

**DEFINING DENDRITIC CELLS AND MACROPHAGES IN LYMPH NODES AND
GUT MUCOSA DURING SIMIAN IMMUNODEFICIENCY VIRUS INFECTION**

by

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The gut mucosa is an important site of virus replication and immune activation in individuals infected with human immunodeficiency virus (HIV), but the impact of infection on innate immune cells within the gut and gut-draining mesenteric lymph nodes (LN) is ill-defined. To address this gap in knowledge, we studied rhesus macaques infected with pathogenic simian immunodeficiency virus (SIV), which is a highly relevant model of HIV infection and AIDS. We analyzed macrophage, myeloid dendritic cell (mDC), and plasmacytoid dendritic cell (pDC) subsets in LN and terminal ileum samples taken from naive rhesus macaques and macaques infected with SIVmac251 with and without antiretroviral therapy (ART) to reduce virus load. CD163⁻CD68⁺ macrophages did not fluctuate with disease, while putative anti-inflammatory CD163⁺CD68⁺ macrophages were significantly increased in mesenteric and peripheral LN, which may be suggestive of the host's attempt to repress inflammation. CD11c⁺ mDC and CD123⁺ pDC in peripheral LN exhibited a modest decrease during acute SIV infection and AIDS. Lastly, we determined the percentage of CD103⁺ mDC, which have been shown to induce regulatory T cell (Treg) differentiation in mesenteric LN and imprint Treg with gut-homing markers. CD103 was expressed on 31% of mDC in mesenteric LN of healthy macaques and a similar proportion of mDC in SIV-infected animals receiving therapy, whereas CD103⁺ mDC were depleted in untreated SIV infection, suggesting a role for CD103⁺ mDC loss in

altering Treg homeostasis. Our results highlight the complexity of the innate immune response to SIV in mucosal tissues and point to contributions of macrophages and DC in virus control and AIDS pathogenesis. These findings could lead to discoveries of novel innate immune therapeutic targets and therefore have significant public health value.

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1.0 INTRODUCTION

1.1 HIV-1: EPIDEMIOLOGY

Human immunodeficiency virus (HIV) type 1, the etiologic agent of acquired immunodeficiency syndrome (AIDS), infects over 34 million people worldwide with 2.7 million new cases reported in 2011. Prevalence rates are highest in parts of Africa, particularly Sub-Saharan Africa, where 67% of infected individuals reside (1, 2). HIV is a lentivirus in the family *Retroviridae* with an RNA genome and the capacity to integrate into host DNA. Due to the error-prone nature of its reverse transcriptase step during synthesis, HIV exhibits high genomic heterogeneity that allows for rapid evasion of host defense mechanisms and the selection of therapy resistant mutations (3). Currently, a preventative vaccine does not exist. Today, public health efforts to stem the spread and morbidity of HIV/AIDS are geared primarily towards educating the public of safe sex practices, teaching pre-exposure prophylaxis strategies to high risk communities, and employing secondary prevention methods to decrease years of potential life lost (4, 5). However, these efforts are costly. In 2002, new HIV infections in the United States resulted in over \$36 billion dollars in medical expenses and lost worker productivity (6). Antiretroviral therapy (ART), commonly a cocktail of non-nucleoside reverse transcriptase inhibitors, nucleoside reverse transcriptase inhibitors, and protease inhibitors that directly target different stages of the viral life cycle, has been critical to postponing the onset of AIDS and prolonging the life expectancy of

HIV⁺ individuals (7). Yet, despite these advances in therapy, our understanding of what drives HIV pathogenesis is still very limited and ultimately crucial to uncovering how to therapeutically minimize new infections and prevent the onset of AIDS.

1.2 THE SIV MODEL

Numerous animals have been used as models to forward HIV research (8). Mice are a sensible animal model to study various pathogens because they are inexpensive and easy to manipulate. However, they are not susceptible to HIV, nor do they progress to AIDS naturally (8). Moreover, attempts to genetically alter or humanize mice and adapt HIV strains to mimic the human disease course obscure the complexities of the human immune response to HIV, making them unsuitable (9-11). Other animal models, such as cats, have similar disadvantages to being used (12). No perfect model exists, but at present, non-human primates have offered the best surrogate for HIV studies. Rhesus macaques are non-natural hosts of the HIV simian analog, SIV, that progress to AIDS and develop pathologic features similar to human disease, including CD4⁺ T cell depletion and chronic immune activation. Alternatively, SIV-infected sooty mangabeys and African green monkeys, each natural hosts of SIV, lack these pathologic disease hallmarks and therefore provide critical insight into the healthy co-existence of host and virus (13). Expectedly, there are a few drawbacks to using the rhesus macaque model to study pathogenic infection. One is that the SIV challenge dose typically used is much larger than the amount of an average human HIV exposure, therefore an assumption must be made that macaques and humans respond similar to infection regardless of dose (14). A second drawback is that many SIV studies are conducted using cell-free virus, which does not mimic natural, cell-

associated viral transmission (15). Nevertheless, research conducted using non-human primates has contributed tremendous scientific advancements to the field and continues to be a preferable model for HIV studies (16).

1.3 HIV/SIV PATHOGENESIS

Sexual contact is the most common mode of HIV transmission, though a large number of cases each year occur by exposure to contaminated blood and vertically, from mother to newborn (17). Upon infection, HIV replication predominately takes places in CD4⁺ T lymphocytes, key players of the adaptive immune system (18). The pathogenesis of HIV and SIV can be divided into an acute stage, a clinically latent stage, and AIDS. While rhesus macaques share these disease stages with humans, macaques generally progress to AIDS at a more accelerated rate (19, 20). In humans, acute infection manifests roughly 2-4 weeks post-exposure and is characterized by high plasma viremia and rapid CD4⁺ T cell death (17, 21). Soon after, cytotoxic T lymphocytes of the adaptive immune system are mobilized to regulate the spread of virus and CD4⁺ T counts rebound slightly, establishing a period of clinical latency. The duration of latency can differ dramatically from person to person depending largely on host genetics but on average lasts 10 years without treatment (22, 23). Overtime, however, the virus overwhelms the immune system putting it into a state of chronic activation. Massive CD4⁺ T cell depletion and chronic immune activation, defined by high levels of serum pro-inflammatory cytokines and an increased frequency of activated T and B cells in circulation, are strong indicators of disease progression (24-28). According to the Centers for Disease Control and Prevention, a clinical diagnosis of

AIDS is defined as CD4⁺ T cells falling below 200 cells/mm³ and accounting for less than 14% of all white blood cells and the emergence of at least one opportunistic infection (29).

An important component of HIV/SIV pathogenesis is the dysfunction of gut immune homeostasis (30). The gastrointestinal (GI) tract is both the body's largest lymphoid organ and its most exposed surface to the external environment (31). It also contains the largest reservoir of CD4⁺ T cells, which are particularly enriched in the intestinal lamina propria and gut-associated lymphoid tissues, such as Peyer's patches, which serve as important immune induction sites to the gut (32-34). Mucosal columnar epithelial cells at the interface of the GI tract and lumen are home to millions of commensal microorganisms that are crucial to fundamental GI processes such as digestion, metabolism, and host defense (35, 36). Figure 1 demonstrates the structure of gut terminal ileum, a region of the small intestine with a high density of Peyer's patches, taken from a rhesus macaque and stained with Hoechst dye.

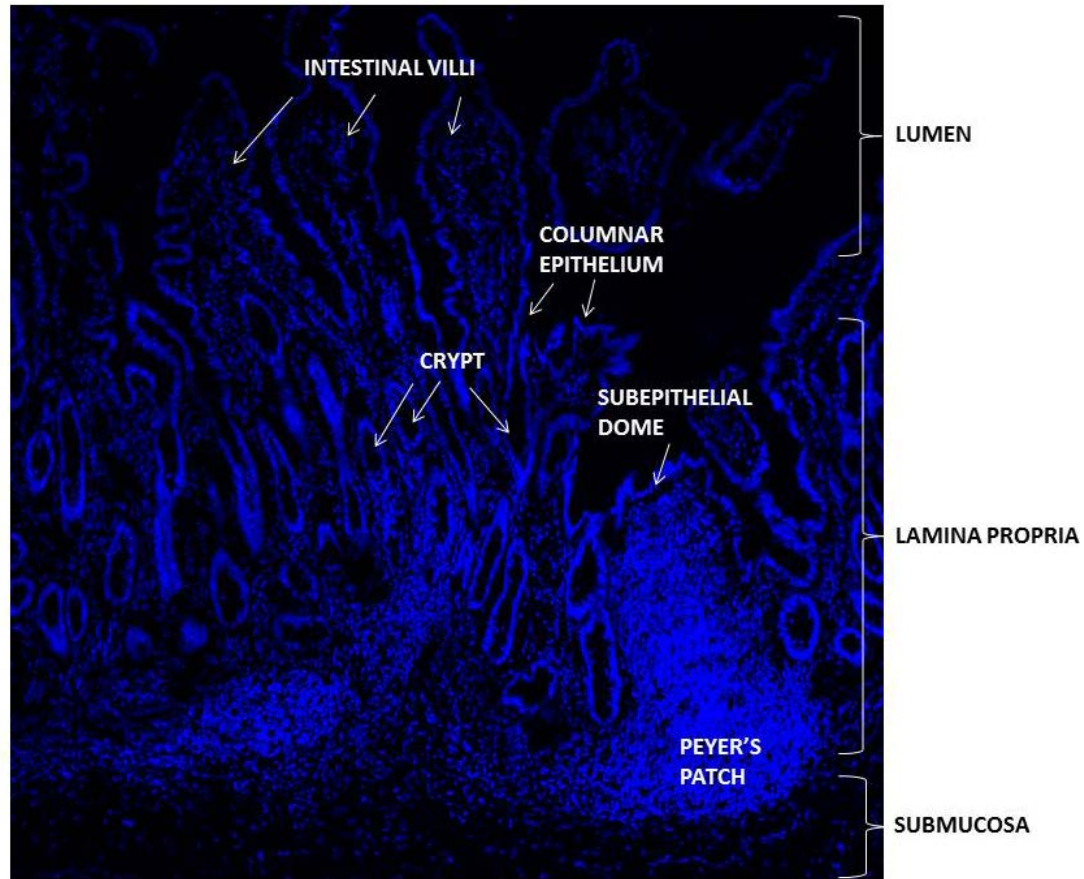


Figure 1. Basic structural features of rhesus macaque terminal ileum.

PFA fixed terminal ileum was sectioned to a thickness of 7 μ m, stained, and visualized by confocal microscopy. Cell nuclei (blue) are stained using membrane permeable Hoescht dye. Image was taken at 100x magnification.

Several studies have demonstrated that gut integrity is essential to the host's ability to prevent pathogen infection and sustain physiological wellness (37-39).

A signature characteristic of HIV/SIV infection that occurs during the early acute stage of infection regardless of transmission route is the rapid depletion of CD4⁺ T cells in the gut lamina propria. This depletion exceeds and precedes the decline of CD4⁺ T cells in blood and peripheral LN (40, 41). Additionally, some studies report that regulatory T cells (Treg), key modulators of excessive immune responses, are lost in the gut of macaques throughout the course SIV

infection, though contrasting evidence suggests their role in HIV/SIV infection may be unclear (42-47). Microbial translocation is one proposed mechanism by which systemic, chronic immune activation may arise. This proposal hypothesizes that the rapid depletion of gut CD4⁺ T cells contributes to a breakdown of the mucosal barrier causing the translocation of bacterial lipopolysaccharide (LPS) from the gut into circulation, which then incites persistent immune cell activation throughout the body (48). Nevertheless, these crucial pieces of evidence establish the GI tract and mucosal immunity as a paramount target for HIV therapeutic and vaccine studies (14, 28, 30, 48-50).

1.4 INNATE IMMUNITY

Despite our growing understanding of the role of the adaptive immune response in HIV/SIV pathogenesis, we know less about the innate immune cells that shape that response. Innate immunity is the first line of defense towards protecting the host from invading pathogens that have breached mechanical barriers, such as the mucosal epithelium, and is absolutely critical to the establishment of a healthy, antigen-specific adaptive immune response. The innate immune response is rapid, antigen non-specific, and does not demonstrate immunological memory, though NK cells might (51-53). NK cells, macrophages, and dendritic cells (DC) are all traditional sensors of the innate immune system that utilize pattern-recognition receptors, such as toll-like receptors (TLR), to detect conserved motifs on foreign pathogens, known as pathogen-associated molecular patterns. Upon activation via pattern-recognition receptor mediated signaling, innate immune cells mature to either eliminate the pathogen directly or communicate with T and B cells locally or at lymphoid induction sites via cytokine signaling and the

upregulation of co-stimulatory molecules to orchestrate an adaptive immune response (54). Antigen-presentation, the process of captured antigen being processed and presented to T cells underlies and begins this orchestration and thus plays an immense role in the polarization of adaptive immunity. Macrophages and DC are each professional antigen-presenting cells of the innate immune system. Importantly, this targeted interaction of innate and adaptive immune cells throughout the body is facilitated by the expression of chemokine receptors and ligands, which mediate cell trafficking (55, 56).

NK cells are innate immune lymphocytes that recognize host cells with abnormal MHC class I expression, typically virally infected cells, and target them for death by releasing cytotoxic granules. Their role in HIV/SIV pathogenesis is not well understood, however studies have shown NK cells from HIV infected humans are anergic and have decreased cytotoxicity (57, 58). On the contrary, some studies have suggested that NK cells may mediate antiviral immune pressure by favoring the selection of non-MHC class I modulatory HIV strains, implying their anti-viral activity is intact (59).

Our studies here have focused on the role of the mononuclear phagocytes of the innate immune system (60) in SIV pathogenesis in LN and gut mucosa; specifically, macrophages, myeloid dendritic cells (mDC), and plasmacytoid dendritic cells (pDC).

1.4.1 Macrophage characteristics

Macrophages are a highly heterogeneous and plastic population of antigen-presenting cell derived from circulating monocytes (61, 62). They can be found in a wide variety of tissues such as lung, spleen, intestine, brain, and LN, among others, and generally mature in each tissue to respond accordingly to the nuances of their environment (63, 64). Classically, macrophages are

robust phagocytic cells that ingest foreign pathogens and cellular debris to present antigen to helper T cells and augment the adaptive immune response (61). Fully polarized macrophages have been loosely defined as having either M1- or M2-like properties (64-66). M1 macrophages promote inflammation through the secretion of IL-1 β , TNF, and IL-23 and carry out microbicidal functions by producing factors such as reactive oxygen species. They are differentiated in the presence of IFN- γ , TNF, and microbial stimuli and the absence of environmental IL-4 and IL-13, which act as antagonists of the M1 pathway and drive differentiation toward the M2 pathway (67). M2 macrophages, in contrast to M1 macrophages, are therefore considered alternatively activated and are typified by their anti-inflammatory properties and ability to promote cell growth, angiogenesis, and tissue repair (64, 68, 69). They express mannose receptor for enhanced endocytosis, are generally poor presenters of antigen to T cells, express low levels of the co-stimulatory molecule CD86, and have limited microbicidal activity (70, 71).

1.4.1.1 The effects of HIV/SIV infection on macrophages

Macrophages may be one of the first immune cells HIV infects, and similar to CD4⁺ T cells, they can serve as reservoirs for latent and infectious virus (72-75). However, their role in HIV/SIV pathogenesis has been clouded by their site-specific phenotypic and functional diversity. CD163, CD68, HAM56, and MAC387 are some markers that have been used to describe macrophages *in vivo* and *in vitro*, however few HIV/SIV studies have exploited the capacity of these markers to delineate functionally distinct macrophage subsets (76, 77). Hence, the role of the macrophage in HIV/SIV pathogenesis is likely underappreciated.

Several macrophage subsets have been brought to attention as playing potential roles in HIV/SIV disease progression and immune regulation. For example, CD163 is a transmembrane hemoglobin scavenger receptor that is putatively used to identify M2 macrophages (64, 68).

Studies have addressed the role of CD163⁺ macrophages in HIV-encephalitis and its simian analogue SIV-encephalitis (78-80). CD163⁺ macrophage accumulation and productive replication around the perivascular cuff of the brain is reported to be partly responsible for SIV-induced encephalopathy (81). Furthermore, soluble CD163 is increased in the plasma of macaques with SIV-encephalitis (78). In mesenteric LN, Hasegawa et al. reported that high rates of CD163⁺ macrophage turnover as indicated by an increased density of BrdU⁺ and TUNEL⁺ CD163⁺ cells was a strong predictor of disease progression (82).

A second macrophage subtype critically involved in mucosal immunology is the intra-intestinal lamina propria macrophage. Because of their location, their role in HIV/SIV disease progression has been subject to much scrutiny. At present, the M1/M2 paradigm is not yet defined with intestinal macrophages (83-85). Functionally, intestinal macrophages have been characterized as highly phagocytic and inflammation anergic due to their inability to secrete pro-inflammatory cytokines and their absence of plasma membrane LPS co-receptor CD14 (86), which is crucial to their co-existence with LPS-rich gut flora (85). Moreover, they have been shown to be non-permissive to HIV replication (87), unlike extra-intestinal macrophages (88), though evidence does exist to the contrary (89). Some studies suggest that the antigen CD68 is a pan-macrophage marker for many tissue macrophage subsets including macrophages of the gut (69, 76, 90, 91). CD68 is a type 1 transmembrane glycoprotein located within late endosomes that is expressed by macrophages, monocytes, some non-myeloid cells (77), and DC precursors (92). Its function is not well known. Interestingly, based on a comparison of several reports, it can be inferred that the properties of intestinal macrophages do not seem to overlap with those of CD163⁺ macrophages, suggesting intestinal macrophages and CD163⁺ macrophages may potentially play unique roles in SIV pathogenesis.

1.4.2 mDC characteristics

DC are critical to bridging the gap between the innate immune response and the adaptive immune response (93, 94). DC are a highly diverse population of professional antigen-presenting cell that derive from hematopoietic bone marrow progenitor cells and occupy blood and peripheral tissues including lungs, intestine, skin, kidneys, and LN (93). Phenotypically, they can be broadly classified as CD11c⁺CD123⁻ myeloid DC (mDC) or CD11c⁻CD123⁺ plasmacytoid DC (pDC) that are HLA-DR⁺ lineage (Lin)-negative (CD14⁻CD3⁻CD20⁻CD56⁻) (95). mDC originate from a myeloid precursor and play a critical role in both host tolerance to self-antigens as well as recognition of foreign antigens (94, 96, 97). mDC express TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, and TLR8 for pathogen sensing and activation from an immature to mature status (98-101). Antigen captured and presented by mDC, such as bacteria and viruses, can originate exogenously or endogenously. Upon pathogen uptake, activated mDC migrate to LN where they process and present antigen peptides via MHC class II molecules to naïve T cells. This interaction provides the first signal towards promoting effector T cell status (99). Additionally, mDC communicate with cells via cytokines to influence the outcome of the immune response. Which cytokines are released is determined in part by the micro-organism and inflammatory stimuli they encounter, ensuring that the appropriate immune response is mounted to protect the host (102). Consequently, due to their potent capacity to manipulate adaptive immunity, mDC have been exploited to function as a vaccine to generate cell-mediated and humoral immunity against bacteria, viruses, and tumors (103-105).

1.4.3 Characteristics of CD103⁺ mDC and lymphocytes

In addition to the two primary DC types, functionally specialized subsets exist (97). CD103⁺ mDC, a subset of CD11⁺ mDC, have recently come to attention as critical regulators of intestinal immune homeostasis. $\alpha E(CD103)\beta 7$, first described on a population of mucosally-located T cells, is a heterodimeric integrin molecule that binds ligand E-cadherin on epithelial cells (106). Studies have shown $\alpha E\beta 7$ knock-out mice have significantly reduced intraepithelial lymphocytes in the gut (107). CD103⁺ mDC are found primarily in intestine, colon, and mesenteric LN of mice and humans, and are thought to be constitutively in flux to and from these sites as demonstrated by studies using CCR7 deficient mice, a chemokine receptor expressed at high levels on this cell population (108-110). No reports have extensively detailed CD103⁺ mDC in non-human primates. Functionally, CD103⁺ mDC trigger the differentiation of Foxp3⁺ Treg in mesenteric LN and mediate trafficking of Treg, T cells, and B cells to gut mucosa (111-113). The expression of Foxp3, a defining determinant of Treg (114), and imprinting of gut-migratory markers CCR9 and $\alpha 4\beta 7$ on naïve T cells are induced by CD103⁺ mDC secreted TGF- β and retinoic acid. In addition, studies have shown TGF- β and retinoic acid produced from the CD103⁺ mDC murine analog can drive naïve B cell production of IgA, an antibody isotype important to mucosal immunity (115). Interestingly, CD103 signaling is not itself responsible for any of these mechanisms but appears to act solely as a marker for mDC endowed with the aforementioned properties (109).

1.4.4 Characteristics of CD141⁺ mDC

CD141, a type 1 membrane glycoprotein, also known as thrombomodulin and BDCA-3 (116), denotes another specialized mDC subset of myeloid lineage (97). The discovery of CD141⁺ mDC in humans, believed to be a functional analog of CD8 α ⁺ mDC in mice, holds a wealth of promise for the development of mDC-based vaccines (117-119). The literature on this subset is extremely limited, but some studies suggest CD141⁺ mDC are Th1 polarizing cells with superior antigen cross-presentation activity, which is the ability to uptake and process extracellular virus particles using the endocytic pathway and present them by MHC class I molecules to CD8⁺ T cells (120). CD141⁺ mDC also exhibit high expression of endosomal TLR3, a pattern recognition receptor critical to the detection of viral double-stranded RNA (121, 122). Poly I:C activation of this subset has been shown to trigger robust levels of IFN- β , IL-12p70, and CXCL10 expression, each of which promote a shift to a more Th1-centric immune response (117, 121). In addition, human blood CD141⁺ mDC express XCR1, a CD8⁺ T cell chemoattractant which may facilitate mDC-mediated antigen presentation (122). CD141⁺ mDC present antigen to both CD8⁺ and CD4⁺ T cells, but to a lesser extent CD4⁺ T cells (121). Currently, there exists no literature on the function of CD141⁺ mDC in non-human primates, although they have been detected in macaque blood by flow cytometry (123, 124).

1.4.5 pDC characteristics

pDC derive from lymphoid precursors and function as major producers of type 1 interferons, specifically IFN- α , upon pathogen recognition by TLR7 and TLR9 which recognize ssRNA and unmethylated CpG motifs (54, 97, 101, 125). IFN- α binds receptors on host cells triggering a

cascade of molecular events that culminate in the upregulation of host cellular defense mechanisms and enhanced function of NK cells, mDC, and T and B cells (99, 126). pDC can also induce Th1 polarization and in some studies have been demonstrated to promote immune tolerance and regulation (127, 128). pDC generally present antigen that originates endogenously, however their presentation capacity is less efficient than traditional antigen-presenting cells due to low levels of MHC class II expression, a diminished ability to endocytose antigen, and the minimal presence of important antigen-presenting cell lysosomal proteases (129, 130). Some studies also suggest pDC are capable of antigen cross-presentation (131-134). Lastly, the role of pDC in immune activation due to potent IFN- α secretion has been proposed as an important clinical target and is currently under study (135).

1.4.5.1 The effects of HIV/SIV infection on mDC and pDC

mDC have been implicated in both the transmission and pathogenesis of HIV/SIV. Several studies suggest HIV/SIV interacts first with intraepithelial mDC or lamina propria mDC via c-type lectin DC-SIGN and other mechanisms like direct infection (16, 89, 136-140). It is believed that mDC disseminate virus to CD4⁺ T cells at T cell rich lymphoid induction sites (141-146). This transmission facilitates the replication and spread of newly synthesized virions. pDC have been shown to play a minor role in HIV/SIV transmission, possibly due to their inability to process and present antigen effectively and their infrequency at pathogen entry sites (125, 147), although they have been shown to cross-present HIV peptides to CD8⁺ T cells (133). Similar to mDC, pDC are susceptible to HIV infection (125, 148, 149).

In humans, mDC and pDC are well established to be lost in blood and to be functionally and phenotypically altered in response to HIV infection (150). This loss, in turn, has been shown to correlate with an increase in viral load and disease progression (151-153). Their roles in SIV

pathogenesis have been addressed in blood, peripheral LN, and to a lesser extent, the mucosa (154-159). During SIV infection, mDC are reported to have decreased responsiveness to TLR ligand stimulation and contribute to impaired T cell function via a deficient antigen-presentation capacity and increased expression of B7-H1, the ligand for inhibitory molecule PD-1 which is upregulated on lymphocytes from SIV⁺ macaques (160-162). Reports on the impact of SIV infection on pDC function are varied, however studies suggest pDC IFN- α production is hyperactive in LN and gut and may contribute to chronic immune activation (163-166). Using the pathogenic SIV rhesus macaque model, our lab and others have found that mDC and pDC are significantly lost in blood of animals with progressive disease but not animals with stable infection (167). pDC, in particular, are mobilized from bone marrow into blood and recruited to peripheral LN during acute infection, but die at a faster rate than they are replenished (154). Our lab has also documented a loss of mDC and pDC in peripheral LN isolated from macaques with AIDS (155); however some studies have found otherwise (168). A recent report found that pDC are lost in peripheral LN but are recruited to ileum and rectum in SIV-infected animals that progressed to AIDS. Additionally, no difference in mDC proportions was found in blood, LN, or intestine (157). A separate group reported an influx of pDC with increased expression of MIP-3 α , a marker for pDC migration, in the endocervix of macaques during acute stage SIV infection and has proposed a role for pDC in partly triggering chronic immune activation (159).

2.0 STATEMENT OF THE PROJECT AND SPECIFIC AIMS

The LN and gut mucosa are important sites of inflammation during HIV/SIV replication and spread. To date, knowledge of the immune response in the gut mucosa is strong in regards to adaptive immunity, while little focus has been placed on cells of the innate immune response. The purpose of this project was to better elucidate which cells of the innate immune system might contribute to the pathogenesis of or protection against SIV infection in LN and gut mucosa. In the present study we analyzed macrophage and DC subsets in LN and gut mucosa of rhesus macaques pre- and post-SIV infection to determine how disease influences the dynamics of these cell populations. Specifically, we used peripheral LN, mesenteric LN, and gut terminal ileum from archived samples to carry out these studies. Gut-draining LN, such as mesenteric LN, offer an opportunity to study the impact of disease on gut immunity. The use of peripheral LN, which drains extremities peripheral to the gut, complemented these studies and provided a picture of the peripheral immune response relative to the gut. Understanding the emerging complexities of macrophage and DC subset biology may provide insight into how these cell types could contribute to disease control, ultimately leading to a treatment that could mimic natural control.

We **hypothesized** that given the specialized compartmentalization of the immune system

- a) macrophages and DC in healthy rhesus macaques differentially occupy the gut and gut-

draining mesenteric LN relative to peripheral LN and b) their proportions are differentially impacted by SIV as a function of infection, disease status, and ART.

Aim 1: Identification of macrophage and dendritic cell subsets in lymph nodes and gut mucosa of healthy rhesus macaques. Rationale: To determine the impact of SIV disease

status on macrophage and DC subsets in gut-draining mesenteric LN, periphery-draining peripheral LN, and gut, we first characterized their healthy levels in uninfected macaques.

Hypothesis: We hypothesized that there would be differences in the proportions of macrophage and DC subsets in mesenteric LN relative to peripheral LN from healthy macaques that reflect the compartmentalization of the immune system. To test this hypothesis we used flow cytometry to quantify macrophages, mDC, CD103 and CD141 expressing mDC subsets, and pDC in mesenteric LN and peripheral LN from healthy rhesus macaques. Additionally, we detected each cell subset in terminal ileum from uninfected macaques by immunofluorescence analysis.

Aim 2: Characterize the effect of SIV infection, disease, and antiretroviral therapy on the proportions of macrophage and dendritic cell subsets in lymph nodes and gut mucosa.

Rationale: Based on the findings from Aim 1, we then mapped out the effect SIV infection and disease status had on the healthy-state proportions of macrophage and DC subsets. **Hypothesis:** We hypothesized that SIV infection, disease status, and ART would differentially impact macrophage and DC proportions in gut and gut-draining mesenteric LN relative to peripheral LN. To test this hypothesis we quantified different macrophage and DC subsets by flow cytometry in mesenteric and peripheral LN isolated from SIVmac251 infected rhesus macaques sacrificed at different stages of infection and with or without ART. Furthermore, we performed

immunofluorescence staining of terminal ileum taken from SIV⁺ animals sacrificed at acute and chronic stages of infection to determine an unprocessed snapshot of macrophage and DC frequency during SIV infection.

3.0 MATERIALS AND METHODS

3.1 ANIMAL SAMPLES

Indian-origin rhesus macaque LN suspensions and frozen terminal ileum archived from published and unpublished studies were used for analyses (154-156). Only tissue harvested at the day of sacrifice was used. Table 1 provides the characteristics of each cohort. The animals in this study defined as Progressor (P) and Stable (S) were prior enrolled in a vaccine study (169). Briefly, they received either a control vector or an adenovirus-based vector expressing or not expressing IL-15 from weeks 16 to 22 and from weeks 36 to 42 post SIV inoculation. In addition, each macaque received treatments of ART as specified in Table 1. Despite boosting T cell responses, upon completion of the study it was ascertained that the adenovirus-based vectors did not have a significant impact over controls on viral load and progression to AIDS. Independent of the immunotherapy received, the animals enrolled in the vaccine study were subsequently found to separate into two groups, which we have defined as Progressor and Stable, based on the determinants described in Table 1.

Table 1. Rhesus macaque cohort characteristics.

Animal Cohort	Status at sacrifice	Animal Cohort Status		Infection	TCID ₅₀ *	ART treatment	ART period	Vaccination	Status determinants
		Time of sacrifice	n						
Uninfected (UI)	uninfected control	-	5	no infection	-	-	-	-	sacrificed at set time point
Day 14 (D14)	acute infection	day 14	6	SIVmac251	1000	-	-	-	sacrificed at set time point
Day 36 (D36)	post-acute infection	day 36	5	SIVmac251	100	-	-	-	sacrificed at set time point
Day 36 +ART (D36 +ART)	post-acute infection (virus not detected)	day 36	5	SIVmac251	100	PMPA, FTC, and L870812	day 7 to 36	-	sacrificed at set time point
Progressor (P)	AIDS	mean: 30 weeks range: 11 to 43 weeks	8	SIVmac251	1000	PMPA and FTC	weeks 12 to 24 and weeks 32 to 44	Received Ad-based vector or control from weeks 16 to 22 and from weeks 36 to 42	mean set point viral load = 1×10^7 (RNA copies/mL) \emptyset , lymphadenopathy, persistent weight loss and anorexia, with opportunistic infections
Stable (S)	infected but no signs of disease	mean: 60 weeks range: 56 to 63 weeks	6	SIVmac251	1000	PMPA and FTC	weeks 12 to 24 and weeks 32 to 44	Received Ad-based vector or control from weeks 16 to 22 and from weeks 36 to 42	mean set point viral load = 2×10^6 (RNA copies/mL) \emptyset , no signs of AIDS

* inoculum was administered intravenously

\emptyset values from weeks 8 to 12 post infection

3.2 FLOW CYTOMETRY

Cryogenically frozen LN suspensions were thawed at 37°C and then re-suspended in FACS buffer. DC subsets were analyzed using CD11c-APC, CD123-PE-Cy7, CD141-PE, and CD103-FITC antibodies. For analysis of macrophage subsets, antibodies CD163-PerCP-Cy5.5 and CD68-FITC were used. All subsets were measured as a percentage of the HLA-DR-APC-Cy7 positive and CD20-eFluor 450 and CD3-PacBlue negative (Lin) population. PE and FITC-conjugated antibodies to nuclear protein Ki-67 were used to determine recently divided cells. CD25-PE, Foxp3-PE-Cy5, CD4-APC, and CD8-APC-Cy7 were used to identify Treg. Further antibody details can be found in Table 2. Extracellular markers CD11c, CD123, CD141, CD103, CD163, HLA-DR, CD20, and CD3 were applied to LN cell pellets and stained for 1 hour at 4°C and then washed with FACS buffer. Next, viable cells were excluded by staining with a violet

fluorescent LIVE/DEAD cell stain (Invitrogen). For analysis of intracellular antigens CD68 and Ki-67, following LIVE/DEAD cell stain, cells were permeabilized for 15 minutes with BD Cytotfix/Cytoperm and then stained for 30 minutes in BD Perm/Wash buffer. Lastly, cells were fixed using 2% paraformaldehyde (PFA) or Cytotfix/Cytoperm in the case of intracellular antigen detection. Data were acquired using a BD LSR II flow cytometer and analyzed using FlowJo software. Fluorescence minus one controls were used for positive detection of each cell type.

Table 2. Antibodies used for flow cytometric analysis of cells in LN suspensions.

Antigen	Conjugate	Isotype	Clone	Species	Company
HLA-DR	APC-Cy7	IgG2a	L243 (G46-6)	mouse	BD 335796
CD11c	APC	IgG2b	S-HCL-3	mouse	BD 340544
CD123	PE-Cy7	IgG2a	7G3	mouse	BD 560826
CD163	PerCP-Cy5.5	IgG1	GH1/61	mouse	Biolegend 333608
CD3	PacBlue	IgG1	SP34-2	mouse	BD 558124
CD141	PE	IgG1	1A4	mouse	BD 559781
CD103	FITC	IgG2a	2G5	mouse	Beckman Coulter IM1856U
CD68	FITC	IgG1	KP1	mouse	Dako F7135
CD20	eFluor 450	IgG2b	2H7	mouse	eBio 48-0209
Ki-67	PE	IgG1	B56	mouse	BD 556027
Ki-67	FITC	IgG1	B56	mouse	BD 556026
CD8	APC-Cy7	IgG1	RPA-T8	mouse	BD 557760
CD4	APC	IgG1	L200	mouse	BD 551980
Foxp3	PE-Cy5	IgG2a	PCH101	mouse	eBio 15-4776
CD25	PE	IgG1	2A3	mouse	BD 341009

Table 2 Continued

isotype	APC-Cy7	IgG2a	G155-178	mouse	BD 557751
isotype	APC	IgG2b	27-35	mouse	BD 555745
isotype	PE-Cy7	IgG2a	G155-178	mouse	BD 552868
isotype	PerCP-Cy5.5	IgG1	MOPC-21	mouse	BD 550795
isotype	PacBlue	IgG1	MOPC-21	mouse	BD 558120
isotype	PE	IgG1	MOPC-21	mouse	BD 555749
isotype	FITC	IgG2a	G155-178	mouse	BD 555573
isotype	PE	IgG1	MOPC-21	mouse	BD 559320
isotype	FITC	IgG1	B56	mouse	BD 556026
isotype	APC-Cy7	IgG1	MOPC-21	mouse	BD 557873
isotype	APC	IgG1	MOPC-21	mouse	BD 555751
isotype	PE-Cy5	IgG2a	G155-178	mouse	BD 555575

3.3 IMMUNOFLUORESCENCE MICROSCOPY

Terminal ileum and small intestine tissue isolated from rhesus macaques was fixed in 2% PFA, infused with 30% sucrose overnight, and then snap-frozen. Frozen tissue was then embedded in OCT and sectioned to 7 μ m sized slices using a cryotome. Sections were hydrated in PBS on Superfrost Plus(+) glass slides and then blocked for 1 hour with 10% serum matching the species of the secondary antibodies (goat and/or donkey serum.) Next, endogenous peroxidase was quenched using 0.15% hydrogen peroxide in PBS for 10 minutes. Sections were then incubated for 1.5 hours with biotinylated primary anti-human CD163 or CD123 and/or purified primary

anti-human CD163, CD123, CD3, or CD103 antibodies. All staining included an appropriately concentrated isotype-matched IgG-antibody. Further antibody details can be found in Table 3. The remaining steps were conducted in accord with the standard Tyramide Signal Amplification (TSA) kit protocol (Invitrogen T20932 and T20913). TSA kits enhance the signal of target antigens by using an enzyme-mediated detection method that relies on the interaction of a horseradish peroxidase (HRP) conjugated secondary antibody with a fluorescently labeled Tyramide substrate. Excluding CD3, all antigens were detected using TSA. Following addition of the substrate, cells were washed in PBS and nuclei were stained for 1 minute using membrane permeable Hoescht dye. Lastly, slides were mounted in gelvatol mounting medium and viewed on an Olympus Fluoview 1000 confocal microscope. Representative images were selected for presentation and brightness adjustments were made equally to images across some figures to improve clarity.

Table 3. Antibodies used for immunofluorescence analysis of cells in frozen gut tissues.

Antigen	Conjugate	Isotype	Clone	Species	Company
CD163	purified	IgG1	5C6-FAT	mouse	NovusBio BM4041
CD163	biotinylated	IgG1	GHI/61	mouse	eBio 13-1639
CD123	purified	IgG2a	7G3	mouse	BD 554527
CD123	biotinylated	IgG2a	7G3	mouse	BD 554528
CD3	purified	polyclonal	-	rabbit	Dako A0452
CD103	purified	IgG2a	2G5.1	mouse	AbD MCA70T
isotype	purified	IgG1	MOPC-31C	mouse	BD 550878
isotype	biotinylated	IgG1	MOPC-21C	mouse	BD 555747
isotype	purified	IgG2a	X39	mouse	BD 349050

Table 3 Continued

isotype	biotinylated	IgG2a	G155-178	mouse	BD 553455
isotype	purified	polyclonal	-	rabbit	GeneTex GTX35035

3.4 STATISTICAL ANALYSIS

Statistics for all analyses were calculated using a nonparametric, two-tailed Mann-Whitney test with a 95% confidence interval. A P-value <0.05 was considered significant.

4.0 RESULTS

4.1 AIM 1: IDENTIFICATION OF MACROPHAGE AND DENDRITIC CELL SUBSETS IN LYMPH NODES AND GUT MUCOSA OF HEALTHY RHESUS MACAQUES

We used flow cytometry to determine the relative percentages of different macrophage and DC subsets in mesenteric and peripheral LN isolated from healthy, uninfected rhesus macaques. A representative flow cytometry gating scheme for detection of each subset is depicted in Figure 2.

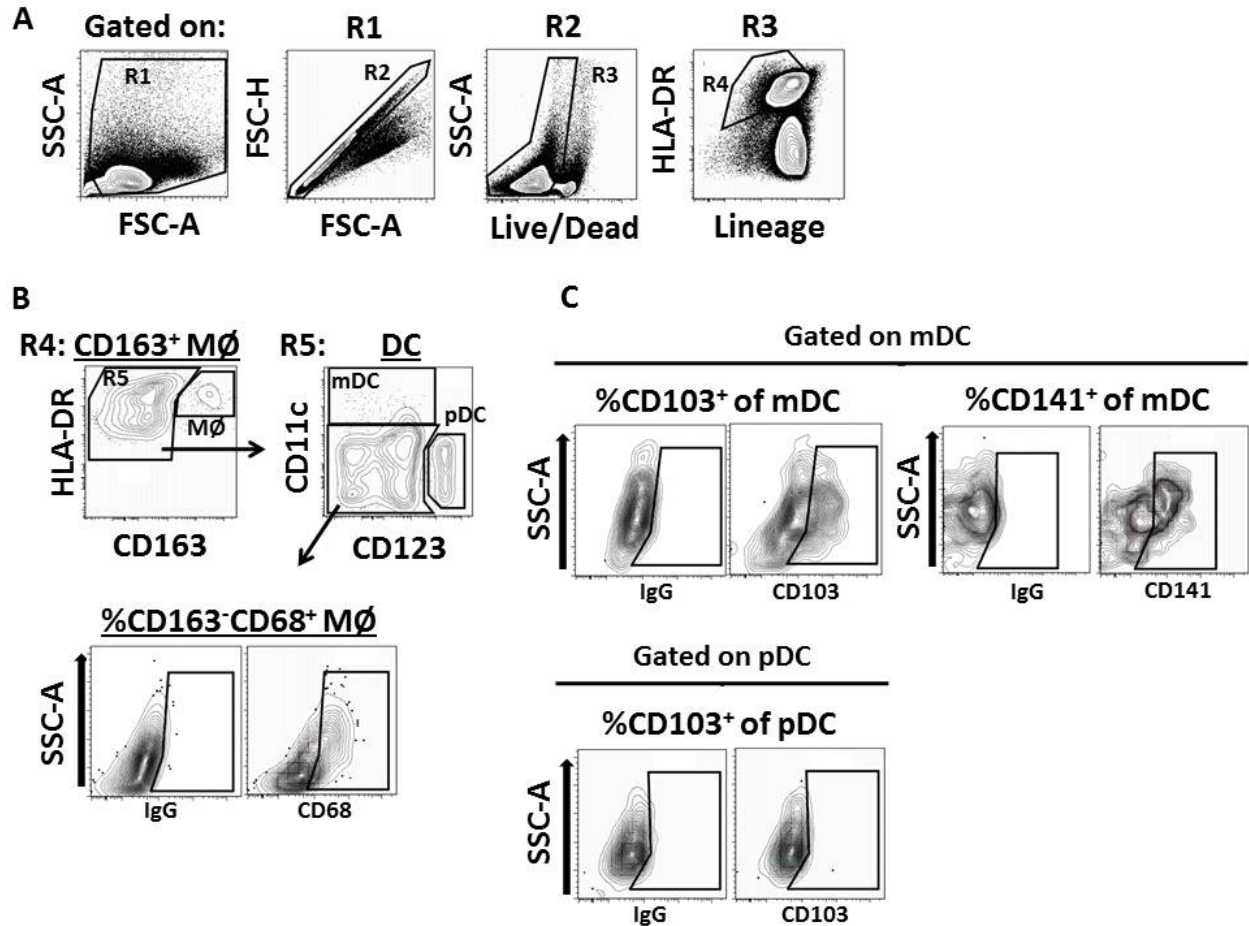


Figure 2. Representative flow cytometry gating strategy to define macrophage and DC subsets in mesenteric and peripheral LN.

(A) LN cells were gated by forward and side scatter (R1) and live cells were determined based on exclusion of an amine reactive dye (R3). HLA⁺DR⁺ cells and lineage (CD20, CD3) negative cells were then gated. (B) Macrophage populations were subdivided into two subsets: the CD163⁺CD68⁺ subset (CD163⁺ macrophages) and the CD163⁻CD68⁺ subset, which also lack dendritic cell markers. DC subsets, mDC (CD11c⁺) and pDC (CD123⁺), were identified within the CD3⁻CD20⁻CD163⁻HLADR⁺ gate (R5). (C) Detection of mDC (top panels) expressing CD103 and CD141 and pDC (bottom panels) expressing CD103 as compared to an isotype control.

Figure 3 demonstrates the proportion of each cell subset calculated as a percentage of the HLA-DR⁺Lin⁻ population (R4) from Figure 2 in healthy macaque mesenteric and peripheral LN. A group size of 5 was used on most occasions, however due to the scarcity of naïve LN samples

available to us and human error, the size for some groups was as few as 3. No significant differences were observed in the proportions of macrophages, mDC, and pDC in healthy mesenteric LN relative to peripheral LN from the same animals, however we observed a trend towards LN compartmentalization between mDC and pDC.

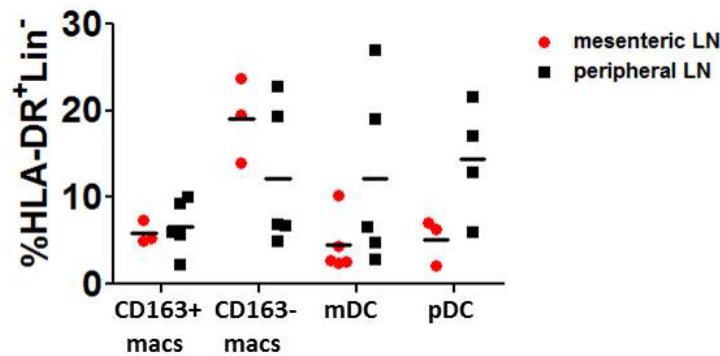


Figure 3. Proportion of macrophages, mDC, and pDC in healthy mesenteric and peripheral LN.

Macrophage and DC subsets from LN suspensions were gated on as described in Figure 2 and calculated as a percent of the HLA-DR⁺Lin⁻ population (R4). Each point represents one animal. Horizontal lines represent means.

We next determined the basal expression of CD141 and CD103 on mDC from healthy macaque LN. In Figure 2 we showed by flow staining that pDC in rhesus macaques do not express CD103. As of yet, we are uncertain if pDC express CD141, though studies suggests CD141⁺ DC are of myeloid origin, not lymphoid origin like pDC (97, 116). Figure 4A is a representative flow plot used to determine the relative percentage of CD103 and CD141 single positive and double positive cells within the CD11c⁺ mDC population. CD103 and CD141 were mostly found on two disparate populations of cells, though a small population may be double positive for these markers. Next, we quantified these two mDC subsets in LN from healthy macaques. In Figure 4B, we found that expression levels of CD103 and CD141 on mDC in mesenteric LN were significantly different relative to expression levels of CD103 and CD141 on mDC in peripheral LN. Separately, CD141 was expressed on 37% of mDC in mesenteric LN

and 54% in peripheral LN. Integrin CD103 was expressed on 31% of the mDC population in mesenteric LN and was not expressed on mDC in peripheral LN (Fig. 3B).

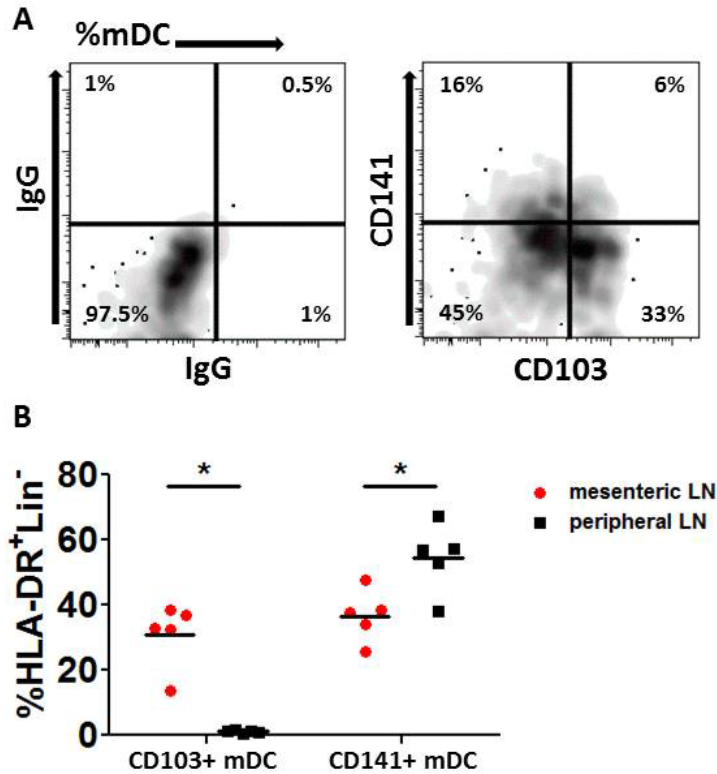


Figure 4. Expression of CD103 and CD141 on mDC in healthy mesenteric and peripheral LN.

mDC (CD11c⁺) were gated as described in Figure 2. (A) Representative gating of CD103 and CD141 single positive and double positive mDC. (B) Scatter plots demonstrate the percentage of CD103 and CD141 positive mDC in mesenteric and peripheral LN suspensions from healthy animals. Each point represents one animal. Horizontal lines represent means and * = p<.05.

Lastly for Aim 1, we sought to detect each subset in terminal ileum from healthy macaques by immunofluorescence staining. Successful detection of CD163⁺ macrophages, CD123⁺ cells, and CD103⁺ cells is shown in Figures 5A, B, and C, respectively.

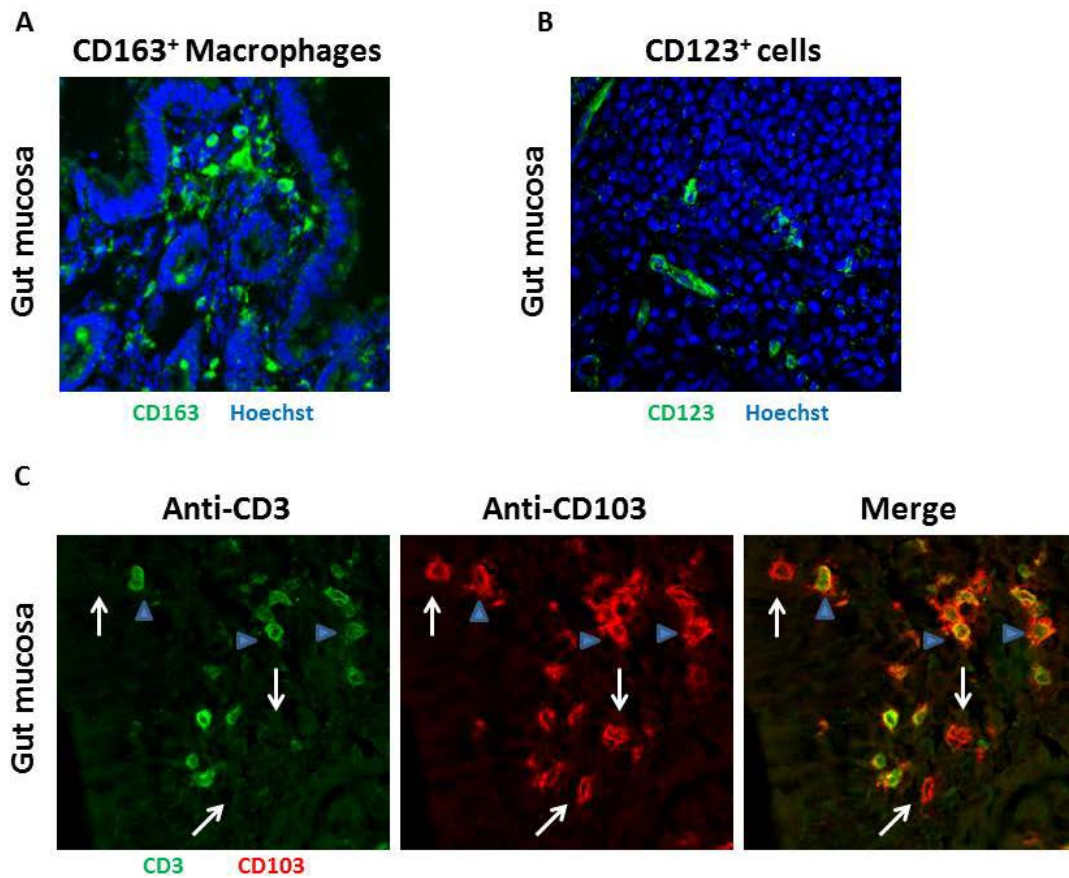


Figure 5. Detection of CD163⁺ macrophages, CD123⁺ cells, and CD103⁺ cells in healthy gut mucosa.

Terminal ileum was sectioned and stained with anti-CD163 (A), anti-CD123 (B), and anti-CD103 and anti-CD3 (C) antibodies, followed by TSA detection (excluding CD3), and visualized by confocal microscopy. White arrows indicate CD3⁺CD103⁺ mDC and blue arrow heads indicate CD3⁺CD103⁺ T cells (C). Cell nuclei (blue) were stained with Hoechst dye (A and B). Images were taken at 200x magnification and electronically zoomed in. Representative images are shown from 2-3 macaques.

Fluorescent antibody staining for CD11c for mDC detection and CD141 was unsuccessful. Other groups have reported similar struggles identifying CD11c⁺ mDC by immunofluorescence in rhesus macaque gut tissue (140). CD68 detection was successful but will be explored in future studies. Because we were unable to detect mDC in gut and CD103 has been shown to be expressed exclusively on mDC and T cells in humans and mice (111), we defined mDC as

CD103⁺CD3⁻ cells. Figure 5C displays a population of co-labeled CD103⁺CD3⁺ T cells and singly labeled CD103⁺ cells, likely mDC.

4.1.1 Summary of Aim 1 results

Figures 6A and B present a holistic summary of the composition of HLA-DR⁺Lin⁻ cells in healthy mesenteric and peripheral LN. As described in Figure 2, each cell subset was calculated as a percentage of the HLA-DR⁺Lin⁻ population and here is presented as a mean of several animals in pie graph format. In mesenteric LN of healthy animals, as depicted in Figure 6A, we found mDC and pDC each represented 5% of the HLA-DR⁺Lin⁻ fraction, whereas CD163⁺ macrophages represented 6% and CD163⁻ macrophages 19%. Figure 6B depicts the proportion of each subset in peripheral LN. mDC accounted for 12% of the HLA-DR⁺Lin⁻ fraction, pDC 14%, CD163⁺ macrophages 7%, and CD163⁻ macrophages 12%.

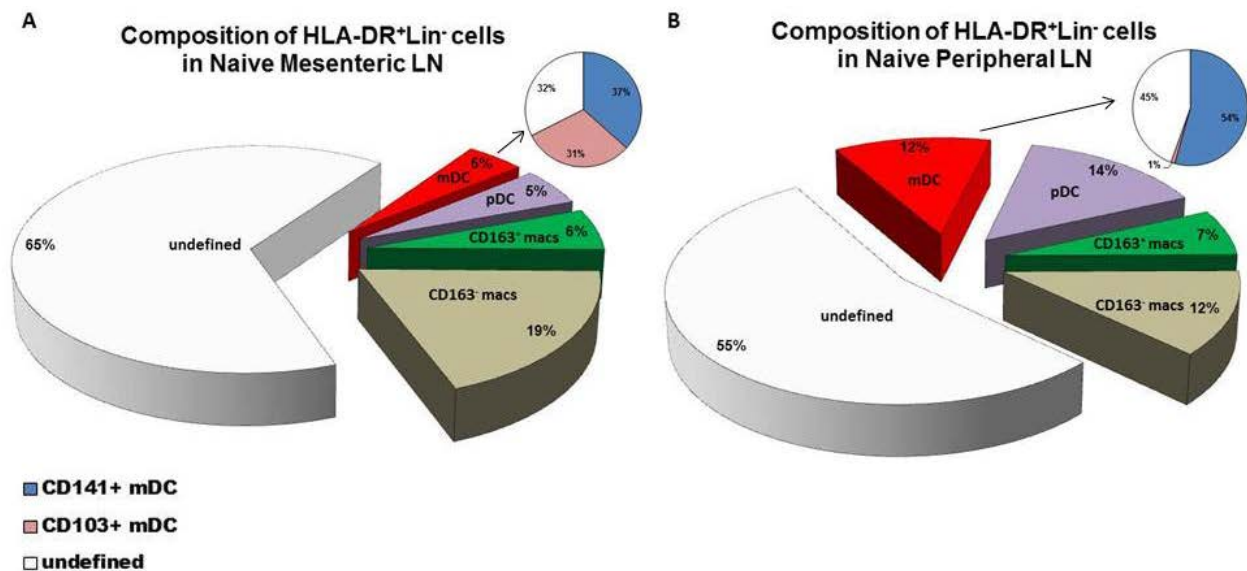


Figure 6. Composition of macrophage and DC subsets in healthy mesenteric LN (A) and peripheral LN (B).

Macrophage and DC subsets from LN suspensions were gated on as described in Figure 2 and calculated as a percent of the HLADR⁺Lin⁻ population (R4). Percentages represent means from at least three healthy rhesus macaques.

Cells labeled as undefined likely represent a variety of immature and undifferentiated populations. No significant differences were observed in these cell types between the two LN compartments, however there was a trend towards LN compartmentalization amongst mDC and pDC, with fewer mDC and pDC found in mesenteric LN relative to peripheral LN. These findings may suggest an immune response generated in the periphery would be more robust than an immune response specific to the gut. Contrary to these results, significant differences were observed between CD103⁺ mDC and CD141⁺ mDC in mesenteric LN relative to CD103⁺ mDC and CD141⁺ mDC in peripheral LN from uninfected animals. CD103⁺ mDC have gut-specific functions, so it is not likely they would be present in peripheral LN. The meaning behind our CD141⁺ mDC findings is currently unclear. Lastly, because we were unable to detect mDC and CD141⁺ cells by immunofluorescence staining, subsequent studies with terminal ileum were conducted only on CD163⁺ macrophages, CD103⁺ cells, and CD123⁺ cells.

4.2 AIM 2: CHARACTERIZE THE EFFECT OF SIV INFECTION, DISEASE, AND ANTIRETROVIRAL THERAPY ON THE PROPORTIONS OF MACROPHAGE AND DENDRITIC CELL SUBSETS IN LYMPH NODES AND GUT MUCOSA.

4.2.1 Animal cohorts and disease stages

To determine how SIV infection, disease status, and the effect of ART impact the healthy-state proportions of these cell subsets, we used archived LN tissue taken from animals intravenously infected with SIVmac251 and sacrificed at acute (day 14), post-acute (day 36), and chronic (> day 36) stages of infection with or without ART to reduce viral load. ART consisted of two nucleoside reverse transcriptase inhibitors (PMPA and FTC) and one protease inhibitor (L870812), a cocktail commonly used to suppress HIV replication in humans (170). ART was administered on day 7 post-infection and continued until day 36 post-infection, the day of sacrifice. Animals categorized as chronically infected were divided into two groups: those that progressed to AIDS status and those that stably controlled infection and did not progress to AIDS status as elaborated in Table 1 and defined previously (156). In addition to flow cytometric analysis of mononuclear phagocyte subsets in peripheral LN and gut-draining mesenteric LN, immunofluorescence analysis was employed to discern the impact of infection and disease on macrophage and DC levels in gut mucosa. We proceeded to analyze the impact of SIV infection on each cell type individually in order of macrophages, mDC, CD103⁺ cells, CD141⁺ mDC, and lastly, pDC.

4.2.2 SIV disease status impacts CD163⁺, but not CD163⁻CD68⁺ macrophages in LN and gut mucosa

First, we sought to address the impact of SIV infection and disease on the kinetics of putative anti-inflammatory CD163⁺ macrophages and CD163⁻ macrophages in peripheral LN, mesenteric LN, and terminal ileum. We used antibodies against CD68 and CD163 to identify and distinguish these two populations. Figure 7A is a representative flow plot from an uninfected macaque confirming the presence of these two distinct macrophage populations.

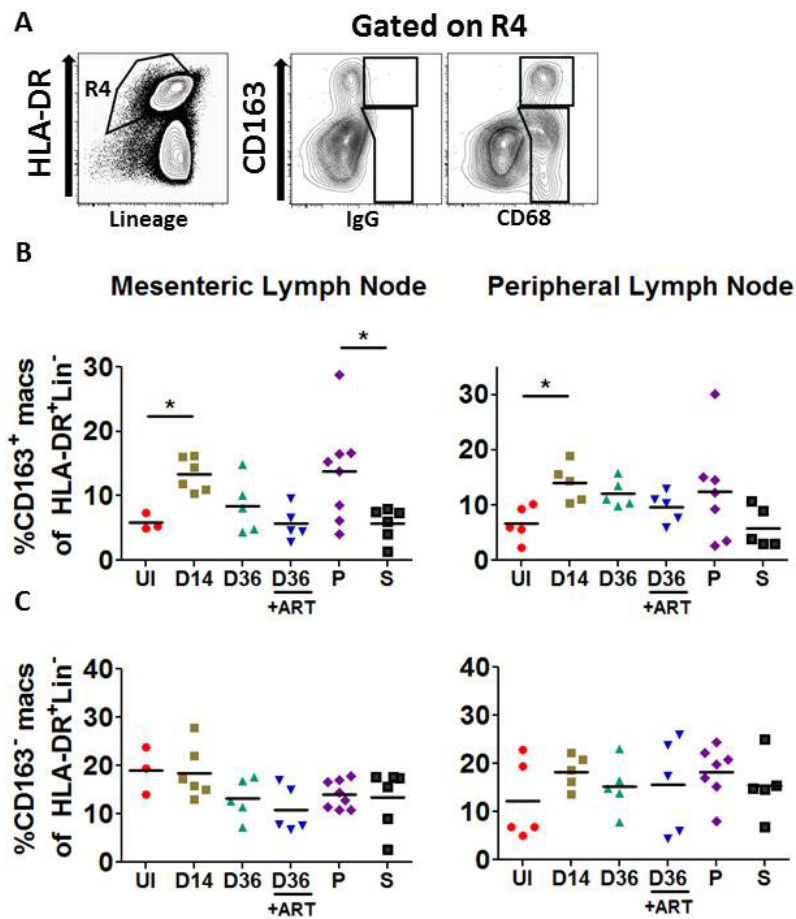


Figure 7. CD163⁺ macrophages are increased in mesenteric and peripheral LN of acutely and progressively SIV-infected animals, while CD163⁻ macrophage proportions remained unchanged between groups.

(A) Representative gating strategy for detection of CD163⁺CD68⁺ and CD163⁻CD68⁺ macrophages in an uninfected animal from the R4 population. (B) Percentage of CD163⁺CD68⁺ macrophages in mesenteric LN (left) and peripheral LN (right) as determined by gating in Figure 2. (C) Percentage of CD163⁻CD68⁺ macrophages in mesenteric LN (left) and peripheral LN (right) as determined by gating in Figure 2. Each point represents one animal. Horizontal lines represent means and * = p<0.05. UI = uninfected animal. D14 = day 14 post SIV infection. D36 = day 36 post SIV infection. D36 +ART = animals started ART on day 7 post SIV infection and ended on day 36 post SIV infection. P = animal that progressed to AIDS based on viral load. S = animal that stably controlled infection based on viral load.

We found that nearly 100% of CD163⁺ macrophages expressed CD68, a widely accepted pan-macrophage marker (76, 91), and that a separate HLA-DR⁺Lin⁻ population negative for CD163, CD11c, and CD123 expressed CD68, potentially LN resident macrophages (Fig. 7A, Fig. 2B) (76, 78, 91).

We next addressed the impact of SIV disease status on these two macrophage subsets. In Figure 7B, we found that CD163⁺ macrophages were significantly increased in mesenteric and peripheral LN of animals with acute infection as compared to LN from healthy animals. In addition, we observed a significant spike in the percentage of CD163⁺ macrophages in mesenteric LN from animals that progressed to AIDS, but not in rhesus macaques that stably controlled infection. Conversely, we found no statistically significant differences in CD163⁻CD68⁺ macrophage proportions throughout infection and in response to ART compared to healthy control animals (Fig. 7C).

Our findings that CD163⁺ macrophage proportions were increased in acutely SIV-infected and progressor animals led us to ask whether they represented a newly mobilized population of macrophages. Thus, we next used Ki-67, which is only expressed during the active stages of the cell cycle, to determine if these cells had recently divided. As seen in

Figures 8A and B, Ki-67⁺CD163⁺ macrophages were modestly increased in peripheral and mesenteric LN during acute infection and significantly increased in progressively and stably infected macaques, and therefore may represent a recently divided population.

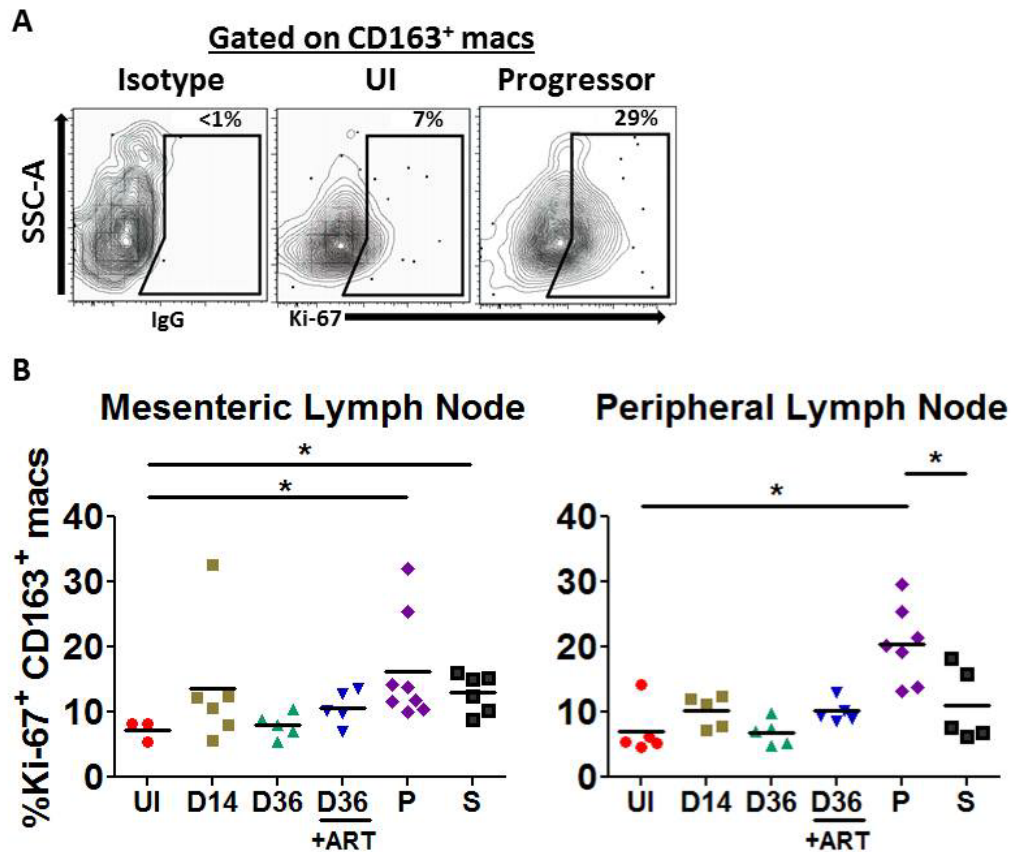


Figure 8. Increased proliferative response of CD163⁺ macrophages in progressive SIV infection.

(A) CD163⁺CD68⁺ macrophages were gated and the percentage of Ki-67 determined by comparing to staining with an isotype antibody. (B) Percent of CD163⁺CD68⁺ macrophages expressing Ki-67 in the different stages of infection. Each point represents one animal. Horizontal lines represent means and * = p<0.05. UI = uninfected animal. D14 = day 14 post SIV infection. D36 = day 36 post SIV infection. D36 +ART = animals started ART on day 7 post SIV infection and ended on day 36 post SIV infection. P = animal that progressed to AIDS based on viral load. S = animal that stably controlled infection based on viral load.

Next, we investigated the impact of SIV disease status on CD163⁺ macrophage proportions in terminal ileum by immunofluorescence staining. CD163⁺ macrophages were

primarily confined to the lamina propria of the terminal ileum but could also be identified in the submucosa (Fig. 9). We observed a noticeable increase in the density of CD163⁺ macrophages in Peyer's patches from animals with chronic stage disease compared to healthy animals (Fig. 9). There also appeared to be a slight increase in the density of CD163⁺ macrophages in the lamina propria and submucosa of animals with acute and chronic SIV infection that was not as evident in terminal ileum from control animals.

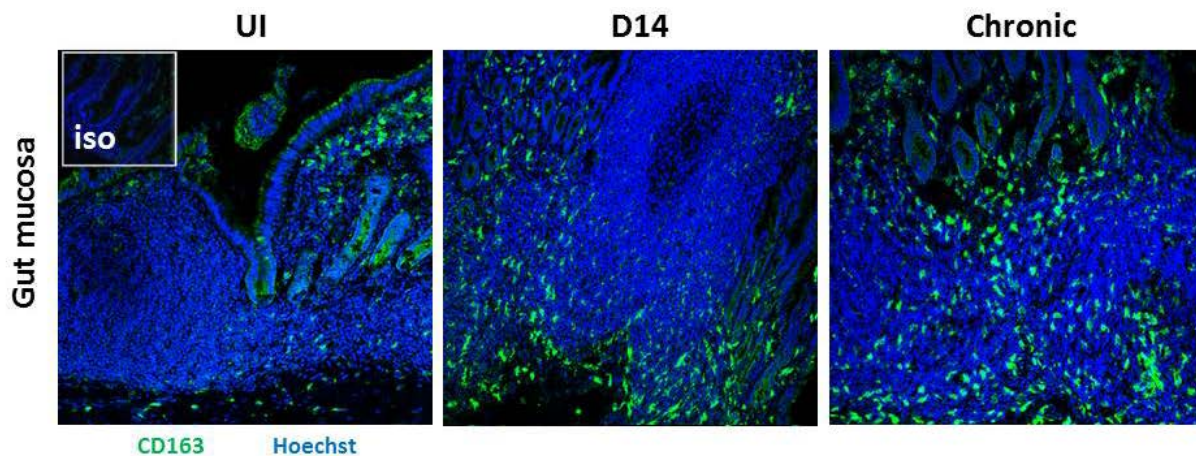


Figure 9. CD163⁺ macrophage density increases in Peyer's patches of chronically SIV-infected macaques.

Immunofluorescence staining with anti-CD163 antibody was performed on terminal ileum isolated from uninfected, acute, and chronically infected macaques. PFA fixed tissues were sectioned to a thickness of 7 μ m, stained, and visualized by confocal microscopy. Isotype control staining is shown in the inset. The CD163 marker was used to define macrophages (green). Cell nuclei (blue) were stained with Hoechst dye. TSA was used to detect anti-CD163 antibody staining. Images were taken at 200x magnification. Representative images are shown from 2-3 macaques per group.

Together, these data suggest that CD163⁺ macrophages are being recruited to LN and gut Peyer's patches during SIV infection.

4.2.3 mDC are modestly lost in peripheral LN during SIV infection

mDC dynamics have been investigated in peripheral and mesenteric LN of rhesus macaques with acute and chronic stages of SIV infection (167). mDC findings have been conflicting: some studies suggest depletion in LN is correlated with disease progression and others report no significant changes or an accumulation (157, 168).

Here we aimed to investigate the effect of SIV infection and disease status on mDC kinetics in peripheral and mesenteric LN. Figure 10A shows a representative flow plot used to define CD11c⁺ mDC as compared to an isotype control.

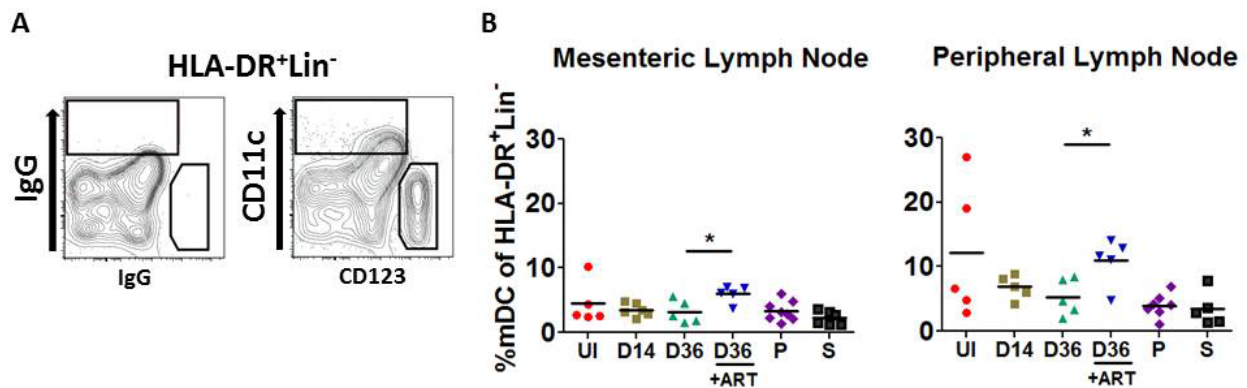


Figure 10. Proportions of mDC are modestly decreased in peripheral LN of SIV-infected macaques.

mDC were analyzed within the HLA-DR⁺Lin⁻ gate (R4) as described in Figure 2. (A) Contour plots represent gating for CD11c⁺ mDC as compared to an isotype control. (B) Percent mDC of total HLA-DR⁺Lin⁻ cells in mesenteric (left) and peripheral (right) LN. Each point represents one animal and horizontal lines represent means. * = p<0.05. UI = uninfected animal. D14 = day 14 post SIV infection. D36 = day 36 post SIV infection. D36 +ART = animals started ART on day 7 post SIV infection and ended on day 36 post SIV infection. P = animal that progressed to AIDS based on viral load. S = animal that stably controlled infection based on viral load.

In mesenteric LN we found few significant deviations in mDC proportions during acute, post-acute, or chronic stages of SIV infection as compared to healthy animals. However, there was a

significant increase of mDC proportions within the HLA-DR⁺Lin⁻ population in mesenteric LN of ART treated animals compared to untreated animals (Fig. 10B). In contrast, mDC proportions exhibited a modest decrease in peripheral LN during acute and chronic SIV infection (Fig. 10B).

From these findings, we can conclude that there is a modest loss in peripheral LN mDC proportions in response to SIV infection relative to no change in mDC proportions in mesenteric LN.

4.2.4 CD103⁺ mDC are lost in mesenteric LN and gut during SIV infection, an effect not seen in ART treated animals

Recently, a subset of mDC that expresses α E integrin, or CD103, has been implicated in playing a significant role in the differentiation and homing of Treg to mucosal sites in humans and mice (111). This molecule has also been found expressed on T cells and mDC and has been reported to facilitate effector T cell migration to the gut (113). Based on these reports, we first set out to detect the level of CD103 expression on T cells. Figure 11A is a representative flow gating scheme that was used to gate CD3⁺ T cells and determine what proportion express CD103. Roughly 2% of mesenteric LN CD3⁺ T cells from uninfected animals expressed CD103. Furthermore, this percentage did not fluctuate at acute, post-acute, or chronic stages of SIV infection and was not altered by ART (Fig. 11B).

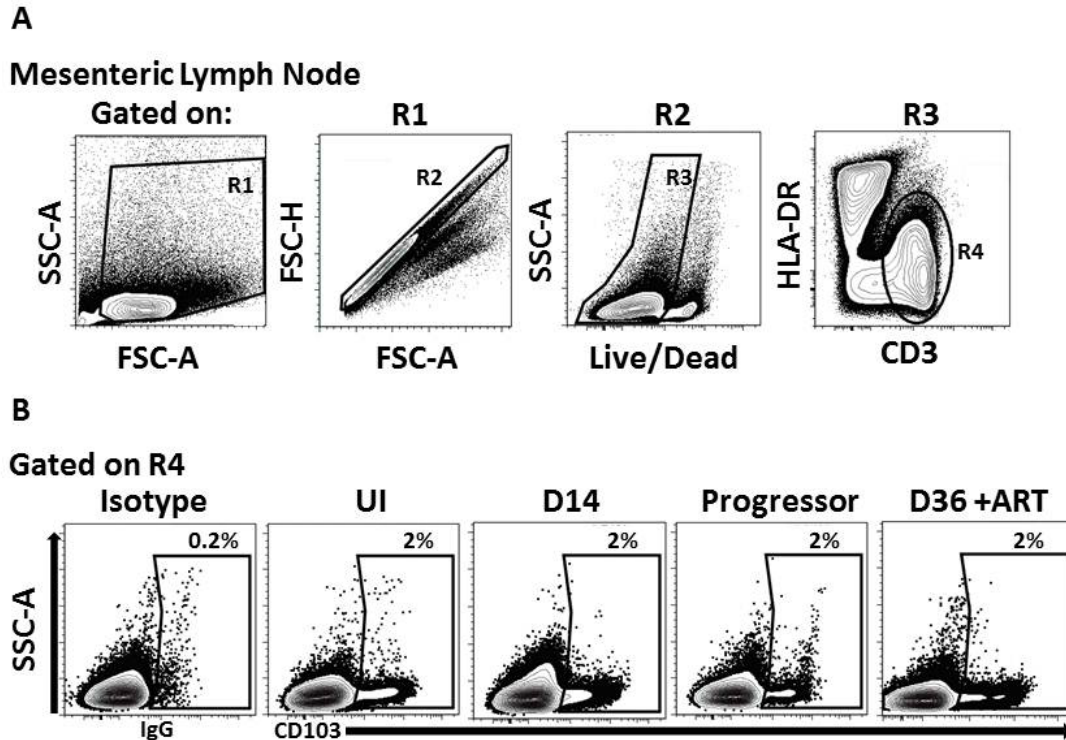


Figure 11. SIV disease status does not change the frequency of CD103⁺ T cells in mesenteric LN.

The percentage of CD103⁺ T cells was determined in all cohorts by gating T cells as described in (A) and analyzing CD103 expression on the R4 population. (B) Dot plots show one representative experiment from each cohort as compared to an isotype control. UI = uninfected animal. D14 = day 14 post SIV infection. D36 +ART = animals started ART on day 7 post SIV infection and ended on day 36 post SIV infection. P = animal that progressed to AIDS based on viral load.

Next, we determined if CD103⁺ pDC and CD103⁺ mDC proportions are influenced by SIV disease status in peripheral LN, mesenteric LN, and terminal ileum from rhesus macaques. In Figure 12, we found less than 1% of pDC from the HLA-DR⁺Lin⁻ population expressed CD103 in peripheral and mesenteric LN of healthy macaques, nearly equivalent to the isotype. We show in Figures 12A and B that this percentage does not change throughout SIV infection, which strongly implies that CD103 is not expressed on CD123⁺ cells.

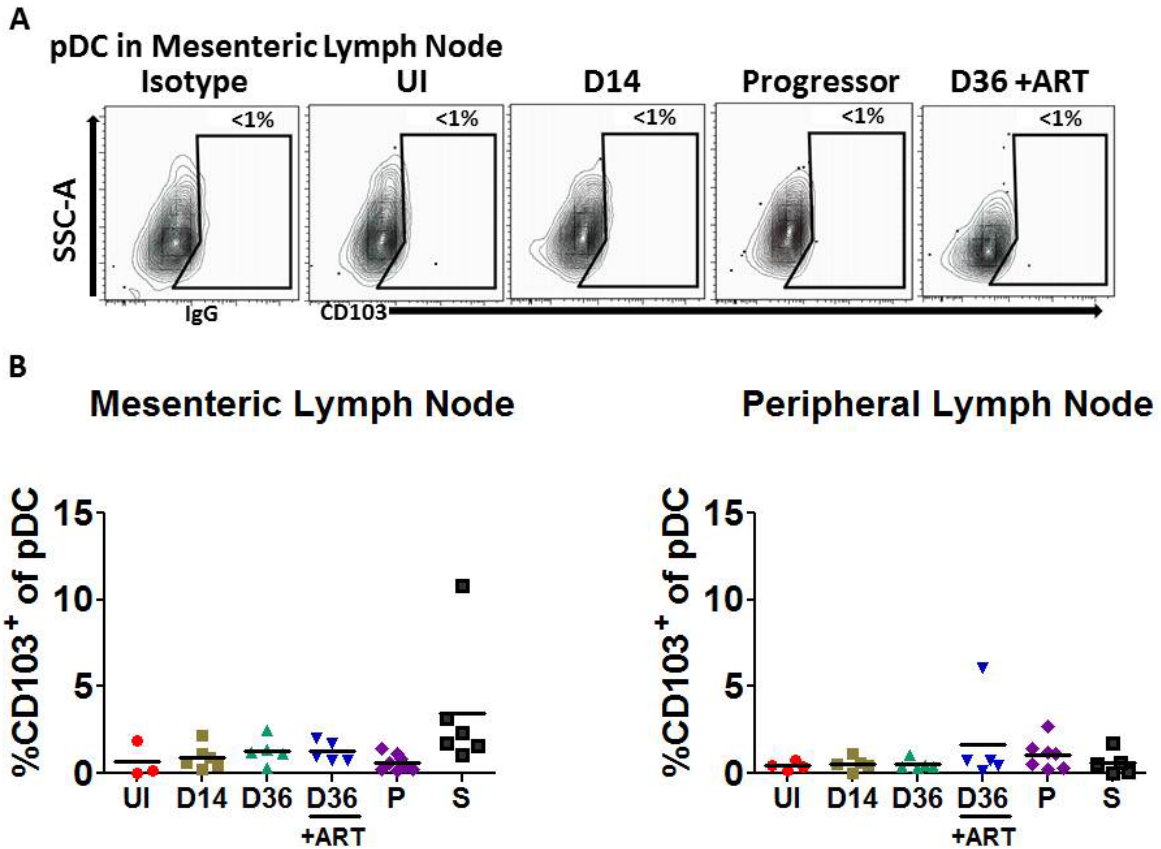


Figure 12. pDC do not express integrin CD103.

(A) Representative flow cytometry contour plots to depict the expression of CD103 on pDC in the different cohorts as compared to an isotype control. (B) Percent of pDC expressing CD103 in the different cohorts in mesenteric (left) and peripheral (right) LN. Each point represents one animal and horizontal lines represent means. UI = uninfected animal. D14 = day 14 post SIV infection. D36 +ART = animals started ART on day 7 post SIV infection and ended on day 36 post SIV infection. P = animal that progressed to AIDS based on viral load.

Finally, we analyzed the impact of SIV infection and disease status on the percentage of CD103⁺ mDC in mesenteric and peripheral LN. In mesenteric LN from naïve macaques, we found that roughly 31% of mDC expressed CD103. Strikingly, as disease progressed we observed a near two-fold decrease of CD103⁺ mDC in mesenteric LN from acutely SIV-infected animals and an even greater decrease in chronically infected animals that was not seen in ART

treated animals (Fig. 13A and B). We also observed that CD103 was not expressed on mDC in peripheral LN (Fig. 13B).

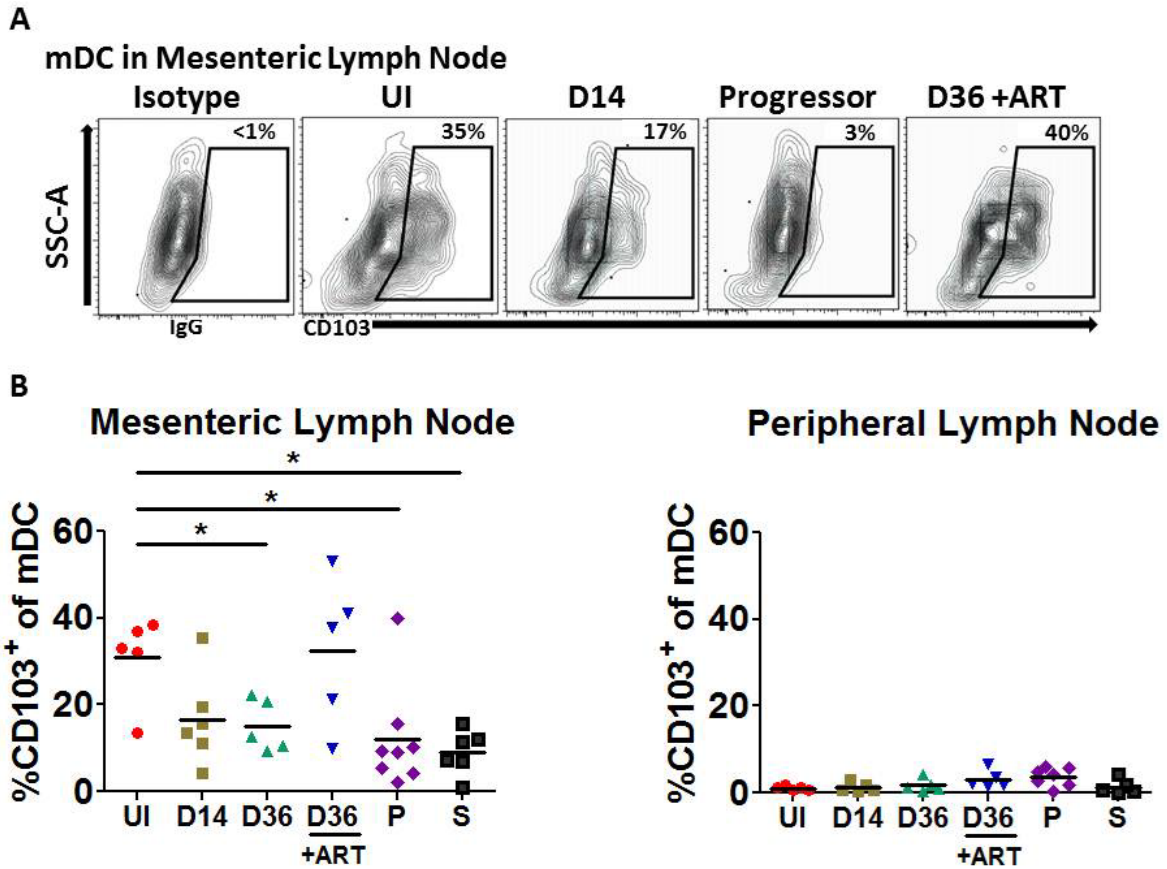


Figure 13. CD103⁺ mDC are lost in mesenteric LN as SIV infection progresses, an effect not seen in ART treated animals.

mDC were defined as positive for expression of CD11c as described in Figure 2B and analyzed for expression of CD103 (Fig. 2C). (A) Representative flow cytometry contour plots to depict the expression of CD103 on mDC in the different cohorts. (B) Each point represents the percentage of CD103⁺ mDC in mesenteric (left) or peripheral (right) LN of one animal. Horizontal lines represent means and * = $p < 0.05$. UI = uninfected animal. D14 = day 14 post SIV infection. D36 = day 36 post SIV infection. D36 +ART = animals started ART on day 7 post SIV infection and ended on day 36 post SIV infection. P = animal that progressed to AIDS based on viral load. S = animal that stably controlled infection based on viral load.

We next used immunofluorescence staining to detect CD103 expressing cells in parallel with CD3⁺ T cells in terminal ileum. We observed that the majority of CD103 expressing cells also expressed CD3 (Fig. 14). In uninfected macaques, CD103⁺ cells were localized primarily to the subepithelial dome of Peyer's patches and villi of the terminal ileum. However, during the course of infection, we observed a slight decrease in the density of CD103⁺ cells in the subepithelial dome and intestinal villi (Fig. 14).

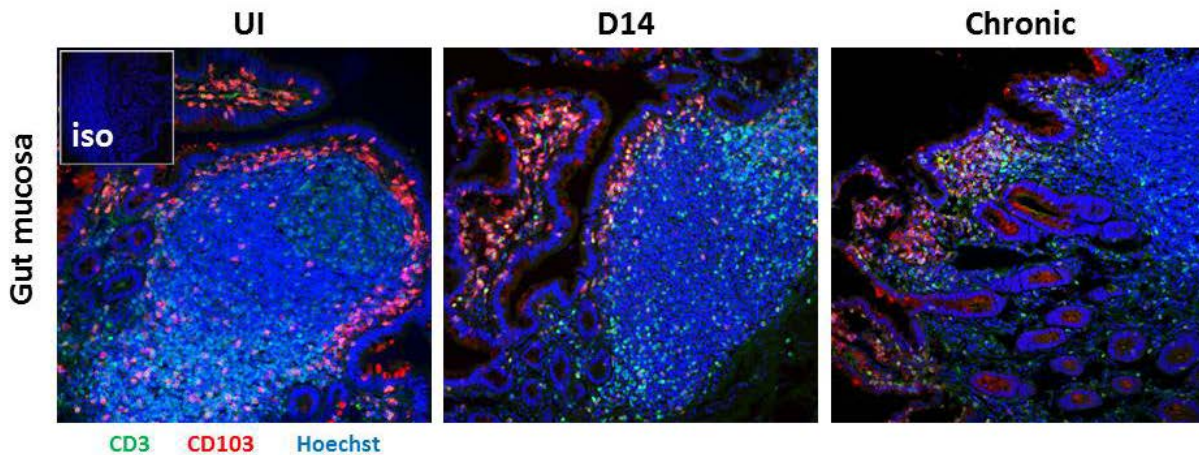


Figure 14. CD103⁺ cells are lost from the subepithelial dome of Peyer's patches as SIV infection progresses.

Immunofluorescence staining with anti-CD103 and anti-CD3 antibodies was performed on terminal ileum isolated from uninfected and SIV-infected macaques. Expression of CD103 is visualized in red and CD3 is visualized in green. Isotype control staining is shown in the inset. CD103⁺ cells were localized to the villi and subepithelial dome region within Peyer's patches in healthy animals. Cell nuclei (blue) were stained with Hoechst dye. TSA was used to detect anti-CD103 antibody staining. Images were taken at 200x magnification. Representative images are shown from 2-3 macaques per group.

These findings of lost CD103⁺ mDC in mesenteric LN and possibly the gut mucosa would suggest that, based on findings in mice and humans, Treg differentiated from CD103⁺ mDC in rhesus macaques are also decreased or lost at these sites in response to SIV infection.

4.2.5 The proportion of CD141⁺ mDC increases in mesenteric LN during acute SIV infection

CD141 is a type 1 membrane receptor that denotes mDC with superior antigen cross-presentation capacity (122). At this time, no studies have been published on the role of CD141⁺ mDC in SIV pathogenesis. Recognizing the possible importance of this subset in controlling viral infections, we analyzed the impact of SIV infection and disease status on CD141 expressing mDC.

Figure 15A indicates positive flow cytometric detection of CD141 on mDC in naïve animals as compared to an IgG stained control.

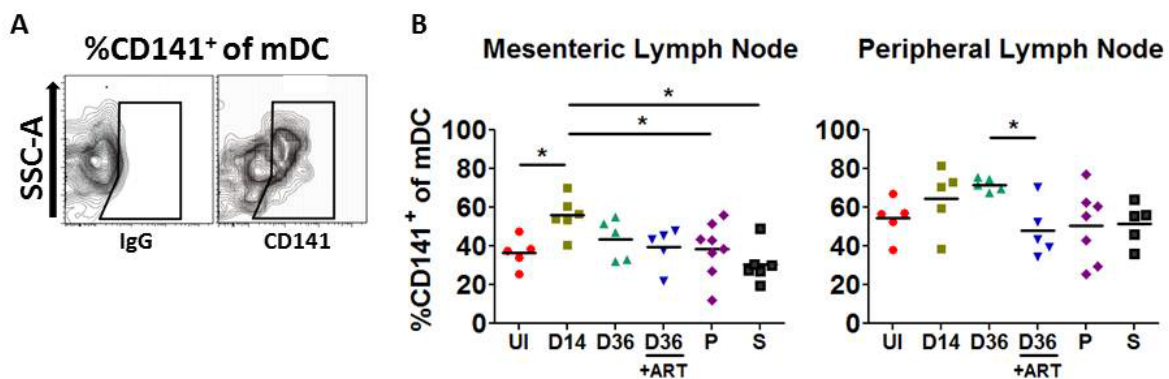


Figure 15. Significant increase in CD141⁺ mDC during acute but not chronic SIV infection in mesenteric LN. (A) Representative flow plots demonstrating expression of CD141 on mDC as compared to an isotype control. (B) Each point represents the percentage of CD141⁺ mDC in mesenteric (left panel) and peripheral (right panel) LN for one animal. Horizontal lines represent means and * = p < 0.05. UI = uninfected animal. D14 = day 14 post SIV infection. D36 = day 36 post SIV infection. D36 +ART = animals started ART on day 7 post SIV infection and ended on day 36 post SIV infection. P = animal that progressed to AIDS based on viral load. S = animal that stably controlled infection based on viral load.

Thirty-seven percent of mDC in mesenteric LN and 54% in peripheral LN from healthy macaques expressed CD141. We observed a significant increase of CD141⁺ mDC in mesenteric

LN and a trend towards significance in peripheral LN from animals with acute SIV infection (Fig. 15B). However, at all later stages of infection in mesenteric and peripheral LN CD141⁺ mDC percentages were roughly equivalent to naïve levels.

4.2.6 SIV disease status differentially impacts pDC in LN relative to gut

Loss of pDC in peripheral LN has been associated with HIV/SIV disease progression (152, 154, 171). Here we investigated the effect of SIV infection and disease status on pDC proportions in peripheral and mesenteric LN. CD123⁺ pDC detection as compared to an isotype control is shown in Figure 16A.

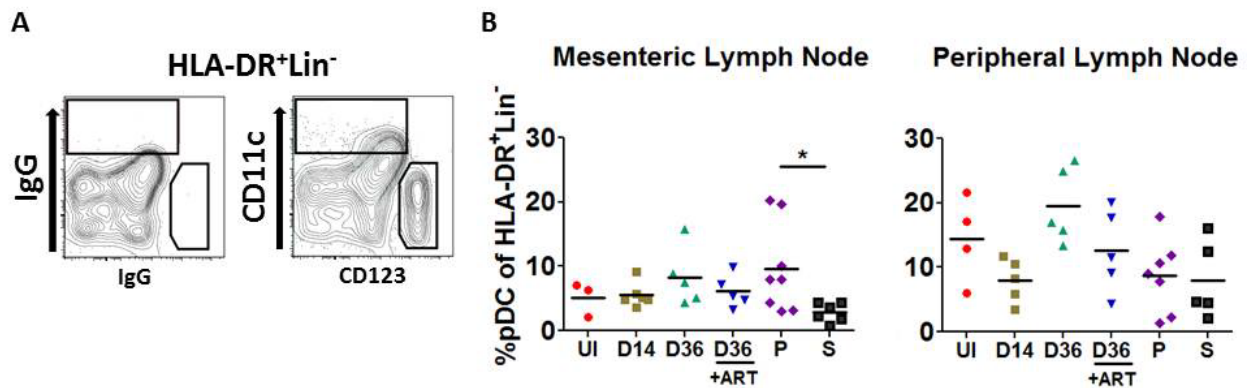


Figure 16. pDC proportions are modestly decreased in mesenteric and peripheral LN from SIV-infected macaques.

pDC were analyzed within the HLA-DR⁺Lin⁻ gate (R4) described in Figure 2. (A) Dot plots represent gating for CD123⁺ pDC as compared to an isotype control. (B) Percent pDC of total HLA-DR⁺Lin⁻ cells in mesenteric (left) and peripheral (right) LN. Each point represents one animal and horizontal lines represent means. * = p<0.05. UI = uninfected animal. D14 = day 14 post SIV infection. D36 = day 36 post SIV infection. D36 +ART = animals started ART on day 7 post SIV infection and ended on day 36 post SIV infection. P = animal that progressed to AIDS based on viral load. S = animal that stably controlled infection based on viral load.

Compared to uninfected macaques, pDC proportions were modestly decreased in peripheral LN during acute SIV infection and AIDS (Fig. 16B). We also observed a significantly greater proportion of pDC in mesenteric LN from the progressor group compared to stably infected animals (Fig. 16B).

We next sought to determine the impact of SIV infection on CD123⁺ cell proportions in terminal ileum. Figure 17 displays successful staining for CD123⁺ cells using a biotinylated anti-CD123 antibody.

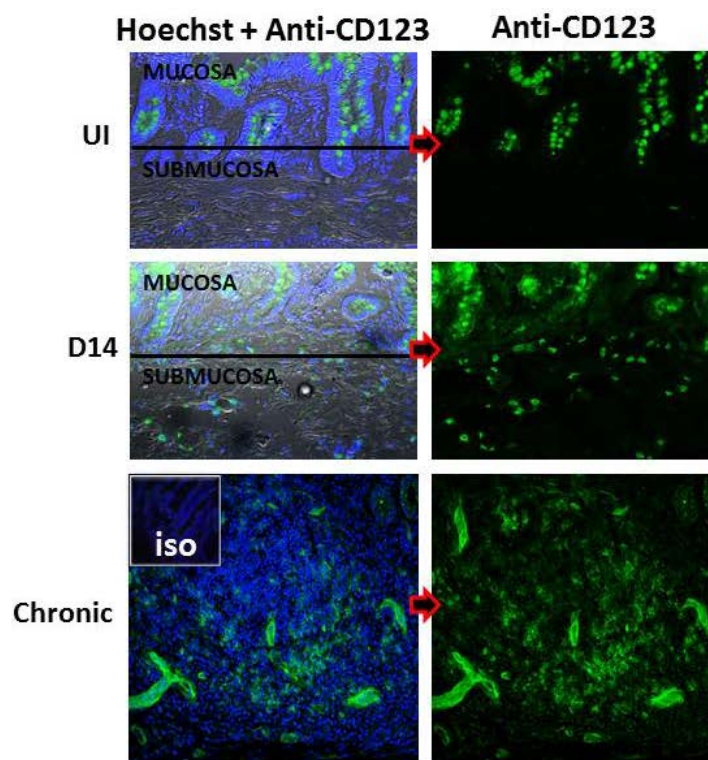


Figure 17. SIV infection of rhesus macaques results in an influx of CD123⁺ cells to the gut mucosa.

Immunofluorescence staining with anti-CD123 antibody was performed on terminal ileum isolated from uninfected and acutely and chronically infected macaques. PFA fixed tissues were sectioned to a thickness of 7 μ m, stained, and visualized by confocal microscopy. Expression of cell-specific markers is visualized in green. CD123⁺ cells were seen predominately localized to the submucosa during acute infection and to the lamina propria during chronic infection. Non-specific staining is seen within the crypts and outer lining of the villi in the mucosa. Differential interference contrast light was used to provide texture and delineate mucosa from submucosa regions of ileum from

uninfected and acute infected macaques. Cell nuclei (blue) were stained with Hoechst dye. TSA was used to detect anti-CD123 antibody staining. Images were taken at 200x magnification and electronically zoomed in. Representative images are shown from 2-3 macaques per group.

CD123⁺ cells appeared to be increased in terminal ileum submucosa during acute SIV infection and increased during chronic infection. Positive staining lining the intestinal crypts and villi of the columnar epithelial cells is believed to be anti-CD123 antibody bound non-specifically to mucus-secreting goblet cells, which cluster around gut epithelium. This non-specific staining pattern was inconsistently present in the isotype control stained tissue.

These data show that pDC proportions may be differentially impacted by SIV infection in peripheral LN and gut mucosa. While pDC results in peripheral LN were not significant, their trends confirm current knowledge of pDC kinetics in LN in response to HIV/SIV infection (154, 155, 157).

4.2.7 Summary of Aim 2 results

Figures 18 and 19 below display a holistic summary of our findings from Aim 2. In Figure 18, the most robust changes found were with the CD163⁺ macrophage subset which our data suggest are being recruited to LN and possibly gut during SIV infection and disease. Only minor changes in mDC, pDC, and CD163⁻ macrophage proportions were observed throughout each of our cohorts, although we did see a trend towards a loss of mDC and pDC in peripheral LN of SIV-infected animals compared to healthy uninfected animals.

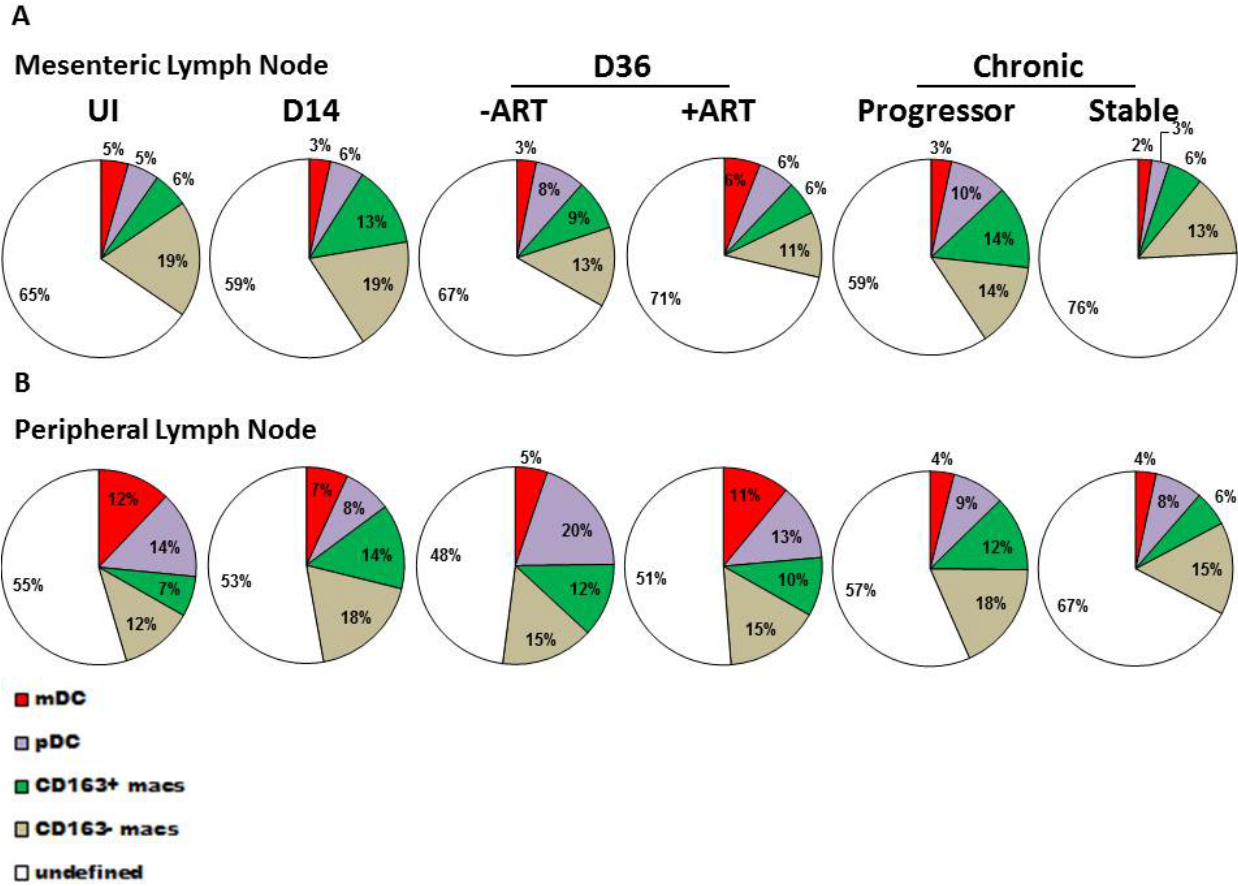


Figure 18. Summary of mDC, pDC, and macrophage proportions in healthy and SIV-infected LN.

Macrophage and DC subsets from mesenteric (A) and peripheral (B) LN suspensions were gated on as described in Figure 2 and calculated as a percent of the HLADR⁺Lin⁻ population (R4). Percentages represent means from at least three healthy rhesus macaques.

In Figure 19, we summarized our findings described earlier on CD103⁺ mDC and CD141⁺ mDC in LN from healthy and SIV-infected macaques. Each subset was detected by gating on the CD11c⁺ mDC population and analyzing for expression of CD103 or CD141 as compared to an isotype control (Fig. 2C). CD103⁺ mDC were found to be exclusive to mesenteric LN, whereas CD141⁺ mDC were found in mesenteric and peripheral LN. We observed that CD103⁺ mDC were lost in gut-draining mesenteric LN as SIV infection progressed but not in animals that received ART. In comparison, CD141⁺ mDC were increased in both

mesenteric and peripheral LN during acute stage SIV infection but not during the later stages of infection.

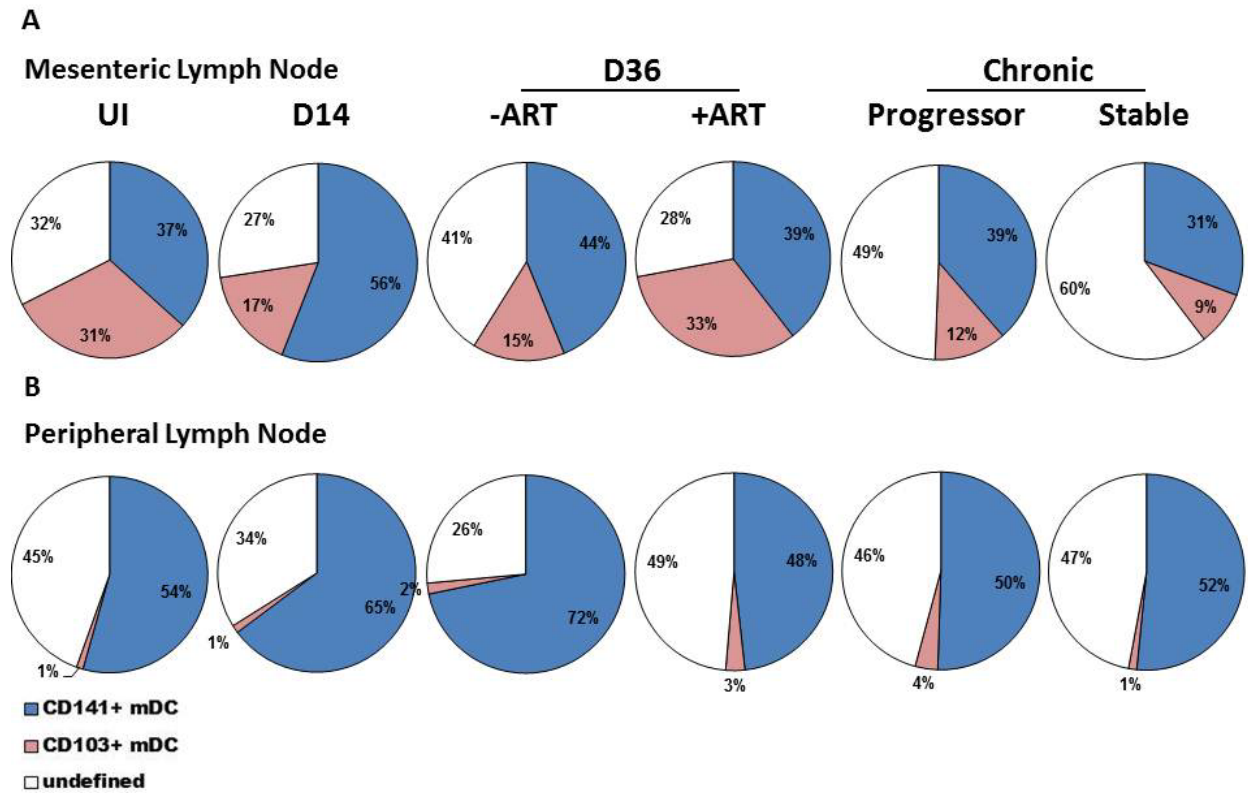


Figure 19. Summary of CD103⁺ and CD141⁺ mDC proportions in healthy and SIV-infected LN.

mDC were gated on and analyzed for expression of CD103 and CD141 as described in Figure 2. mDC subsets analyzed from mesenteric (A) and peripheral (B) LN suspensions were calculated as a percent of the HLADR⁺Lin⁻ population (R4). Percentages represent means from at least three healthy rhesus macaques.

4.2.8 Preliminary investigation of Treg

We have demonstrated that CD103⁺ mDC proportions, shown to be critical modulators of Treg differentiation in mice and humans, are progressively lost in mesenteric LN from SIV⁺ rhesus macaques. As an extension to the aims of this project, based on our CD103⁺ mDC findings, we were encouraged to investigate Treg proportions in mesenteric and peripheral LN from each

cohort. We hypothesized that $\text{Foxp3}^+\text{CD25}^+$ Treg proportions would positively correlate with CD103^+ mDC levels in mesenteric LN of macaques throughout the different stages of SIV infection.

Figure 20 below diagrams our gating strategy for detection of $\text{Foxp3}^+\text{CD25}^+$ Treg in LN suspensions, as well as expression of Ki-67 on Treg cells to determine cellular proliferation.

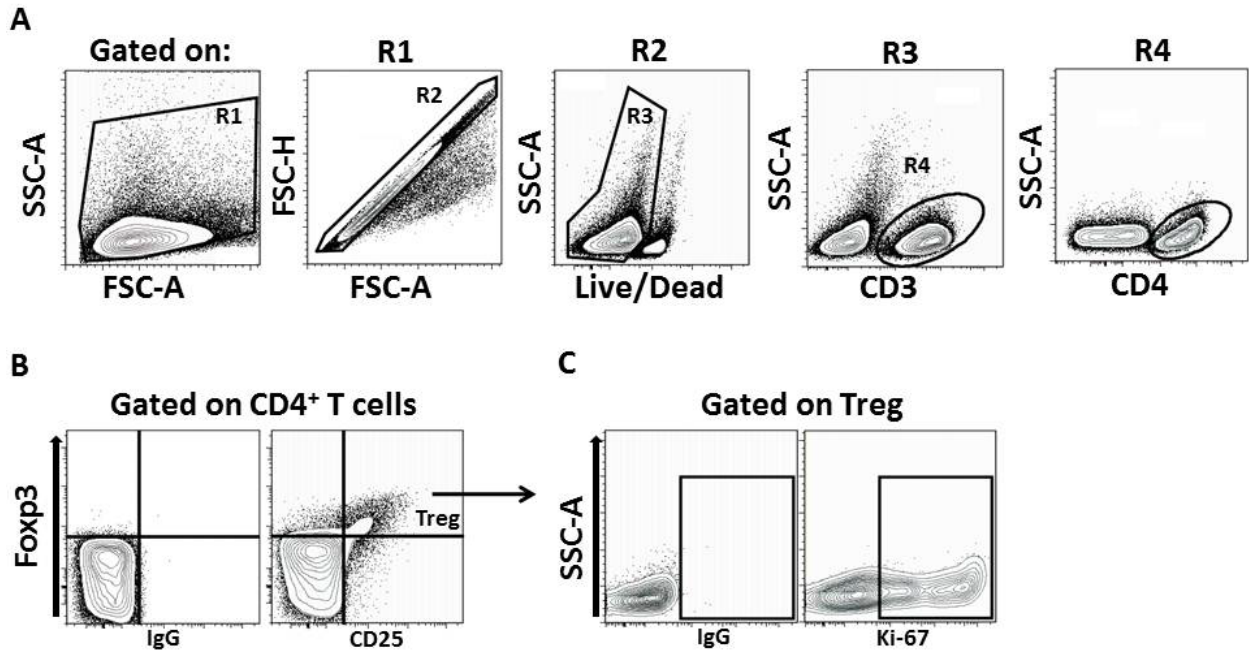


Figure 20. Representative flow cytometry gating strategy to define Treg in mesenteric and peripheral LN.

(A) LN cells were gated by forward and side scatter (R1) and live cells were determined based on exclusion of an amine reactive dye (R3). CD3^+ cells were then gated (R4) and stained for CD4. (B) Proportion of CD4^+ T cells co-expressing Foxp3 and CD25 (Treg) as compared to an isotype control. (C) Proportion of Treg expressing Ki-67 as compared to an isotype control.

Figure 21A displays the proportion of Treg detected in peripheral and mesenteric LN from uninfected and SIV-infected rhesus macaques calculated as a percentage of the CD4^+ population.

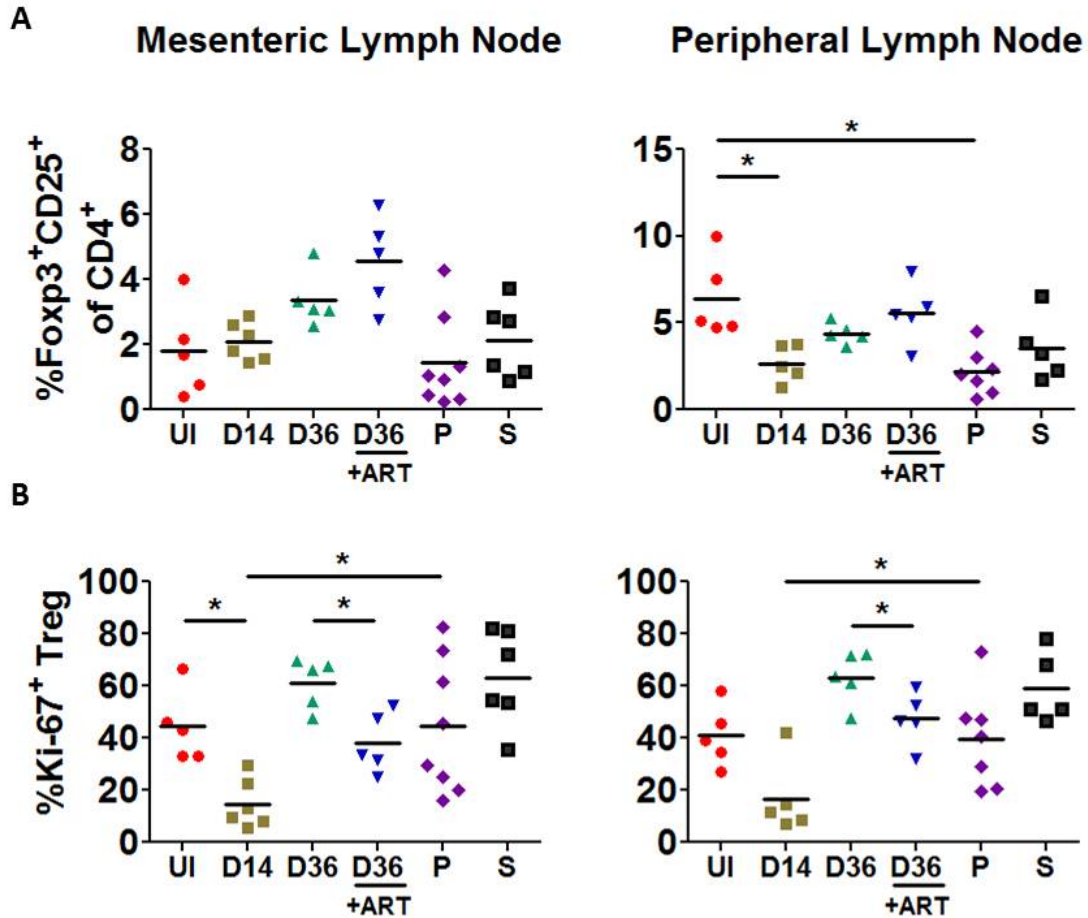


Figure 21. Treg are lost in peripheral LN of diseased animals, but not mesenteric LN.

Each point represents the percentage of Treg as detected in Figure 20B (A and B) and Ki-67 as detected in Figure 20C (C and D) for one animal. Horizontal lines represent means and * = $p < 0.05$. UI = uninfected animal. D14 = day 14 post SIV infection. D36 = day 36 post SIV infection. D36 +ART = animals started ART on day 7 post SIV infection and ended on day 36 post SIV infection. P = animal that progressed to AIDS based on viral load. S = animal that stably controlled infection based on viral load.

We observed no significant change in the percentage of Foxp3⁺CD25⁺ Treg in mesenteric LN in response to SIV infection and disease status (Fig. 21A). Conversely, we found that Foxp3⁺CD25⁺ Treg were lost from peripheral LN in animals with acute and chronic stages of SIV infection, a finding in accord with some previous reports (43, 44, 46). To determine the

percentage of newly proliferated Treg, we stained for Ki-67 in Treg (Fig. 20C). In mesenteric and peripheral LN, shown in Figure 21B, Ki-67 expressing Treg were lost during acute SIV infection but seemingly increased to slightly beyond healthy levels at later stages of infection, possibly suggesting delayed mobilization of Treg.

In summary, these preliminary results show that Treg proportions are not lost in mesenteric LN during SIV infection. This is in contrast with our finding in peripheral LN, in which Treg were significantly lost in SIV-infected cohorts as compared to uninfected macaques. Based on our findings in Figure 13 that CD103⁺ mDC are lost in mesenteric LN throughout SIV infection, these results could indicate that the link between Treg and CD103⁺ mDC in macaques may be more complex than in humans and mice.

5.0 DISCUSSION

In this study we have addressed some gaps in knowledge of how specialized innate immune cell proportions in LN and gut mucosa are impacted by SIV infection and disease status. These results have called attention to the potential significance of specific antigen-presenting cell subsets in the pathogenesis of SIV. Here we examined the impact of SIV infection, disease status, and ART on the frequency of macrophage and DC subsets in peripheral LN, mesenteric LN, and gut mucosa relative to healthy macaques.

First, we investigated the effect of SIV disease status and ART on CD163⁺CD68⁺ and CD163⁻CD68⁺ macrophages. CD163 is a transmembrane protein scavenger receptor reported to be present on M2 macrophages, and CD68 is a type 1 transmembrane glycoprotein believed to be a pan-macrophage marker (64, 68, 91). We found that CD68 was expressed by 100% of CD163⁺ macrophages, but was also expressed on a non-overlapping population negative for CD163 expression. Putative anti-inflammatory CD163⁺ macrophage proportions were significantly increased in gut-draining mesenteric LN during acute SIV infection and AIDS, which may represent a manifestation by the host to attenuate a local or systemic inflammatory response and preserve LN integrity. We also found that CD163⁺ macrophage proportions were increased in peripheral LN of SIV⁺ animals with acute infection. Our assumption that CD163⁺ macrophages function to diminish inflammation is based on reports that CD163 denotes M2 macrophages and thus will need to be validated in future studies to definitively reach this conclusion. One study

that could be conducted to determine their function would be to stimulate CD163⁺ macrophages sorted by FACS *in vitro* and assay for the presence or absence of pro- and anti-inflammatory cytokines. Theoretically, rather than serving an anti-inflammatory function, the influx of CD163⁺ macrophages we observed may instead be promoting inflammation. Intriguingly, we found that stably infected SIV⁺ macaques that did not progress to AIDS had healthy CD163⁺ macrophage levels. Given that M2 macrophages are more phagocytic than M1 macrophages (61, 172) and a reasonable cause of chronic immune activation has been attributed to increased levels of systemic LPS due to gut microbial translocation (48, 173), it is likely CD163⁺ macrophages are M2-like in function. Moreover, these findings do correlate with reports of increased CD163⁺ macrophage frequency in plasma with detectable HIV (174) and increased turnover in mesenteric LN (82). In addition, preliminary findings from our lab show CD163⁺ macrophage proportions are increased in bronchoalveolar lavage fluid from SIV-infected macaque lungs (M. Kader). Cumulatively, these findings would suggest that this macrophage subset may function to attenuate inflammation, not exacerbate it. In contrast, the fact that we observed no difference in the proportion of mesenteric LN CD163⁻CD68⁺ macrophages, which may be similar in function to intestinal macrophages, shown to be highly phagocytic, CD14 deficient and non-permissive to HIV infection, downplays their role in SIV infection, but does not rule out their potential importance in providing host protection from or vulnerability to opportunistic infection (85). We also observed no change in CD163⁻CD68⁺ macrophage proportions in peripheral LN between our cohorts. However, without more detailed studies, the role and function of CD163⁻CD68⁺ macrophages, which may represent one or many macrophage subtypes, in mesenteric LN and peripheral LN is unclear. Future studies should clarify these questions.

mDC and pDC are widely reported to be lost and dysfunctional in blood during HIV/SIV infection, however reports on mDC and pDC in LN are conflicting (150, 167). Furthermore, studies on the role of mDC and pDC in SIV pathogenesis in the gut are lacking. Our findings on mDC and pDC dynamics in LN of healthy and SIV⁺ rhesus macaques confirm some previous reports (154, 155, 157). We found that SIV disease status differentially impacted mDC and pDC proportions in peripheral LN relative to mesenteric LN. mDC and pDC proportions were modestly decreased in peripheral LN during acute and chronic stages of SIV infection, but were unaltered in mesenteric LN. In terminal ileum, we observed an increased density of CD123⁺ cells in macaques with acute and chronic stage SIV infection as compared to uninfected animals. Kwa and colleagues recently used flow cytometry to quantitatively demonstrate that pDC are recruited to gut mucosa of SIV-infected animals with AIDS and that recruitment and type 1 interferon immune activation could be reversed by *in vivo* blockade of $\alpha 4\beta 7$. Furthermore, this reversal of pDC recruitment resulted in decreased T cell proliferation and recruitment to the colorectum. No change in mDC proportions was observed in LN or gut (157). Nevertheless, the roles of mDC and pDC in SIV pathogenesis are still emerging; however based on ours and others' findings it is becoming clear that pDC may be a contributing factor to immune activation in the gut and that loss of pDC and mDC in peripheral LN is predictive of disease progression (156, 157, 159, 166).

Next, we explored the influence of SIV infection on CD103 expressing T cells, pDC, and mDC in LN and gut mucosa isolated from healthy and SIV-infected macaques. Recent evidence indicates that CD103⁺ mDC in mice and humans promote mucosal immune regulation by stimulating Treg differentiation and imprinting effector T and B cells with gut-homing markers CCR9 and $\alpha 4\beta 7$ (108). T cells have also been shown to express CD103, which is likely

important to their retention in the gut (107). Currently, the role of CD103 expressing cells in SIV infection and disease progression is undefined. In healthy animals, we found CD103 was expressed on 2% of CD3⁺ T cells and 31% of mDC in mesenteric LN, but not on mDC or pDC in peripheral LN. We did not determine the expression level of CD103 on T cells in peripheral LN. CD103⁺ mDC were dramatically lost by day 14 post SIV infection and continued to be depleted in the later stages of infection. We also observed that this depletion was not present in ART treated macaques. Additionally, a slight decrease in the density of CD103⁺ cells in Peyer's patches and intestinal villi of terminal ileum as disease progressed was observed; the vast majority of which were CD3⁺ T cells. Why this subset was not present at healthy levels in mesenteric LN from our stably SIV-infected cohort that did not progress to AIDS is unclear but could signify a direct or indirect consequence of sustained SIV infection and/or inflammation in the gut. Two studies that looked at the effect of gut inflammatory diseases on CD103⁺ mDC proportions found opposing results; intestinal tissue from Crohn's disease patients was found to have healthy CD103⁺ mDC levels, whereas mice with colitis had significantly fewer CD103⁺ mDC in their intestine (109, 175). These findings offer some clues to our observation of a sustained loss of CD103⁺ mDC in mesenteric LN from macaques with and without signs of AIDS. GI problems, such as colitis, are common manifestations in HIV/SIV-infected individuals and non-human primates, and thus, at the moment, may explain our results (176). However, the animals separated into the stably infected cohort exhibited no signs of diarrhea or other clinical complications associated with AIDS, suggesting there may be a better explanation. Our finding that ART treated macaques had healthy CD103⁺ mDC levels in mesenteric LN suggest their loss may be linked to direct viral infection, which may be inducing cell death. This hypothesis could be validated by staining CD103⁺ mDC with caspase markers that determine cell death and

assaying for SIV in CD103⁺ mDC by RT-PCR, immunofluorescence, or flow cytometry. In addition to microbial translocation, based on studies conducted in mice and humans on the critical nature of CD103⁺ mDC in driving Treg differentiation and T lymphocyte homing to the gut, our findings may suggest one mechanism by which gut immune homeostasis is disrupted during SIV infection (108).

Lastly, we analyzed the impact of SIV disease status on CD141⁺ mDC. CD141⁺ mDC have been shown in humans to trigger Th1-centric immune responses (122). We found that 37% of total mDC in mesenteric LN and 54% in peripheral LN from healthy rhesus macaques expressed CD141. In response to SIV infection, CD141⁺ mDC proportions were significantly increased in mesenteric LN and modestly increased in peripheral LN during acute stage infection, but were returned to naïve levels during the subsequent post-acute and chronic stages of infection. Lastly, we were unable to detect CD141⁺ cells by immunofluorescence staining in macaque terminal ileum. The relative contribution of this subset to SIV disease progression is, at this time, completely unknown. Numerous reports have documented that an apparent switch from a Th1 cell-mediated immune response to a Th2 humoral immune response underlies AIDS onset (177-179). Interestingly, our findings of increased Th1-inductive CD141⁺ mDC proportions in LN during acute SIV infection, but not during chronic infection, tend towards this same interpretation, suggesting that this dynamic may be in part responsible for mediating this switch. However, this theory implies the proportion of CD141⁺ mDC in stably SIV⁺ animals would be equally as high as those in the acutely infected group, and our data show this is not the case. At this time, we can only speculate about the role of CD141⁺ mDC in SIV pathogenesis when so little is known phenotypically and functionally about this subset in rhesus macaques.

As an extension to the aims of this thesis, our CD103⁺ mDC findings prompted us to undertake a preliminary investigation of Treg in our healthy and SIV-infected cohorts. The loss of CD103⁺ mDC proportions in mesenteric LN we observed would suggest a correlating loss of Treg based on CD103⁺ mDC functional reports in humans and mice (108, 111). Surprisingly, no change was observed in the proportion of Foxp3⁺CD25⁺ Treg in mesenteric LN from SIV-infected animals relative to controls, but we did see a significant decrease in peripheral LN Treg proportions at acute and chronic stages of infection. These findings are confounding. The function of CD103⁺ mDC in rhesus macaques is undefined, and consequently, the meaning and implications of decreased CD103⁺ mDC frequency in mesenteric LN during SIV infection are unclear. On the surface our data would suggest there is no correlation between CD103⁺ mDC and Treg in rhesus macaques in response to SIV infection. Presecci and colleagues recently demonstrated that mDC sorted by FACS from mesenteric LN of SIV-infected animals with AIDS and cultured with autologous CD4⁺ T cells promoted Treg induction better than mDC from healthy animals. However, they observed that CD103 expression was reduced on the same sorted mDC population from AIDS animals compared to controls, which they concluded suggests CD103 expression does not correlate with Treg induction (180). A more definitive study we hope to conduct in the future would be to sort CD103⁺ mDC from mesenteric LN of healthy and SIV⁺ macaques, culture those cells with autologous naïve CD4⁺ T cells, and assay for Foxp3 expression by flow staining.

Taken together, this work provides a cross-sectional analysis of the proportions of macrophage and DC subsets in tissues from uninfected and SIV⁺ macaques sacrificed at different stages of infection. Our results align with models of excessive immune activation in LN and the GI tract due to HIV/SIV infection and possibly, in part, may suggest a mechanism behind this

phenomenon (48, 181). We found that CD123⁺ cells, which have been shown to be chronically activated in HIV/SIV studies and correlated with disease progression (157, 164), were increased in density in the gut as infection progressed, supporting studies that suggest they may promote intestinal inflammation. Conversely, our finding of increased anti-inflammatory CD163⁺ macrophage proportions in mesenteric LN and gut alludes to a counter-attempt by the host to attenuate an inflammatory response. Lastly, SIV-infected rhesus macaques had significantly lower Treg-driving CD103⁺ mDC proportions as compared to healthy uninfected macaques, which may be a contributing factor to loss of gut immune homeostasis. Unfortunately, our Treg findings in LN complicate this model; however some evidence suggests Treg proportions are significantly depleted in gut during SIV infection (42). In the future we aim to quantify Treg in gut tissues from our cohorts.

As with all research, there are limitations to this study. One limitation of this study was its inability to demonstrate cause-and-effect. A longitudinal study design would have been a better way to demonstrate the effects of infection overtime and establish causality.

Importantly, these studies highlight the impact of SIV infection on innate immune cells in LN and gut mucosa and point to their potential contributions in virus control and chronic immune activation. Continued research on the role of macrophages and DC in SIV pathogenesis could bolster strategies to design novel anti-HIV therapeutics that dually target the viral life cycle and the innate immune response, and thus have significant public health value. Further studies towards elucidating the impact of disease on macrophages and DC are critical to understanding the complexity of the immune response to HIV/SIV infection.

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