].

For this study, we elected to use CD163 to define a subset of macrophages based on a growing body of evidence of the involvement of CD163-positive cells in HIV/SIV infection but an absence of information on their contribution to disease pathogenesis [89, 148, 151, 160, 190, 191, 193, 194, 208, 259, 289, 305-308]. CD163 is a transmembrane glycoprotein and high affinity scavenger receptor for complexes of hemoglobin and haptoglobin in plasma and in tissues [139, 309]. Removal of free hemoglobin is a principal function of macrophages due to its inflammatory and oxidative properties, and CD163's unique ability to mitigate hemoglobin-

induced damage originally contributed to its distinction of recognizing an “anti-inflammatory” macrophage [309, 310]. CD163 has since been reported to also function as an innate sensor for the recognition of some bacteria and viruses and binds and sequesters TNF- $\alpha$  superfamily molecule TWEAK, preventing TWEAK-associated pathologic changes, such as apoptosis [311-313]. Macrophages expressing CD163 have been linked to a myriad of M2-like functions, including the promotion of Th2 polarized responses and the secretion of arginase-1 as well as various angiogenic and tissue growth factors [136, 314]. However, expression of CD163 is not restricted to M2 macrophages, as demonstrated in tissues from patients with chronic venous leg ulcers, where CD163+ cells populate wounds and express TNF- $\alpha$ , iNOS, and IL-12 [315]. In health, certain tissue resident macrophages constitutively express high levels of CD163, including those located in the gastrointestinal tract, bone marrow, liver, and lung [310]. In addition to HIV/SIV infection, increases in CD163+ macrophages and/or macrophage shed sCD163 are associated with acute hepatitis, malaria, and tuberculosis infections, as well as in chronic non-infectious illnesses including Crohn’s disease, rheumatoid arthritis, coeliac disease, and scleroderma [316-321].

While the use of CD163 does not likely reflect the activity or responses of all macrophages in the SIV-infected macaques used in our study, it does allow for the analysis of a discrete population of macrophages, based on a common identifier, that are unmistakably a feature of various inflammatory settings. Others have reported that CD14+CD68+ macrophages accumulate and promote inflammation in colon from AIDS patients, and that CD169+ macrophages capture HIV and trans-infect CD4+ T cells in spleen and lymph nodes [170, 196]. Whether these cells also express CD163 or represent entirely unique subsets of macrophages acting in parallel is not clear and should be the focus of the future hypotheses.

## **6.2 DIFFERENTIAL RESPONSES BY MACROPHAGES EXPRESSING CD163 IN LYMPH NODES AND GUT MUCOSA**

In our model of SIV infection, CD163 defined a population of macrophages that were activated and pro-inflammatory in lymph nodes but not activated and non-inflammatory in small intestine. Furthermore, we found that there was differential recruitment of macrophages between lymph nodes and gut mucosa depending on the stage of infection and disease outcome. These data indicate a compartmentalized response to SIV infection that warrants continued investigation if we are to effectively intervene and alter macrophage activity for the wellbeing of HIV-infected patients.

In the small intestine, we found that quantitative changes in macrophage numbers mirrored plasma viral load kinetics in acute and chronic SIV infection, suggesting a role for viral burden in mediating divergence in macrophage recruitment. Interestingly, CD163<sup>+</sup> and CD68<sup>+</sup> macrophages also accumulate in mucosa from SIV-naive macaques with experimentally-induced colitis and in lamina propria of colon and ileum from individuals with Crohn's disease, ulcerative colitis, and spondyloarthritis [265, 322, 323]. Moreover, while viral RNA in plasma was statistically significant between groups in our study, the level of cell-associated virus in intestine from the same animals was comparable. These data instead point towards a relationship between CD163<sup>+</sup> macrophage recruitment and pathologic changes in gut, such as microbial translocation and/or endogenous cues pertaining to tissue inflammation and injury, and not to the presence of virus [91]. Consistent with this hypothesis, in a pathogenic model of SIV, macaques administered a TNF- $\alpha$  inhibitor subcutaneously had reduced CD163<sup>+</sup> macrophage numbers in peripheral lymph nodes but no change in plasma viremia compared to untreated control animals [190]. It is also possible that the persistent recruitment of macrophages in SIV progressors

reflects a compensatory response by the host to overcome gut macrophage phagocytic defects. Similar observations have been made of CD4<sup>+</sup> T cells, where the continued proliferation of memory CD4<sup>+</sup> T cells in blood and subsequent relocation to tissues counteracts SIV-associated CD4<sup>+</sup> T cell loss and differentiate macaques with stable infection from those that progress rapidly to disease [324]. In conflict with our findings, a separate study found a strong positive correlation between chronic phase plasma viral load and SIVmac251 replication in macaque ileum and rectum but not spleen, peripheral lymph nodes, or mesenteric lymph nodes [264]. Given that the controller sample size was 3 animals and there was a convincing trend towards a similar relationship in our study, it is possible that with the addition of more data points the SIV controller group would have significantly less cell-associated virus in intestine than SIV progressors at week 20.

Although we found changes in gut macrophage numbers that distinguished controllers from progressors, CD163<sup>+</sup> macrophages were not activated or responsive to viral stimuli in either group. In an uninfected healthy gut, the constitutive expression of cytokines TGF- $\beta$  and IL-10 play an obligate role in preventing LPS-induced intestinal damage through their interactions with local cell populations [115, 269]. As illustrated in murine models with gut macrophages engineered to be nonresponsive to IL-10 or TGF- $\beta$  and with macrophages isolated from human intestine, these cytokines avert harmful M1-like responses by suppressing macrophage proinflammatory but not phagocytic or bactericidal activity [116, 270, 325, 326]. In chronic HIV and SIVmac infections, the frequency of TGF- $\beta$  producing T regulatory cells is markedly increased in gut mucosa and corresponds with increased TGF- $\beta$  tissue expression [271, 272, 327]. Mucosal IL-10 levels are also increased in models of SIV infection, however one study reported no change, and in HIV-infected colonic tissues IL-10 is significantly reduced

following antiretroviral treatment [273, 328, 329]. Based on these data, our results may indicate that newly recruited macrophages are adequately tolerized upon arrival to gut, possibly due to robust mucosal IL-10 and TGF- $\beta$  responses to SIV infection and in spite of other alterations in the intestinal cytokine milieu. HIV-associated immuno-suppressive responses have been shown to have unintended consequences, however, such as dampening CD8<sup>+</sup> T cell-mediated antiviral immunity, and is manifest in findings that TGF- $\beta$  levels in intestinal lymphoid organs are positively correlated with rapid progression to AIDS [327]. One limitation of our study is that we did not assess gut macrophage cytokine responses to TLR4 ligands, such as bacterial LPS, after SIV infection. Stimulation of intestinal macrophages from AIDS patients with LPS elicits increased macrophage TNF- $\alpha$ , IL-1 $\beta$ , IFN- $\gamma$ , and IL-18 mRNA expression [196]. IRAK-M, a negative regulator of TLR4-mediated signaling, promotes tolerance towards microbial products in macrophages and is upregulated by macrophages in a human endotoxemia model [330, 331]. To date, no studies have investigated IRAK-M expression in macrophages in the context of HIV and SIV, and it is possible IRAK-M plays a central role in influencing macrophage activity towards gut translocated microbes at different stages of infection.

In contrast to gut mucosa, macrophages in lymph nodes were activated, apoptotic, and inflammatory, primarily in acute infection, however macrophage cytokine production persisted after viral set-point to an extent in both groups. Increases in cell surface CD40 and CD86 associated with macrophage emigration to lymph nodes suggest macrophage participation in antigen-presentation and T cell responses. However, a recent study from our group found that the ability of lymph node macrophages in SIV infection to stimulate naïve allogeneic CD4<sup>+</sup> T cell proliferation is significantly impaired due to defective IL-12 and IFN- $\alpha$  production and not co-stimulatory molecule expression [100]. In agreement with these data, lymph node

macrophages in our study were also poor producers of IFN- $\alpha$ , even after additional *ex vivo* activation. Interestingly, alterations in TGF- $\beta$  and IL-10 expression in HIV/SIV-infected lymph nodes are similar to those reported in intestinal tissues [332-334]. When compared within the same study, however, IL-10 levels in gut mucosa exceeded those in matched lymph nodes from patients with symptomatic acute HIV infection, and therefore might explain in part the differences in macrophage activation between the two compartments [335]. We found that lymph node macrophage frequency declined after viral set-point, following an acute increase in caspase-1 and caspase-3 expression, suggesting cell death. Cell death is a well-defined mechanism of myeloid cell loss in HIV/SIV-infected lymph nodes, mediated by direct virus infection and/or bystander killing depending on the cell type, and is proposed to be the primary mode of macrophage clearance from inflamed tissues [83, 84, 336]. Paradoxically, macrophages are reported to be resistant to the cytopathic effects of virus, contributing to their longevity as a viral reservoir during infection, even after depletion of CD4<sup>+</sup> T cells [173, 174, 337, 338]. It is possible our findings reflect a transient and beneficial reaction by the host to clear an overabundance of macrophages in acutely-infected lymph nodes and that the residual macrophage population is more resilient to death [336].

### **6.3 MONOCYTES AS A SOURCE OF GUT MACROPHAGES**

Integrin  $\alpha 4\beta 7$  provides an important pathway for circulating CD4<sup>+</sup> T cells and other blood leukocytes to home to mucosal tissues [339]. Recently, its expression on plasmacytoid DCs in the context of HIV/SIV infection was shown to associate with plasmacytoid DC accumulation in colorectum and intestine [88]. In our study, the induction of  $\alpha 4\beta 7$  on monocytes in response to

SIV infection coincided with a prominent increase in intestinal CD163<sup>+</sup> macrophages. We further found that expression of CD163 on  $\alpha 4\beta 7$ <sup>+</sup> monocytes, specifically the CD16<sup>+</sup>CD14<sup>-</sup> subset, paralleled differences in macrophage numbers between groups. Collectively, these observations suggest that accumulating CD163<sup>+</sup> macrophages in SIV-infected intestine are derived from monocyte extravasation mediated by  $\alpha 4\beta 7$ .

The mechanisms by which infection influences the recruitment of CD16<sup>+</sup> monocytes to gut mucosa in SIV infection are currently not clear [91]. Studies investigating the effects of tolerance in cancer patients show that intravenous administration of LPS alters the ratio of monocyte subsets in favor of CD16-positive monocytes, suggesting a role for microbial translocation [340]. In mice, CD16-equivalent monocytes with attenuated inflammatory activity are recruited to heart with myocardial ischemic injury and promote angiogenesis and collagen deposition, suggesting tissue damage may also be driving recruitment [341]. Interestingly, the balance of non-inflammatory CD16<sup>+</sup>CD14<sup>-</sup> monocytes, which dominant blood in acute and chronic SIV infection as reported by others, is skewed in favor of the inflammatory CD14-expressing monocyte subsets in SIV/AIDS [161]. This would be consistent with the progression from a non-inflammatory CD163<sup>+</sup> macrophage found in acute and chronically SIV-infected intestine to a TNF- $\alpha$ <sup>+</sup> macrophage in gut with AIDS in our study. Continued research into the signals that elicit the differential migration of specific monocyte subsets to SIV-infected mucosa could expose novel avenues for therapeutic manipulation of downstream macrophage responses.

Our study does not address whether monocytes utilize other mechanisms in unison with the  $\alpha 4\beta 7$  pathway to traffic to intestine. Altered CCR2/CCL2 and CD31/ICAM-1 expression also induce monocyte emigration to inflamed mucosal tissues, as demonstrated in inflammatory bowel disease (IBD) and tuberculosis models, and associate with quantitative increases in

macrophages [248, 342, 343]. In chronic HIV-infected duodenum, CCL2 protein levels are elevated in conjunction with intestinal macrophage numbers and blood monocyte CCR2 expression [89]. Chemokine receptor CCR9 also plays a role in peripheral leukocyte trafficking to mucosa, responding to its ligand CCL25, however CCL25 is expressed poorly in terminal ileum, the segment of small intestine examined in our study [344, 345]. Interestingly, SIV infection results in the upregulation of the CCR5 ligand CCL5 in lymph nodes, lung, and intestine [346]. It is possible that CD16<sup>+</sup> monocytes, which selectively express CCR5, are drawn to lymph node or gut contingent on the expression of  $\alpha 4\beta 7$  in HIV/SIV infection [159-161]. The homing of  $\alpha 4\beta 7$ <sup>+</sup> cells to gut is governed by its interaction with MAdCAM-1 on gut endothelial venules [347]. While we did not investigate MAdCAM-1 in our study, its levels are markedly elevated in duodenum from AIDS patients with or without ART as well as in other mucosal inflammatory conditions [347, 348]. Lastly, our conclusion that macrophages are derived from  $\alpha 4\beta 7$ -expressing monocytes is further supported by findings that *in vivo* treatment with an anti- $\alpha 4$  integrin antibody significantly reduces CD68<sup>+</sup> macrophage burden in gut, which independent studies have shown also express CD163, in addition to heart and brain of SIV-infected macaques, and has similar effects in mice and humans [162, 349-352].

In addition to being an important pathway for peripheral cells to home to mucosal tissues,  $\alpha 4\beta 7$  can also serve as a receptor for HIV binding and facilitate dissemination of HIV/SIV to gut-associated target cells [353, 354]. Recent studies in SIV-infected macaques reveal that ART treatment in combination with an anti- $\alpha 4\beta 7$  antibody suppresses viral replication in plasma and gastrointestinal tissues to undetectable levels compared to ART alone, even after discontinuation of  $\alpha 4\beta 7$  antibody therapy [280]. Moreover, the authors report the restoration of intestinal CD4<sup>+</sup> T cell populations, including Th17 and Th22 cells, important for intestinal barrier health,

however they do not comment on the integrity of the intestine following therapy discontinuation [92, 95, 165]. These findings could have implications for  $\alpha 4\beta 7$ -driven macrophage recruitment to gut in SIV progressors. Our data suggest that a reversal of gut epithelial damage in pathogenic SIV infection, potentially a function of reconstituted Th17 and Th22 cell subsets, could associate with diminished monocyte/macrophage recruitment to intestine and restored phagocytic function.

#### **6.4 ROLE OF MACROPHAGE INFLAMMATORY ACTIVITY IN CONTROLLED AND PROGRESSIVE SIV INFECTION**

Macrophages produce low levels of inflammatory mediators in health and quantitative increases in their numbers and cytokine activity feature prominently in inflamed tissues. Inflammation plays a vital role in accelerating host defense and pathological healing but can simultaneously exacerbate these processes if inadequately controlled [355]. Bacterial infections, for example, benefit from an early and robust inflammatory response by M1-like macrophages, producing nitric oxide, TNF- $\alpha$ , and IL-1 $\beta$  upon activation to stymie growth of *Salmonella typhi*, *Mycobacterium tuberculosis*, and *Listeria monocytogenes*, among others [127-129]. Alternatively, excessive stimulation of macrophages in the context of bacterial infection can result in macrophage-mediated sepsis via cytokine storm [356]. Newly-emigrated macrophages are often a source of inflammation in disease, supplemented by monocytes responding to chemotactic cues either derived from the pathogen itself or in response to pathogen-induced inflammatory mediators [357]. Studies in murine models demonstrate that inhibition of monocyte recruitment, either through antibody-blockade or vector-mediated suppression, reduces macrophage burden in atherosclerotic lesions and regresses disease pathology [358, 359].

In HIV/SIV infection, monocytes and macrophages can be activated by certain pathogen-associated antigens to produce inflammatory mediators within the environment. LPS is an outer membrane component of gram-negative bacteria that is present in tissues and in circulation during infection, can activate macrophages through TLR4, and is strongly correlated with measures of immune activation and progression to disease [91]. Similarly, HIV-derived ssRNA nucleotides activate monocytes/macrophages through TLR8, and *in vitro* co-culture of monocytes with naïve CD8<sup>+</sup> T cells in the presence of HIV oligonucleotides significantly increases CD8<sup>+</sup> T cell activation, an effect not seen when monocytes are excluded [360]. Nevertheless, to date, few studies have addressed the macrophage response in the context of stable infection, leaving the question of the extent of macrophage involvement in disease-associated inflammation and AIDS pathogenesis ill-defined.

In our study, we find an increased frequency of proinflammatory macrophages coincident with macrophage influx to lymph nodes in acute SIV infection compared to in health. Even with the onset chronic infection, macrophages continued to produce TNF- $\alpha$ , IL-6, and IFN- $\alpha$ , however macrophage frequency had declined to pre-infection levels, reducing the relative magnitude of their contribution to overall lymph node inflammation. A hallmark of acute HIV/SIV infection is the pronounced expression of proinflammatory genes and cytokines in lymphoid organs, including TNF- $\alpha$ , IL-6, and IFN- $\alpha$ , which our data suggest are partially macrophage in origin [361]. While these results point towards a pathologic function for lymph node macrophages in SIV infection, this effect was observed in both controllers and progressors, and therefore may instead reflect an orderly inflammatory response intended to amplify surrounding immune activity and mediate antiviral host defense. The sustained spontaneous production of inflammatory mediators by macrophages above pre-infection levels in the chronic stage,

however, may offset any benefit of an initial protective effect and ultimately perpetuate inflammation-induced tissue damage and immunopathology. Interestingly, when compared to macaques with controlled SIV infection, we found that Env976 stimulated macrophages in SIV progressors became increasingly responsive in the chronic stage. These findings demonstrate that lymph node macrophages may eventually contribute to tissue inflammation through chronic virus stimulation of TLR8. Conversely, lymph node macrophages were not responsive to influenza *ex vivo*, which activates innate cells through TLRs 3,7/8 and RIG-I, suggesting macrophages after SIV infection may be unable to contribute an innate response towards opportunistic pathogens.

In contrast to macrophages in SIV-infected lymph nodes, newly recruited CD163+ macrophages in small intestine were functionally non-inflammatory. Patients with IBD present with similar enteropathies as found in HIV infection and in SIV progressors from our study, such as intestinal breach, microbial translocation, and a pronouncement of macrophages in mucosal tissues, particularly around blood vessels suggesting monocyte origin [322, 362, 363]. In IBD, however, aberrant gut macrophage cytokine activity is a key proinflammatory pathway driving disease pathology, and our data would suggest the contrary for gut macrophages in acute and chronic SIV infection [364]. One limitation of our study is that use of CD163 may preclude the possibility of the recruitment of multiple macrophage subsets to gut mucosa during infection, some of which may participate in SIV-associated intestinal inflammation. Indeed, CD163+ cells also become abundant in intestinal tissues from IBD patients, however whether they convey a pro- or anti-inflammatory function was not addressed [322, 323]. Instead, IBD-related inflammation appears to be amplified by an accumulation of CD14-high TREM-1+ macrophages, consistent with one finding in colon from AIDS patients, and which do not express

CD163 [196, 250, 263, 365, 366]. Another possibility could be that macrophage subsets are differentially recruited along the gastrointestinal tract in response to infection. The aforementioned studies on inflammatory macrophages in AIDS or IBD focused on a region of the large intestine, which differs considerably in function, structure, and microbiota composition compared to small intestine [86, 367]. Consistent with this hypothesis, in health and in chronic SIV infection, colonic macrophages were found to differ phenotypically and functionally from macrophages in jejunum [153].

In spite of this finding, in the context of AIDS, we found evidence that intestinal CD163+ macrophages express TNF- $\alpha$  and IFN- $\alpha$ , consistent with an inflammatory function. Indeed macrophage function at one stage of disease is not always reflective of their function in a later stage, as demonstrated in a model of inflammatory lung disease, suggesting macrophages have the capacity to switch phenotypes as disease progresses [368]. Consistent with this notion, monocyte expansion in acute and chronic SIV infection favors the CD16+CD14- subset but in AIDS switches in favor of CD14-expressing monocytes [161]. Our data may suggest that the persistent accumulation of CD163+ macrophages associated with a progressive disease course gradually becomes pathologic as infection advances and thus participates in mucosal inflammation. This response might be mediated in part by impaired monocyte maturation through decreased CD16 expression and/or a gradual buildup of antigenic debris which could chronically stimulate macrophages, perhaps a consequence of an impaired phagocytic response established earlier in infection. It is also possible that variation in macrophage responses between infection and AIDS in gut mucosa could be a function of the presence of opportunistic infections associated with disease. Although enterocyte loss was evident in SIV progressors during the early chronic stages of infection (week 12), macrophage-secreted TNF- $\alpha$  in AIDS

could perpetuate disruption of the epithelial repair process and prevent reformation of the intestinal barrier [132].

A limitation of our study is that we did not investigate the production of anti-inflammatory (TGF- $\beta$ , IL-4, IL-10, IL-13, arginase-1) or other pro-inflammatory (IL-1 $\beta$ , IL-12, iNOS) cytokines by lymph node and gut macrophages. CD163+ macrophage accumulation in SIV-infected lymph nodes and heart is associated with increased tissue fibrosis, which could be driven by macrophage-derived TGF- $\beta$  [151, 190]. Moreover, it is possible that gut macrophage production of tissue growth factors, such as HGF, played a role in the differential maintenance of the intestinal barrier between controllers and progressors [144]. Future studies should aim to provide a more comprehensive and complete assessment of macrophage cytokine responses in the context of controlled and progressive disease outcomes to fully appreciate the role of macrophages in HIV/AIDS pathogenesis.

## **6.5 CHANGES IN MONOCYTES/MACROPHAGES THAT PRECEDE DISEASE PROGRESSION**

In chronic HIV and SIV infections, the frequency of CD163+CD16+ monocytes in blood is proposed as a biomarker for AIDS progression given its strong correlation to plasma viral load [156, 160, 161, 369]. Based on similar correlations, other groups have reported increases in monocyte/macrophage activation as measured by plasma sCD163 levels to be an early indicator of disease prognosis and AIDS-associated dementia [191, 194, 195, 370]. While these data are seemingly contradictory given sCD163 is shed by monocytes/macrophages, studies have shown that sCD163 levels inversely correlate with surface CD163 on CD16-CD14+ monocytes and not

CD16<sup>+</sup>CD14<sup>-</sup> monocytes [371]. Notably, monocyte upregulation of CD163 may reflect maturation into tissue macrophages [259, 372]. Our data expand on this body of knowledge and demonstrate that macrophage activation in mesenteric lymph nodes and increased CD16<sup>+</sup> monocyte CD163 expression are apparent prior to viral set-point in animals that eventually develop progressive infection. Furthermore, we found that CD163 expression on CD16<sup>+</sup> monocytes remained elevated after set-point and associated with viral burden, consistent with the previously mentioned studies. These data indicate that early changes in monocytes/macrophages might influence or predict disease outcome and therefore could have prognostic value after HIV infection. Although isolating mesenteric lymph nodes for evaluation of macrophages is not feasible, an assessment of monocytes in blood could conceivably be paired with other predictive measures of disease progression, such as T cell proliferation and viremia, to more reliably inform drug treatment commencement and efficacy [373]. In addition, with insight into the relationship of monocyte CD163 expression and tissue CD163<sup>+</sup> macrophages in SIV infection, particularly in the small intestine, clinicians hoping to target macrophages for therapeutic purposes could potentially use monocytes as a window into gut-associated macrophage activity prior to and after treatment.

## **6.6 MECHANISMS OF MACROPHAGE DYSFUNCTION**

The mechanisms underlying macrophage dysfunction in HIV/SIV infection are not well understood and are likely complex. A popular hypothesis is that infection of macrophages contributes to defects in function. Monocytes and macrophages are permissive to HIV *in vitro* and infected macrophages are readily found in tissues, including lymph nodes, brain, and vagina

[374-376]. *In vitro* macrophage exposure to viral proteins p24, Nef, and Tat are independently implicated in reducing phagocytic function, possibly by downmodulating Fc $\gamma$ R-mediated signaling and/or mannose receptor expression [377-382]. Relative to CD4<sup>+</sup> T cells, however, the proportion of SIV gag DNA<sup>+</sup> macrophages across tissues is considerably minor [163]. Only in the absence of CD4<sup>+</sup> T cells do macrophages emerge as the primary targets of viral infection, as established in separate macaque models involving experimental depletion of CD4<sup>+</sup> T cells or use of a highly pathogenic SIV/HIV chimera [383, 384]. Interestingly, macrophage susceptibility to viral infection differs across tissues, with myeloid cells in spleen being highly susceptible and macrophages in the small intestine being almost entirely resistant due to TGF- $\beta$  mediated downregulation of macrophage CCR5, CD4, and Nf- $\kappa$ B [163, 268, 385, 386].

Information concerning the effects of HIV/SIV infection on macrophages ability to secrete cytokines and the mechanisms underlying these responses are inconsistent. Gene expression of LPS-related pattern recognition receptors TLR4 and CD14 are downregulated from unseparated PBMC after SIV infection, and alveolar macrophages isolated from HIV-infected lung have reduced responsiveness to TLR2/4 stimulation [203, 387]. Similarly, LPS-mediated activation and secretion of proinflammatory cytokines by monocyte-derived macrophages is inhibited by exposure to HIV Tat [201]. Instead, Tat and gp120 stimulation of monocyte-derived macrophages from healthy individuals is shown to induce anti-inflammatory IL-10 production [206, 207]. Interestingly, monocytes isolated from healthy donors and stimulated with LPS after *in vitro* infection with HIV, however, display heightened proinflammatory potential [201]. A separate study in untreated HIV infection showed that PBMCs from viremic patients have increased TLR6, 7, and 10 expression, and that individuals with advanced disease also upregulate TLR2, 3, and 4, which is reversed after viral suppression [388]. Moreover,

stimulation of monocytes from HIV-infected individuals through TLR2 results in increased TNF- $\alpha$  production [389].

In our study, macrophage function was assessed within the first 20 weeks of infection, a timepoint when tissue CD4+ T cell reservoirs are not as depleted as in AIDS. Macrophages in gut mucosa, but not mesenteric lymph nodes, had impaired phagocytic activity that was associated with progressive SIV infection, despite both sites harboring detectable SIV gag RNA. Although macrophages are not permissive to HIV/SIV infection in small intestine, some evidence suggests newly recruited macrophages can be infected as monocytes prior to differentiation [182, 183]. CD16+ monocytes are more susceptible to infection than other monocyte subsets due to their expression of CCR5, and our data indirectly suggest that recruitment of this subset to gut mucosa is associated with phagocytic impairment [160, 184]. Overall however monocytes represent an insignificant fraction of infected cells in blood compared to other susceptible cells in circulation, and therefore macrophage abnormalities as a result of direct monocyte infection are probably unlikely [385]. Furthermore, macrophages in both mesenteric lymph node and gut mucosa expressed little to no SIV p27 by flow cytometry in our study. An alternative explanation could be that monocyte/macrophage engagement of viral proteins is negatively affecting their function by impacting the endocytic pathway and/or receptors that mediate phagocytosis. Phagolysosomal fusion is significantly impaired after exposure of monocyte-derived macrophages taken from HIV-infected individuals to recombinant gp120 [390]. Indeed, there is a trend towards more intestinal cell-associated virus in progressors compared to controllers in our study and significantly more virus in plasma for gut-homing monocytes to encounter. Furthermore, macrophage expression of mannose receptor, which facilitates non-opsonic binding and uptake of pathogen debris is reduced in HIV-infected lung

and correlated with an inability to phagocytose *Pneumocystis carinii* [391]. Observations of reduced complement-mediated phagocytic efficiency by monocyte-derived macrophages have also been reported in response to HIV infection [392]. It is possible newly differentiated macrophages might be abnormal even before accumulating in inflamed HIV/SIV-infected mucosa as a consequence of monocyte dysfunction. Monocytes have reduced phagocytic function in the context of HIV infection, before or after TLR4 activation, compared to HIV-naive controls and the extent of monocyte impairment is accentuated in individuals with plasma viral loads  $>10^4$  RNA copies/mL [393]. Interestingly, myeloid DCs and T cells have largely normal function in viremic nonpathogenic SIV models, indicating that environmental factors, such as differential levels of tissue inflammation, might play a more important role in influencing macrophage functionality than the presence of virus [20].

Macrophages in lymph nodes from our study spontaneously produced TNF- $\alpha$ , IL-6, and IFN- $\alpha$  with infection, however comparatively, the levels of cytokines produced after *ex vivo* stimulation with viral agonists remained mostly unchanged. Only with Env976-stimulated macrophages at week 20 did we see differences between controllers and progressors. These data are mostly consistent with findings by others that lymph node macrophages are non-responsive or poorly responsive to TLR7/8 ligands after infection and that macrophages lose the ability to secrete IL-12 important for eliciting Th1 responses [100, 101]. The lack of information on lymph node macrophages, as compared to monocyte-derived or cell-line macrophages, makes it difficult to speculate on the molecular mechanisms underlying these data. Interestingly, one study found that activation of monocyte-derived macrophages after *in vitro* HIV infection occurs through signaling pathways independent of TLRs, including calcium and MAPK, which can also induce Nf- $\kappa$ B expression [394]. It is possible that HIV evasion of TLR-signaling may

simultaneously involve suppressing TLR recognition, resulting in muted responses to external stimulation by TLR agonists seen by us in lymph nodes. However, while we did not quantify macrophage infection in peripheral lymph nodes, macrophages in gut mucosal tissues were mostly devoid of virus. Studies investigating TLR-expression and molecules that inhibit TLRs, such as IRAK-M, are urgently needed using sorted macrophages from tissues in the context of stable and progressive HIV/SIV infection to provide clarity on mechanisms underlying macrophage responses.

## **6.7 ROLE OF GUT MACROPHAGE PHAGOCYTTIC IMPAIRMENT IN PROGRESSIVE INFECTION**

Outside of the gut, reports of macrophage phagocytic defects are numerous in HIV/AIDS patients, depicting a potent inability to uptake and kill opportunistic pathogens such as *Toxoplasma gondii*, *Staphylococcus aureus*, *Pneumocystis carinii*, and *Histoplasma capsulatum* [197-199, 391, 395-397]. These data suggest that the high incidence of opportunistic infections in AIDS is in part a consequence of an inadequate host defense by macrophages. Blood monocytes have also been reported impaired at various stages of HIV infection and thus implicated in inefficient pathogen control, although some groups report normal function [393, 398, 399]. In our study, phagocytic impairment of macrophages in gut mucosa was evident at early chronic infection (week 20) in SIV progressors and was associated with intestinal macrophage burden, enterocyte loss, and plasma viral load. A link between decreased macrophage phagocytic capacity, intestinal integrity, and microbial translocation was established in two recent studies using disparate animal models. In the first study, the authors analyzed LPS-

burden in SIVmac251-infected mucosal (colon, mesenteric lymph nodes) and peripheral (liver, axillary lymph nodes) tissues taken from rhesus macaques necropsied at early acute infection (<10 day post infection), late acute infection (14 to 28 days post infection), chronic infection without disease (168 to 396 days post infection), and AIDS [90]. They found that measures of *E.coli* were present in lamina propria of colon and in mesenteric lymph nodes by late acute infection but were not present in other tissues. In animals with chronic infection and AIDS, however, *E.coli* products could be readily detected in mucosal and peripheral sites and corresponded with reduced *in situ* co-localization with HAM56+ macrophage in the gut. In a separate study, the authors compared outcomes in HIV-infected humanized mice with mice treated with dextran sodium sulfate (DSS) to induce intestinal damage independent of infection [278]. Bacterial LPS levels were elevated in plasma from HIV-infected mice but only detected in DSS-treated mice after macrophages had been selectively depleted. *Ex vivo* analysis of liver macrophage function from both groups revealed that impaired macrophage phagocytic function differentiated HIV-infected mice from DSS-treated mice. Coupled with our findings of intact macrophage function in intestine from SIV controllers, these data collectively suggest a model in which a defective macrophage phagocytic response contributes to the diffusion and subsequent deposition of gut-associated microbes throughout the body in HIV/SIV infection.

Pathogen-mediated inflammation is not likely to be the only consequence of impaired macrophage functionality. Macrophage removal of apoptotic waste, a process known as efferocytosis, averts secondary necrosis caused by the release of noxious and immunogenic contents from within dead and dying host cells [112]. Defective clearance of host-derived apoptotic/necrotic debris by macrophages underlies the pathogenesis of neurological disorders, obesity, atherosclerosis, and inflammatory lung disease [400-403]. A study in mice found that a

deficit in macrophage ability to clear apoptotic cells at the site of diabetes wounds delays the wound healing process and prolongs inflammation [404]. In HIV-infected individuals, monocyte-derived macrophages have a decreased capacity to uptake apoptotic neutrophils, which studies in nonhuman primates demonstrate accumulate in blood from animals with pathogenic but not nonpathogenic SIV infection [405, 406]. Moreover, *in vivo* analysis of cell death by TUNEL staining in HIV-infected lymph nodes revealed TUNEL expression is strongly correlated with follicular hyperplasia and germinal center formation, and the authors posit that a build-up of apoptotic debris in lymph nodes could magnify immune activation through nonspecific stimulation of cells [407]. Consistent with this hypothesis, in our study, intestinal cell loss, a pathology driven by caspase-3 dependent apoptosis, was strongly correlated with gut macrophage abundance, suggesting macrophage recruitment to gut may be driven by increased enteropathy [328, 408]. It remains unclear whether increased cell death in HIV/SIV infection is a cause or consequence of chronic inflammation or reflects a positive feedback loop. A recent study suggesting cell death contributes to the cycle of inflammation found that non-permissive quiescent CD4<sup>+</sup> T cells, as opposed to activated CD4<sup>+</sup> T cells, selectively undergo caspase-1 dependent “pyroptotic” cell death in lymphoid tissue [409]. Caspase-1 activates the release of IL-1 $\beta$  and IL-18 from cells and initiates a highly inflammatory form of cell death intended to restrict bacterial growth but that can be detrimental if unregulated [282]. Although it would be difficult to quantify the relative burden of host cell waste as a source of inflammation and its relationship to macrophage function, it is clear that HIV/SIV infection induces widespread apoptosis of cells, and that macrophage ability to alleviate cellular debris through phagocytosis in animals progressing to disease, as demonstrated by us and others, is significantly compromised.

## 7.0 PUBLIC HEALTH SIGNIFICANCE

Over 37 million people worldwide are currently infected with HIV, a virus that causes prolonged and irreversible damage to immune function and tissue health throughout the body [15, 410-412]. Macrophages are effector cells of the innate immune system that participate in tissue maintenance and host defense by orchestrating inflammatory processes and ingesting cellular and foreign debris [114]. Alterations in macrophage biology are a hallmark of HIV/SIV infection, yet to date their heterogeneity across tissues has complicated interpretations of their role in disease control and progression [413]. This is a critical impediment that undermines our knowledge of HIV/AIDS pathogenesis and highlights the need for comprehensive, comparative, and systematic analysis of the macrophage response in different disease contexts across multiple tissues. In this study, we found evidence that suggests the macrophage cytokine response contributes to the pathologic process of inflammation in lymph nodes in acute and early chronic SIV infection and in small intestine with AIDS, but likely does not significantly influence the course of disease. Moreover, we found in SIV-infected small intestine, but not in mesenteric lymph nodes, that inadequate macrophage phagocytic function strongly correlated with a progressive SIV disease course and therefore could indirectly have proinflammatory consequences. Collectively, these findings suggest that therapeutic strategies that preserve or regenerate macrophage phagocytic capacity may promote reduced pathology in tissues, including macrophage-derived inflammation in lymph nodes, and ultimately facilitate enterocyte renewal

and progress towards immune homeostasis. One approach supported by proof of concept studies in rats proposes exploiting the specificity and function of receptor CD163 as a mechanism for drug entry into CD163+ macrophages [310].

## APPENDIX: PUBLICATION LIST

**Swan ZD**, Wonderlich ER, Barratt-Boyes SM. Macrophage accumulation in gut mucosa differentiates AIDS from chronic SIV infection in rhesus macaques. European Journal of Immunology. 2016 Feb;46(2):446-54.

Wonderlich ER, **Swan ZD**, Bissel SJ, Hartman AL, Carney JP, O'Malley KJ, Obadan AO, Santos J, Walker R, Sturgeon TJ, Frye Jr LJ, Maiello P, Scanga CA, Bowling JD, Bouwer AL, Duangkhae PA, Wiley CA, Flynn JL, Wang J, Cole KS, Perez DR, Reed DS, Barratt-Boyes SM. Widespread virus replication in alveoli drives acute respiratory distress syndrome in aerosolized H5N1 influenza infection of macaques. Journal of Immunology. 2016. Accepted.

**Swan ZD**, Bouwer AL, Wonderlich ER, Barratt-Boyes SM. Gut macrophage accumulation and phagocytic dysfunction is predictive of progressive disease in SIV-infected rhesus macaques. European Journal of Immunology. 2016. In the process of being submitted.

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