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Investigating the contribution of CD4+ T cell subsets to SIV disease progression and persistence

By

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Investigating the contribution of CD4+ T cell subsets to SIV disease progression and persistence

By

Colleen Shannon McGary
B.S., University of North Carolina at Chapel Hill, 2010

Advisor: Mirko Paiardini, Ph.D.

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Abstract

CD4+ T-cells are progressively depleted from the blood and mucosa of individuals infected with Human Immunodeficiency Virus-1 (HIV), resulting in a massive disruption of T-cell homeostasis. The severity of HIV infection is coupled not only to the magnitude but also to the quality of CD4+ T-cells lost. Moreover, a small subset of infected CD4+ T-cells persists during antiretroviral therapy (ART), constituting the latent viral reservoir and the major barrier to an HIV cure. Determining the phenotypic and functional profile of virally infected CD4+ T-cells is critical for designing effective HIV therapeutic interventions.

To investigate which CD4+ T-cell subsets contribute to HIV pathogenesis, we utilized the pathogenic and non-pathogenic Simian Immunodeficiency Virus (SIV) infection models of rhesus macaques (RMs) and sooty mangabeys (SMs), respectively, and first examined how the preferential infection of central memory (TCM) CD4+ T-cells affects CD4+ T-cell homeostasis. Increased proliferation of CD4+ TCM cells was found only in SIV-infected RMs, yet was unable to restore CD4+ T-cell homeostasis, demonstrating how aberrant TCM proliferation facilitates disease progression. We then examined the impact of anatomic localization of CD4+ T-cells by investigating the loss of circulating CCR6+ and CD161+ CD4+ T-cells during SIV infection. While CD161+CCR6-CD4+ T-cells were preferentially infected, we found that CCR6+CD4+ T-cells accumulated in the gut mucosa of SIV-infected RMs, but not in SMs, demonstrating how these alterations are damaging to the host and advance disease progression.

Finally, to investigate the immunophenotype of latently infected cells, we examined ART-suppressed, SIV-infected RMs and identified CTLA-4 and PD-1-expressing cells as the major contributors to the viral reservoir across multiple anatomic sites. Importantly, we showed, for the first time, that CTLA-4+PD-1- regulatory T-cell-like (Treg) CD4+ T-cells contained the highest SIV-DNA levels, harbored replication competent virus, and localized outside the follicle in the T-cell zone. These data illustrate how SIV establishes and maintains viral persistence by targeting Tregs and follicular helper T-cells, two subsets critical for immune function.

Taken together, these findings identified CD4+ T-cell subsets critical for HIV/SIV pathogenesis and persistence, and ultimately, will provide insight into designing therapeutics aimed at restoring CD4+ T-cell homeostasis and achieving a functional cure.

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Chapter One

Introduction

HIV Epidemiology and Origins

The Human Immunodeficiency Virus (HIV) epidemic represents one of the most historic public health crises of our time. The World Health Organization (WHO) estimates that, as of 2015, 36.7 million individuals are infected with HIV worldwide, with 2.6 million of those infections representing children under the age of 15 years old (1). By these estimates, nearly 0.5% of the world's population (7.2 billion) is infected with HIV. While the disproportionate burden of this epidemic falls in Sub-Saharan Africa (24.7 million infections), HIV is still estimated to affect 1.2 million adults in the United States (2). Epidemiologically, HIV infections in the US are concentrated in major urban centers; however, the number of individuals living with HIV/AIDS in the US is highest in the South, accounting for nearly 52% of new AIDS diagnoses in 2010 (3). Nevertheless, despite the availability and efficacy of antiretroviral drugs in reducing HIV morbidity and mortality, only 30% of HIV-infected individuals in the US are currently achieving viral load suppression through use of antiretroviral therapy (ART), demonstrating that medicine alone is unlikely to end the HIV epidemic and emphasizes the need for increasing knowledge and decreasing the stigma surrounding HIV. However, in developing nations where access to ART is not universal, HIV/AIDS continues to pose a significant public health threat.

The landscape of the HIV/AIDS epidemic, though, has dramatically changed since the first reported cases of AIDS (Acquired Immunodeficiency Syndrome) in the US in 1981 (4). Following the isolation of HIV-1 (5, 6) and the later determination of HIV as the causative agent of AIDS, the disease was retrospectively demonstrated to originate in areas of central and east Africa (7, 8). HIV originated from the cross-species transmission of Simian Immunodeficiency Virus (SIV) from African non-human primates (NHPs) (9). While the means by which humans acquired HIV is still debated, phylogenetic analysis revealed that HIV-1, the more common and

pathogenic of the two HIV strains, originated from SIV harbored in a chimpanzee species, *Pan troglodytes troglodytes* (10, 11). In fact, HIV-1 comprises 4 distinct lineages, each resulting from a separate cross-species transmission event (represented by M, N, O, and P lineages), of which HIV-1 group M is the pandemic form. Each of these lineages can then be classified by its subtype- for example, HIV-1 group M has nine subtypes. Subtype B virus constitutes the majority of infections in the Americas and in Europe, while Subtype C is responsible for the greatest burden of the epidemic- that is, southern Africa and other Asian nations (12). HIV-2, a less pathogenic strain resulting in lower plasma viral loads, resulted from the transmission of SIV from sooty mangabeys. HIV-2 has at least eight distinct lineages (designated as groups A-H), yet only two groups (A and B) have supported human-to-human spread; the other groups represent a single individual who were each infected following independent host transfers (9).

HIV Biology

The diversity of HIV viral species across the globe results heavily from the unique properties of this virus. HIV is a member of the virus family *Retroviridae* and of the genus *lentivirus*. Each virion contains two copies of a single-stranded RNA genome that make up 9 open reading frames and encode 15 viral proteins (reviewed in (13, 14)). These viral proteins are divided into three classes: the structural and enzymatic proteins, the regulatory proteins, and the accessory proteins. Among the structural proteins, the Gag polyprotein gives rise to the main structural components of the core viral particle- the matrix (p17), capsid (p24), nucleocapsid (p9), and p6- all of which are essential for viral particle assembly (15). The Envelope (Env) polyprotein (gp160) is a heavily glycosylated precursor whose cleavage generates the two major components of the outer membrane envelope, gp120 and gp41, which together exist as a trimer. The gp120 subunit is found on the surface of the virion and mediates virus attachment to a target cell by binding the viral receptor CD4 and subsequently interacting with its co-receptors CCR5 and/or CXCR4 (16-18). Upon attachment, the transmembrane gp41 subunit then mediates the fusion of the viral and

cellular membranes to enable the viral core to enter into the cell's cytoplasm (19). The Pol polyprotein contains 3 enzymes essential for viral replication and maturation- protease, reverse transcriptase, and integrase (20-22). Two viral regulatory proteins, Tat and Rev, control HIV-1 gene expression. Tat activates viral transcription by promoting elongation from the viral long terminal repeat (LTR) region, which allows for the production of full-length viral transcripts (23-25). Rev transports unspliced RNA transcripts from the nucleus to the cytoplasm to avoid their nuclear degradation (26). Among the other viral proteins, the accessory proteins Vif, Vpu, Nef, and Vpr distinguish HIV-1 from other retroviruses, as they predominantly function to mediate viral evasion by antagonizing host proteins (13).

Understanding how HIV-1 replicates has been critical in determining the pathogenesis of the virus, the immune response against HIV, and also in developing ART. HIV-1 infection begins when the gp120 moiety of an HIV virion attaches to its cellular receptor, CD4 (27), and rearranges to allow for binding of one of its co-receptors, CCR5 or CXCR4 (17, 18, 28-30). The pattern of co-receptor usage varies by lentivirus species, with all SIVs and the majority of transmitted HIV-1 primarily using CCR5 (31, 32). The gp41 subunit then fuses with the cell membrane of the target cell, bringing the membranes together to release the viral core into the cell's cytoplasm (19). Upon cell entry, viral uncoating reveals the reverse transcription complex (33). Reverse transcriptase initiates reverse transcription of the RNA genome to produce a full-length double-stranded DNA (reviewed in (34)), which then is compacted into a pre-integration complex (35, 36), docked to the nuclear membrane by the viral protein Vpr (37, 38), and enters the nucleus through a nuclear pore. Integrase integrates the viral DNA genome into the host chromosome; however, pre-integration complexes that do not integrate can form 2-LTR circles by joining their viral DNA ends or single-LTR circles after undergoing homologous recombination. Upon integration, transcription can occur, which results in numerous HIV transcripts as a result of differential splicing (39). Initially, transcription by cellular RNA

polymerase II is inefficient, yielding short, multiply spliced transcripts encoding Nef, Tat, and Rev that are transported to the cytoplasm. Tat is able to improve this transcriptional efficiency, by binding to the transactivation response (TAR) element and rapidly recruiting an elongation complex to produce singly or unspliced viral transcripts (24). The accumulation of Rev facilitates the transport of these incompletely spliced viral transcripts to the cytoplasm (40). These longer transcripts can then be translated into viral proteins that assemble an infectious viral particle or can be packaged as genomic single-stranded RNA. Viral assembly occurs at the plasma membrane. Env proteins, translated and glycosylated within the endoplasmic reticulum, insert into the plasma membrane, along with the Gag and Gag-Pol polyproteins, and the full-length genomic viral RNA (41). Nef, with the assistance of Vpu, is then responsible for the endocytosis of CD4 from the cell surface, which allows for the budding of an immature virion (42). Budding activates the viral protease (43), which completes virion maturation by processing the Gag and Gag-Pol polyproteins into their individual proteins, and thus yields an infectious, mature virion that is able to infect another permissive host cell (44-46).

HIV Transmission and Dissemination

In 2015, 2.1 million people were newly infected with HIV globally, with the majority of new infections occurring in Eastern and Southern Africa (47). In regions, like these, with high rates of HIV transmission, the predominant mode of transmission is through sexual, mucosal transmission (48). Yet, transmission rates by heterosexual contact are estimated to be as low as 0.1% per exposure (and as high as 10%), suggesting that numerous factors, both virologic and immunologic, influence the establishment of new infections in uninfected individuals (49, 50). Chief among these factors are the physical barriers of the genital and gastrointestinal tract. An epithelial layer covers the female cervicovaginal tissues and the male penis (51, 52), preventing access of viral particles to underlying susceptible target cells. The protectant ability of this layer is supported by evidence in the rhesus macaque (RM) model of SIV infection where the

thickening of the epithelium via estrogen treatment reduced SIV vaginal transmission (53). Additionally, the mucus layer of the genital tract can trap virus particles to further limit their access to susceptible cells (54). A second factor that may influence the low HIV-1 transmission rate is the high ratio of defective to replication-competent virus in cervicovaginal fluids and semen, which decreases the exposure events of susceptible target cells (55). The influence of these factors, as well as the rapid HIV-immune system interactions at the portal of entry, likely contributes to the fact that disseminated HIV infection is most frequently established by a single transmitted founder virus (56).

Immediately following HIV/SIV exposure, there is a short period of time known as the "eclipse phase" where viral RNA is undetectable in the blood (57). The duration of this eclipse phase is a product of both the route of exposure as well as the levels of susceptible, target cells located at the portal site of entry. For example, in humans, the eclipse phase following vaginal HIV-1 exposure is approximately 10 days, while the eclipse phase is only 7 days in RMs following vaginal or rectal SIV exposure (55, 58). Studies in RMs have elucidated the influence of target cells on the eclipse time, by demonstrating a direct relationship between the levels of activated CD4+ T cells in mucosal tissues and the speed of viral dissemination to the periphery (59). This finding is consistent with the model in which local inflammation increases the rate of HIV infection. The eclipse phase comprises the initial infection, propagation and amplification of the virus necessary for establishing productive HIV infection. In the first 3 days of infection, virus replication is limited to resting CD4+ T cells, predominantly expressing CCR5, at the portal site of entry- for example, the lamina propria of the genital tract following intravaginal infection (55, 60-62). The virus output of these early foci of infection initiate new foci of infected CD4+ T cells throughout the localized tissue (62). The establishment of this productive viral infection then supports virus dissemination to draining lymph nodes (LNs) via lymphatic drainage by 1 week post-infection. Additionally, dendritic cells and Langerhans cells are able to bind free virus using

the C-type lectin receptor DC-SIGN at the portal site of entry, facilitating the migration of cell-bound and internalized virus to draining LNs (63). The abundance of activated CD4+ T cells and the accumulation of follicular DCs capable of trapping free virions on their surface make the LN an ideal environment for viral amplification (64, 65). High levels of pro-inflammatory cytokines (including IL-6 and TNF-a), produced as part of the innate immune response, further support virus replication within the LN (66). Despite the detection of an immune response at this time (stimulated HIV-specific CD4+ T cells, e.g.), no effector components are able to contain the spread of virus at this point, resulting in the dissemination of virus to distal tissues via peripheral circulation (67). Therefore, the eclipse phase ends as HIV viral RNA becomes detectable in the plasma during this viral spreading, which is referred to as Fiebig Stage I (68).

One of the critical sites of viral replication and seeding following viral dissemination is the gut-associated lymphoid tissue (GALT). In addition to being one of the largest organs within the lymphoid system, the GALT is predominantly populated by CD4+ CCR5+ memory T cells, thus making this site an abundant source of target cells (67, 69-71). Like at the portal site of entry, HIV entering the GALT initially replicates within a small foci of CD4+ T cells (61, 72). However, due to an abundance of target cells densely populating this anatomic location, an exponential expansion of HIV infection occurs, infecting up to 20% of mucosal CD4+ T cells and depleting nearly 80% of these cells, with a preference for CCR5+ CD4+ T cells (67, 73, 74). As a result of this viral amplification, SIV RNA levels peak in the plasma between 10 and 21 days post-infection, at around 10^7 virus particles per mL of plasma (68). HIV/SIV-specific responses, including both CD8-mediated and antibody responses, emerge at this time, leading to a decrease in virally infected cells as well as a post-peak viral decline.

Kinetics of Immune Response

Upon viral dissemination into distal lymphoid tissues, the host faces the nearly insurmountable task of containing further viral replication. Concurrent with the peak of plasma viremia, the initial antibody and cellular immune responses become detectable. Evidence from both humans and NHPs suggests that these initial responses have little effect on viral replication and spreading (56, 75-78). Nevertheless, the immune response mounted by the host during primary HIV infection influences the rate of pathogenesis and disease progression (79). In fact, by 4 weeks post infection, levels of plasma viremia begin to drop until they reach a virologic set point around 12-16 weeks post infection, which marks the transition into the chronic phase of infection (80).

The initial humoral response to HIV infection is marked by the appearance of Immunoglobulin G (IgG) antibodies directed against HIV gp41 (81). Anti-gp41 IgG antibodies can bind HIV virions to form immune complexes, but lack neutralization activity and thus are unable to prevent viral spreading. Following the transition to chronic infection, though, anti-gp120 antibodies with neutralization activity are detected (82). Despite their limited breadth, these anti-gp120 antibodies exert selective pressure on the virus, which is evidenced by the appearance of mutations in the HIV Env region 3 months after infection (82). A more potent group of HIV-directed antibodies- broadly neutralizing antibodies (bNAbs)- are generated in a subset of infected individuals two to four years after infection (83). These bNAbs share a number of unusual characteristics, including increased levels of somatic hypermutation, long immunoglobulin heavy chain complementary determining region 3 (CDR3) regions, and restricted germline use (83-87). Importantly, these isolated bNAbs, whether alone or in combination, can prevent SIV infection if passively infused into NHPs prior to challenge (88-92). As a result, vaccine efforts have focused on strategies to elicit these bNAbs in order to prevent acquisition of HIV; yet, standard vaccine approaches have failed, up to this point, in yielding antibodies with this level of breadth and potency.

Similar to the initial humoral response, the first HIV-specific CD8+ T cells detected have a limited effect on HIV replication, as evidenced by a lack of virus escape mutants during this stage (75). Appearing around the peak of plasma viremia, this population of CD8+ T cells is specific for HIV Env and Nef and accumulates in the blood (93). HIV-specific CD8+ T cells continue to expand from this time, with their peak levels corresponding to a decline in plasma viremia, which most directly results from HIV-infected cytotoxic T-lymphocytes killing HIV-infected CD4+ T cells (94-96). While a reduction in CD4+ T cell targets may also result from viral cytopathic effects and/or activation-induced cell death (AICD), amino acid mutations within CD8 epitopes of the founder virus suggests a direct effect of CD8+ T cell pressure on viral replication and their contribution to post-peak viral decline (75). Further evidence of the critical role of CD8+ T cells in limiting viral replication comes from animal models that demonstrated the rapid loss of viral control in RMs experimentally depleted of CD8+ T cells using a monoclonal antibody (97, 98).

While these antiviral immune responses are unable to fully contain viral replication and prevent disease progression in the majority of HIV-infected individuals, the ability of elite controllers (ECs) and long-term non-progressors (LTNPs) to withstand disease progression suggests that both humoral and cell-mediated immune responses have the capacity to control viral replication and spread. ECs are able to spontaneously control HIV-1 replication below the clinical level of detection (<50 HIV-1 RNA copies/mL of plasma) without ART. LTNPs, on the other hand, represent a cohort of individuals who are able to preserve CD4+ T cell levels in the absence of ART. A larger percentage of LTNPs possess bNAbs than in the overall population of HIV-infected individuals (99-103). Additionally, IgA antibodies directed against the CD4-binding site of HIV-1 gp120 have been found in LTNPs, which may be able to reduce viral burden and transcytosis in the mucosa (104, 105). On the other hand, the CD8+ T cell response of ECs demonstrates the capacity of cell-mediated immunity to control viral replication. Numerous groups have found that CD8+ T cells in ECs possess a superior ability to: (a) maintain

polyfunctionality following infection, as measured by cytokine and chemokine secretion (106); (b) proliferate (107); (c) inhibit HIV replication (108); (d) express perforin, degranulate, and kill target cells (108-110); and (e) target Gag epitopes (111, 112). Furthermore, CD8+ T cells from ECs are primed effectors; several groups have found a subset of CD8+ T cells that are able to inhibit HIV replication in the absence of prior activation, demonstrating their immediate antiviral function (113-116). Finally, there is an association between several HLA class I alleles and disease progression. Whereas HLA-B*2705, HLA-B*5701, and HLA-B*5801 alleles are associated with slower disease progression and protection, HLA-B*35 is associated with faster disease progression, illustrating the essential impact CD8+ T cells can exert on viral replication (102, 117). The CD4+ T cell response of ECs and LTNPs also plays a critical role in viral control. Similar to CD8+ T cells, HIV-specific CD4+ T cells in these two cohorts are often polyfunctional (113, 118-121), secrete more IL-2 and IL-21 (122, 123), and have a higher capacity to proliferate alongside lower expression of co-inhibitory receptors (124). These features are in direct contrast to the functional profile of HIV-specific CD4+ T cells in normal progressors, where CD4+ T cells have reduced production of IL-2 and proliferate less, resulting in part from their increased expression of co-inhibitory molecules (109, 124-129).

HIV Pathogenesis

The hallmark of HIV infection is the massive depletion of CD4+ T cells. As described above, CD4+ T cells are susceptible to HIV upon co-expression of the HIV coreceptors CCR5 and/or CXCR4 (although alternative coreceptors have more recently been described). Interestingly, this massive insult to the immune system is only partially restored with ART, though in the absence of therapy, CD4+ T cell levels decrease between 50-100 cells/uL each year (130). Although direct infection of CD4+ T cells by HIV can lead to their elimination (131), viral load cannot predict CD4+ T cell decline alone (132-134). Instead, CD4+ T cell depletion is additionally supported by bystander effects including viral syncitia formation, immune activation and proliferation. Those

CD4+ T cells residing in the GALT lamina propria experience the earliest and most profound depletion following HIV-1 infection, resulting from the high concentration of susceptible target cells found there. It is well established that this robust depletion of mucosal CD4+ T cells, which includes the preferential depletion of Th17 cells (135-137), as well as enterocyte apoptosis (138, 139) results in the breakdown of intestinal structural integrity during HIV/SIV infections (67, 70, 74). As a result, microbial translocation from the gut into the peripheral blood occurs, which is associated not only with HIV disease progression but also with chronic immune activation (140).

During HIV/SIV infection, increased levels of immune activation are present both in the blood and in tissues. In the blood, higher frequencies of CD4+ and CD8+ T cells express the activation markers HLA-DR and CD38 as well as the proliferation marker Ki-67; in fact, these frequencies can predict the decline in CD4+ T cells in infected individuals (132, 141, 142). Immune activation is also manifested through the increased frequencies of CD4+ and CD8+ T cells expressing co-inhibitory receptors (Co-IRs), including PD-1, CTLA-4, TIM-3, and LAG-3, among others, as well an increased frequency of apoptotic cells (123-126, 143, 144). In tissues, immune activation is demonstrated by LN hyperplasia concomitant with an expansion in plasma B cells (145). However, the mechanisms responsible for chronic immune activation are less clear.

One clear contributor to immune activation is the virus itself. Not only does the presence of virions and viral antigens lead to the continual stimulation of the innate and adaptive immune responses during untreated HIV infection, but HIV is also a ligand for TLR7 and TLR8, which, when triggered on plasmacytoid dendritic cells (PDCs), leads to IFN-a production (146-148). Despite this direct effect of the virus, chronic immune activation is still present in virally-suppressed patients on ART with adequate CD4+ T cell counts; therefore, immune activation likely results from the contribution of several factors that synergize, even in the absence of ongoing viral replication. With microbial translocation, bacterial and fungal products, including

lipopolysaccharide (LPS), flagellin and peptidoglycan, cross from the intestinal lumen into systemic circulation and, as a result, can stimulate numerous TLRs throughout the body to produce pro-inflammatory cytokines (TNF-a, IL-6, and IL-1B) (135, 140, 149-151). This mechanistic link between microbial translocation and immune activation is strengthened by data from sooty mangabeys (SMs) and African Green Monkeys (AGMs), two natural hosts of SIV infection that withstand progressive infection, where there is an absence of immune activation and low levels of LPS during SIV infection (138, 140, 152). HIV-infected individuals are also susceptible to the reactivation of additional latent infections, including Epstein Barr virus (EBV) and cytomegalovirus (CMV), during chronic HIV infection as a result of CD4+ T cell depletion, which may increase chronic immune activation. For example, CMV reactivation has been shown to increase immune activation in HIV-infected patients and increase the pool of HIV target cells (153, 154). In fact, valganciclovir, an antiviral agent against CMV, was shown to decrease activated CD8+ T cell levels in ART-treated, HIV-infected individuals, which supports the contribution of reactivated co-infections toward immune activation (155). Immune activation also increases as a result of the altered ratio of CD4+ T cell subsets that occurs following the preferential targeting and depletion of CD4+ T cells (see *CD4+ T Cell Selection and Depletion*). A decreased ratio of Th17 to regulatory T cells (Tregs) in both the blood and mucosa correlates with increased levels of activated CD8+ T cells, though the mechanism responsible for this relationship remains unclear (156, 157). Increased Treg numbers may promote immune activation by suppressing HIV-specific immune responses and allowing for higher viral replication (158, 159). Alternatively, decreased Th17 cell numbers can reduce intestinal barrier integrity, leading to increased microbial translocation that in turn stimulates immune activation (138). Altogether, these processes coordinate to maintain heightened levels of immune activation in HIV-infected patients, which serves as a driving force of disease progression in untreated patients and continually contributes to immune aging in ART-treated patients.

Animal Models for HIV-1 Infection

Defining the key features of HIV-1 pathogenesis and the determinants of progression to AIDS has largely depended on the use of NHPs. First, the pathogenesis of HIV-1 infection, including the dissemination of virus throughout the host and the kinetics of the immune response, has been elucidated through studying SIV infection in RMs and pig-tailed macaques (PTMs). These NHPs are naïve to SIV; therefore, upon experimental infection with SIV, they develop an AIDS-like disease similar to that observed in HIV-1-infected humans, as defined by a progressive depletion of CD4+ T cells, plasma viremia, and later susceptibility to opportunistic infections. Several features of HIV infection are recapitulated in the RM model of SIV infection, including establishment of viral reservoirs, chronic immune activation, mucosal immune dysfunction, microbial translocation, and heightened infectivity of CD4+ TCM cells (74, 140, 160, 161); thus, the SIV/RM model is widely considered the best animal model for HIV infection. The most common viruses used to infect RMs are SIVmac239 and SIVmac251. SIVmac251 is a polyclonal virus that mimics the genetic diversity of HIV-1 in humans, while SIVmac239 is a clonal virus derived from SIVmac251.

However, not all NHPs experience disease progression following SIV infection. In fact, as many as 40 species of NHPs are naturally infected with SIV in the wild, resulting in high levels of viral replication but never develop AIDS (162); these animals are referred to as natural SIV hosts. As a result, researchers are able to compare the characteristics of SIV infection in SMs and AGMs, two natural SIV hosts, to the characteristics found during pathogenic SIV infection in non-natural hosts in order to define contributors to disease progression and mechanisms of protection. Several features of SIV infection are shared between natural and non-natural hosts, indicating that these features are insufficient to drive disease progression to AIDS. For example, natural hosts experience high levels of viremia following SIV infection, similar to levels seen in HIV-infected individuals during acute infection (133, 163-166). As in pathogenic HIV/SIV infection, the acute

immune response in natural hosts is characterized by high levels of immune activation, which includes the production of type I interferons and upregulation of interferon response genes; however, natural hosts are unique in their ability to resolve this innate immune response after 4-8 weeks of infection, despite ongoing viral replication (167-170). In contrast, the type I interferon response continues throughout chronic infection in non-natural hosts, which suggests that the resolution of innate immune responses is actually protective for hosts and assists in their ability to avoid AIDS (171, 172). While SIV infects and depletes mucosal CD4+ T cells in both natural and non-natural hosts during acute infection, their depletion is not progressive; instead, mucosal CD4+ T cell levels remain relatively stable throughout the course of infection in SMs (69, 152). Interestingly, productively infected cells in both natural and non-natural hosts have a similarly short *in vivo* lifespan- approximately 1 day- suggesting that the cytopathic effects of SIV are similar in these two classes of NHPs. These data suggest that another mechanism is responsible for the preservation of CD4+ T cell homeostasis in natural hosts (173-177).

Investigations have revealed several features which distinguish pathogenic from non-pathogenic SIV infection, thus identifying major determinants of disease progression to AIDS. As described above, SIV infection in the natural host SMs and AGMs is devoid of the hallmark loss of peripheral CD4+ T cells seen in HIV-1 infections (133). Although CD4+ T cells are still susceptible to SIV infection in natural hosts, SMs and AGMs demonstrate a differential pattern of CD4+ T cell infection, which has informed our understanding of how CD4+ T cell homeostasis should be preserved to avoid disease progression. For one, central memory CD4+ T cells (TCM) are relatively spared from direct viral infection in SMs as compared to their heightened frequencies of infection in pathogenic infection (161, 178). Ex vivo analysis of TCM from SMs revealed a mechanism behind their preservation- CD4+ T cells from natural hosts express lower levels of the SIV co-receptor CCR5 on their surface compared to RMs, thus decreasing their susceptibility to infection (161, 179). Natural hosts are also able to preserve their mucosal

homeostasis, despite an acute loss of mucosal CD4+ T cells. Several mechanisms have been proposed to contribute to this mucosal preservation, including data in SMs demonstrating the maintenance of mucosal Th17 cells, which in turn strengthens intestinal barrier integrity and reduces microbial translocation (135, 140, 179, 180). Additionally, SIV-infected natural hosts maintain their LN architecture. Levels of virus replication are lower in the LN of natural hosts as a result of their lower levels of CD4+ TCM infection (181, 182); furthermore, they do not support an expansion of germinal centers, as is seen in pathogenic infection in RMs (145, 183, 184). This finding, in combination with the absence of microbial translocation, may partially explain the lack of chronic immune activation found in SIV-infected natural hosts. Remarkably, SMs and AGMs are able to resolve their acute immune activation following SIV infection, as measured through both cellular activation and apoptosis (167-169), suggesting that systemic immune activation only exacerbates disease progression in susceptible non-natural hosts (133, 163, 176, 185, 186). In agreement with this model, a study of ART-treated, HIV-infected individuals demonstrated that the levels of residual immune activation in virally suppressed patients could predict an ineffective reconstitution of immune function (187). Thus, comparative analysis of SIV infection between natural and non-natural hosts illustrates how peripheral CD4+ T cell loss, chronic immune activation and differential patterns of CD4+ T cell infection and depletion contribute to immune damage to the infected host and disease progression.

CD4+ T cell selection and depletion

Although CD4+ T cell depletion characterizes pathogenic HIV/SIV infection, the targeting of CD4+ T cells by the virus is not equal. As a result, CD4+ T cell subsets can be differentiated by their level of susceptibility, pathogenicity, and contribution to persistence. Research has largely focused on these three areas of characterizing CD4+ T cells as each feature underlines a strategy for therapeutic intervention. In this regard, understanding which CD4+ T cells are increasingly susceptible to HIV/SIV infection, whether characterized by their location or function, informs the

design of preventative vaccines. Comparative analysis of which CD4+ T cells are preferentially depleted in pathogenic SIV infection versus non-pathogenic SIV infection (e.g. RMs versus SMs) elucidates the perturbations in CD4+ T cell homeostasis that advance disease progression. Additionally, understanding which CD4+ T cells harbor latent proviral DNA during suppressive ART informs the development of therapeutic interventions that can reduce the viral reservoir and promote a functional HIV cure. Thus, among each classification of CD4+ T cell subset- memory, functionality, and location- different CD4+ T cells are varied in their susceptibility, pathogenicity, and viral persistence.

Memory CD4+ T cells

Naïve CD4+ T cells, upon recognition of their cognate antigen on an antigen presenting cell (APC), become activated, proliferate, and then differentiate into antigen-experienced memory CD4+ T cells. Within the memory CD4+ T cell compartment, a hierarchy of differentiation exists, in which more immature, long-lived T cells serve as precursors that can repopulate and differentiate into mature, short-lived T cells. A recently described memory CD4+ T cell subset- stem cell memory CD4+ T cells (TSCM)- are considered the least differentiated of memory CD4+ T cells. CD4+ TSCM are unique in that, in addition to expressing the memory T cell markers CD95 and CD62L, they also express naïve T cell markers such as CD45RA and CCR7 (188, 189). Accounting for only a small proportion of the memory CD4+ T cell compartment (between 2-4%), CD4+ TSCM possess self-renewal capacities, allowing them to regenerate their pool of cells after repopulating more differentiated memory T cell subsets (188, 190-192). Central memory (TCM) CD4+ T cells also self-renew, but are more differentiated than CD4+ TSCM. These cells reside in lymphoid compartments as a result of their expression of the lymphoid homing markers CD62L and CCR7. Due to this localization, CD4+ TCM have limited effector functions, but instead, rapidly proliferate following antigenic restimulation, which contributes to their maintenance and long half-life. In contrast, effector memory (TEM) CD4+ T cells lack

CD62L and CCR7 expression but express chemokine receptors that direct their migration to non-lymphoid and inflamed tissues. There, CD4+ TEM produce effector cytokines, such as IFN-G and IL-4, at the site of infection. Of the memory CD4+ T cells, TEM are the shortest-lived, as their functionality is often transient upon pathogen exposure.

The increased susceptibility of memory CD4+ T cells to HIV/SIV infection is in part dependent on their increased expression of the viral co-receptor CCR5. As a chemokine receptor, CCR5 directs the migration of expressing cell subsets to sites of inflammation in response to CCL3, CCL4, and CCL5 production (193, 194). Therefore, CCR5-expressing cells are susceptible to HIV infection both as a result of their viral co-receptor expression, but also due to their increased prevalence at tissue sites that commonly encounter pathogens- for example, the gut mucosa (195-197). The highest levels of CCR5 expression occur on effector and TEM cells, making these subsets key viral targets (161). Moreover, in RMs, the level of CCR5 expression has been shown to correlate with the permissiveness of CD4+ T cell subsets to SIV infection (161, 176, 198). However, due to their short life-spans, CD4+ TEM cells are unlikely to persist during ART, and thus contribute less than other memory CD4+ T cell subsets to disease progression and viral persistence. *In vivo* data from untreated HIV-infected humans and SIV-infected RMs have found that both TSCM and TCM CD4+ T cells are highly permissive to HIV infection, as evidenced by the high levels of virus harbored in these isolated subsets (161, 199). Interestingly, the infection of these memory CD4+ T cell subsets (TSCM and TCM) is a distinguishing feature of pathogenic infection. A pivotal study from Paiardini et al. demonstrated that CD4+ TCM cells from SMs harbor lower levels of cell-associated SIV DNA than RMs, which is in part due to their lower levels of CCR5 expression (161, 179). More recently, Cartwright et al. were only able to detect SIV DNA in CD4+ TSCM from 2 of 10 SIV-infected SMs, compared to all SIV-infected RMs, a finding which was similarly observed in HIV patients who maintain CD4+ T cell counts during infection (199, 200). In both of these examples, low to complete absence of surface CCR5

expression protects these critical memory CD4+ T cell subsets from HIV/SIV infection and demonstrates that the resistance of CD4+ TSCM and TCM is critical for host protection against HIV/SIV disease progression. On the other hand, during pathogenic HIV/SIV infection, the progressive depletion of CD4+ TCM cells determines the rate of disease progression to AIDS, predominantly as a result of their high levels of viral infection (160, 201).

While their loss influences CD4+ T cell homeostasis and promotes disease progression, the ability of CD4+ TSCM and TCM cells to persist during ART influences their major contribution to the viral reservoir. In a landmark study examining viral DNA in long-term ART-treated, HIV-infected individuals, Chomont et al. identified central memory and transitional (TTM) memory CD4+ T cells as the major cellular reservoirs for HIV during ART (202). Viral persistence within these CD4+ TCM cells, the key reservoir in patients with normal CD4+ T cell reconstitution following ART, results from the prolonged T cell survival of this memory subset, while CD4+ TTM cells, the subset more responsible for persistence in patients with low CD4+T cell counts, results more from their homeostatic proliferation. By homeostatically proliferating in response to IL-7, memory CD4+ T cells can expand without viral reactivation, allowing HIV to remain latent (202). Similar to TCM cells, CD4+ TSCM increasingly contribute to the viral reservoir with increasing time on ART, supporting the stability of this cellular subset (199, 203). However, CD4+ TSCM cells only make up between 2-4% of the CD4+ memory compartment; therefore, this cell subset accounts for a smaller proportion of the reservoir than CD4+ TCM cells (199). Nevertheless, recent reports have determined that the decay of HIV DNA in CD4+ TSCM cells is significantly lower compared to other memory CD4+ T cell subsets (199, 203). Therefore, both CD4+ TCM and TSCM cell subsets are critical contributors to disease pathogenesis as well as viral persistence during ART, and thus, should be a key focus of HIV vaccine and therapeutic strategies.

Functional CD4+ T cell subsets

Memory CD4+ T cells can be further characterized from a functional perspective. In contrast to being defined by life spans and anatomic locations like memory CD4+ T cell subsets, functional CD4+ T cell subsets are classified by the group of pathogens for which they have specific functionality, their cytokine secretion patterns, as well as a master transcriptional regulator which coordinates their genetic expression profile (204). The original paradigm for CD4+ T cell differentiation identified two polarized subsets in both mice and humans: Type 1(Th1) and Type 2 (Th2) cells (205, 206). However, since then, additional polarized CD4+ T cell subsets have been identified and characterized, to now include the following: Th1 (antibacterial defense), Th2 (helminth and parasitic defense), Th17 (extracellular bacteria and fungal defense), Th9 (allergy and tumor regulation), regulatory T cells (Tregs; regulating and modulating antimicrobial activity), and follicular helper T cells (TFH; support humoral immunity) (reviewed in (207)). These polarizations are controlled predominantly by the cytokine milieu present during CD4+ T cell priming, which influences the regulation of transcription factors and ultimately the gene expression profile of the functional subset.

During pathogenic HIV and SIV infections, the functionality of CD4+ T cells is altered, as demonstrated by their decreased production of effector cytokines such as IL-2, TNF-α, and IFN-G (106, 208, 209). However, apart from a general functional impairment, HIV can also perturb CD4+ T cell homeostasis by preferentially infecting distinct functional CD4+ T cell subsets. Several lines of evidence point to this viral selection based on CD4+ T cell functionality. For example, HIV has been shown to preferentially infect HIV-specific CD4+ T cells (210) and later work demonstrated a viral preference toward Mycobacterium Tuberculosis-specific CD4+ T cells (211). HIV-specific CD4+ T cells, in particular, are more susceptible to viral infection likely as a result of their increased activation status (62, 210, 212).

Th17 cells, a subset of memory CD4+ T cells that are enriched in mucosal tissues (135), preferentially produce IL-17, in addition to IL-21, IL-22, TNF-α, and IL-2, all of which coordinate to play a critical role in the clearance of extracellular bacteria and fungi (213). Through the secretion of IL-17, Th17 cells are able to promote the recruitment, activation and migration of neutrophils to areas of bacterial infection (214). Th17 cells further promote mucosal defenses by secreting IL-22, which drives the production of antimicrobial peptides (215), the expression of claudins and mucins (216, 217), as well as the proliferation of enterocytes (218, 219). Th17 cells also influence the regulation of functional CD8+ T cells as well as the generation of memory B cells and antibody-producing plasma cells through their IL-21 production. Th17 cells are depleted throughout the entire GI tract of HIV-infected patients. This Th17 cell loss is specific to mucosal tissues and is not seen in the blood or bronchoalveolar lavage (BAL) of HIV-infected individuals (135, 220).

The mechanisms responsible for the loss of Th17 cells during pathogenic SIV and HIV infections have been extensively explored. Th17 cells express the HIV/SIV co-receptor CCR5, although its expression appears to be tissue-specific. Blood Th17 cells express low levels of CCR5, while Th17 cells in the mucosa are predominantly CCR5-positive, thus making them highly susceptible targets of HIV/SIV infection (135). However, despite low CCR5 levels, *in vitro* studies have found that Th17 cells from peripheral blood are highly permissive to HIV infection, compared to Th1 cells (221-223). Investigations into this phenomenon have suggested that the increased susceptibility of Th17 likely results from a gene expression signature that better supports viral replication than other CD4+ T cell subsets (224). Nevertheless, it is less certain whether this increased susceptibility *in vitro* reflects the mechanism by which mucosal Th17 cells are depleted. For example, no correlation was found between levels of plasma viral load and the frequencies of IL-17-producing lymphocytes in the mucosa of SIV-infected RMs, which suggests that viral replication alone is unlikely to be responsible for the preferential depletion of Th17 cells

from the GI tract (225). Investigations of a potential impact of HIV infection on the proliferative capacity of Th17 cells similarly revealed no differences from Th1 cells, illustrating that Th17 cells are not more susceptible to activation-induced cell death (135). Apart from a direct impact of HIV on Th17 cells, one mechanism that may contribute to the preferential loss of Th17 cells is the loss of CD103+ dendritic cells (DCs) from the colon and mesenteric lymph nodes following infection, as was demonstrated in SIV-infected RMs (225). CD103+ DCs have been shown to promote Th17 differentiation of naïve CD4+ T cells *in vitro* and their levels are positively correlated with levels of Th17 cells *in vivo (225).* In addition, intestinal IL-21-producing lymphocytes are significantly depleted during chronic SIV infection of RMs, which is associated with the loss of Th17 cells (226). This finding suggests that the loss of cytokines that are critical for the generation and maintenance of Th17 cells during SIV/HIV infection may also contribute to the preferential depletion of mucosal Th17 cells. Consistent with this model, the administration of recombinant IL-21 to SIV-infected macaques results in an increase in mucosal Th17 cell frequencies(136, 227, 228). Therefore, the depletion of Th17 cells is likely influenced by a dysregulation in the priming environment upon HIV/SIV infection, although additional mechanisms that may contribute to this preferential depletion are continually being explored.

The extent of Th17 cell depletion has been shown to be predictive of chronic immune activation and disease progression in HIV-infected individuals (156). Consistent with this finding, examination of the frequencies of Th17 cells in the GI tract of SIV-infected SMs and AGMs revealed that these cells are maintained in the mucosa of natural hosts, despite an overall loss of mucosal CD4+ T cells (135, 180). Furthermore, LTNPs have higher levels of Th17 cells than normal progressors in the blood and can maintain mucosal Th17 cells at levels that are comparable to those found in uninfected individuals (229, 230). These findings illustrate that the maintenance of Th17 cells in the GI tract is a critical feature for resisting disease progression, both in HIV and SIV infection. The contribution of Th17 cells to viral persistence, though, is still

being investigated. Characteristics of Th17 cells, including their long life-spans, their ability to homeostatically proliferate and their developmental plasticity, combined with their known permissivity to HIV infection, have long supported their hypothesized contribution to viral persistence during ART (231-233). However, investigations into the enrichment of Th17 cells from ART-treated, HIV-infected individuals have been limited by identifying Th17 cells based solely on their expression of surface receptors, and not transcription factors or secreted cytokines. Despite this caveat, CD4+ T cells enriched in Th17 cells, as determined predominantly by chemokine receptor expression, have demonstrated higher levels of HIV DNA than other CD4+ T cell subsets from ART-treated patients, supporting their proposed contribution to viral persistence (234, 235).

Regulatory CD4+ T cells (Tregs), a subset characterized phenotypically by their expression of CD25 (IL-2α receptor), CTLA-4, and the transcription factor FoxP3 (236, 237), are not specific to a particular class of pathogens, but instead, function to regulate the activation, proliferation and effector functions of other immune cells (238). While these functions are essential to regulate self tolerance, Tregs can also have a negative function during chronic infections, where their suppressive activity inhibits antiviral immune responses and subsequent pathogen clearance (239, 240). Similar to Th17 cells, Treg homeostasis is heavily perturbed during HIV infection. Treg frequencies are elevated in the blood of HIV-infected individuals, when compared to healthy controls, yet this increase is not reflected in absolute counts, which decrease with progressive infection (241-244). This expansion of Tregs, though, is unable to significantly reduce chronic immune activation, suggesting that Tregs function more in inhibiting antiviral immune responses during untreated HIV infection (156, 168, 243, 245-248). Tregs secrete the cytokines IL-10 and TGF-β, suppressive cytokines that can dampen viral control by reducing HIV-specific T cell proliferation (249-251). Moreover, Tregs from HIV-infected patients exhibit different phenotypic profiles than those from healthy individuals, including elevated expression levels of FoxP3,

ICOS, and CTLA-4 (252). Given their shared developmental pathway with Th17 cells, the dysregulation of Tregs during HIV/SIV infection is not entirely unexpected. In fact, a change in the Th17 to Treg ratio is predictive of generalized T cell activation and is reflective of disease progression (184, 243, 247, 248, 253).

Tregs are highly susceptible to HIV/SIV infection, with several studies in untreated HIV-infected individuals demonstrating higher levels of HIV DNA (up to 15-fold) in Tregs compared to non-Tregs *in vivo* (242, 246, 254-256). This preferential susceptibility has been proposed to result from the combination of a high proportion of memory cells, proliferating cells, and CCR5-expressing cells (242, 257-260). Additionally, higher levels of *in vivo* proliferation may support the contribution of Tregs to viral persistence, by allowing this subset to give rise to new cell progeny carrying HIV DNA in the absence of HIV reactivation. In support of this model, Tregs from ART-treated, HIV-infected individuals were found to contain higher levels of HIV DNA than other CD4+ T cell subsets (256). Moreover, *in vitro* culture experiments have demonstrated the ability of Tregs to release virus upon restimulation, suggesting that virally-infected Tregs could persistently contribute to viral production (256, 259).

Follicular helper CD4+ T cells (TFH) reside in LN follicles in close proximity to B cells, where they are critical for the formation of the germinal center (GC), and there, provide B cell help, promote Ig class switch, somatic hypermutation and B cell maturation (261-265). TFH cells are characterized by surface expression of CXCR5 and PD-1, are regulated by the transcription factor Bcl6, and functionally, can be characterized by their production of IL-21 (261, 263, 264, 266-269). During HIV/SIV infection, TFH cells expand in the LN, which can be partially reversed following ART-mediated viral suppression, suggesting that the changes in frequency are largely driven by the virus (270-273). In fact, the frequencies of TFH cells correlate with plasma viremia levels in untreated HIV-infected patients (272). TFH cells are highly permissive to HIV and SIV

infection. TFH cells from HIV/SIV-infected humans/RMs harbor higher levels of HIV/SIV DNA than other CD4+ T cell subsets, which confirms *in vitro* experiments demonstrating the ability of TFH cells to be infected and support productive infection (271-277). While their increased susceptibility is likely influenced by an enrichment in virus-specific cells (272, 274), the susceptibility of TFH cells likely also results from their localization within the GC, where they remain in a heightened activation state and are in close proximity to virally-loaded follicular DCs that can promote their infection (64, 145).

The contribution of TFH cells to viral persistence during ART is an area of active investigation. Previous studies have found that HIV DNA levels decline rapidly in TFH cells during the first years of ART (272). However, other studies have found TFH cells to be a major viral reservoir *in vivo*, both in ART-treated HIV-infected individuals and SIV-infected RMs, with viral production still detected in elite controller macaques (278-280). A more recently described subset of peripheral TFH cells (pTFH) with shared B cell helper functions and CXCR5 expression may also be contributing to viral persistence (122, 235, 281, 282). TFH cells sorted from PBMCs have been found to harbor more virus upon *in vitro* infection, a result that is consistent with increased levels of HIV DNA in circulating PD-1+ TCM cells and is irrespective of their CCR5 expression levels (202, 280). Therefore, the contribution of TFH cells to the viral reservoir may be influenced by their ability to exit the LN as memory cells and persist as long-lived TCM cells.

Anatomic Characterization of CD4+ T cells

In addition to characterizing viral reservoirs by memory and functional CD4+ T cell subsets (i.e. cellular reservoirs), viral reservoirs can also be defined anatomically, which results predominantly from the CD4+ T cells found there (i.e. anatomic reservoirs). As previously described, gut associated lymphoid tissue (GALT) constitutes a major site for viral production and replication. As a result, CD4+ T cells residing within this tissue compartment or with

migratory potential there are rapidly and severely depleted during HIV/SIV infection. The preferential localization of HIV in the GALT reflects the high concentration of potential target cells there, since the majority of CD4+ T cells are CCR5-expressing, activated memory CD4+ T cells (283). Due to their high frequency, CD4+ TEM cells harbor the highest levels of infection in the GALT (284). Additionally, Th17 cells reside predominantly in the mucosa. These phenotypes also characterize CD4+ T cells with the potential to migrate to the mucosa.

The main pathway CD4+ T cells use to traffic to the GALT is the α4β7-MAdCAM-1 axis. Mucosal vascular Addressin Cell Adhesion Molecule-1 (MAdCAM-1) is expressed on high endothelial venules throughout the GALT (285) and attracts α4β7-expressing CD4+ T cells, which are often primed in Peyer's patches or intestinal draining lymph nodes. CD4+ T cells expressing high levels of the α4β7 integrin are early targets of HIV and SIV during acute infection, and the level of integrin expression is associated with their frequency of infection (286-290). This preferential infection may result from the direct engagement of HIV gp120 with the α4β7 integrin; this interaction has been shown to facilitate the formation of a virologic synapse and thus promote viral infection of integrin-expressing CD4+ T cells (290, 291). The impact of this preferential infection on disease progression is supported by studies in RM, where animals treated with a monoclonal antibody directed against α4β7, both before and during acute SIV infection, had significant decreases in plasma VL and levels of virus in the GALT (292). Additionally, when given prophylactically, α4β7 blockade significantly protected RMs from SIV transmission, supporting the conclusion that α4β7-expressing cells are early viral targets that become major sites of viral replication (293). Chemokine receptors, including CCR6 and CCR9, also coordinate the migration of T cells to the GALT (294).

Lymph nodes are also considered major sites for HIV production and replication. Yet, unlike the GALT where HIV/SIV infection leads to a marked depletion in CD4+ T cells, the major viral

targets of HIV in the LN- the TFH cells- expand during infection (271, 272). Similar to the GALT, though, HIV preferentially infects this anatomic location as a result of the high frequencies of CD4+ TCM there as well as the heightened state of activation consistently present in the GCs (272). Even within the LN, the compartmentalization of the virus influences the pathogenicity of the infection. For example, in the natural host SMs, SIV can be detected in the LN during acute infection within the paracortex, but not in the GCs, where it is only found during pathogenic SIV infection of RMs (295). Viral RNA and DNA can then be found in the GC throughout chronic infection and even during ART-mediated viral suppression. Understanding why virus persists within LN GCs has been an active area of investigation for the past decade. CD8+ T cells may have limited access into GCs (296), and other evidence suggests that those CD8+ T cells entering the GCs possess limited cytotoxic capacity (297). Nevertheless, the genetic similarity of viral DNA from LN and PBMCs in untreated patients suggests that virus is readily exchanged between these two compartments and that infected cells may be trafficking between sites of preferential infection and other anatomic locations (298).

Antiretroviral therapy (ART) and the treatment of HIV

In the absence of additional therapy, chronic HIV infection invariably progresses to Acquired Immunodeficiency Syndrome (AIDS), defined by a circulating CD4+ T cell count of less than 200 cells/uL (299). As a result of their low frequency of CD4+ T cells, patients become increasingly susceptible to opportunistic infections and other malignancies, often leading to mortality in these AIDS patients. However, the development of ART has significantly reduced the morbidity and mortality associated with HIV infection (300, 301). Now, nearly 30 years after the first approved antiretroviral drug (zidovudine), combination ART (cART) is capable of: (i) achieving suppression of plasma viremia to below the clinical level of detection; (ii) improving CD4+ T cell counts and subsequent immune function; (iii) increasing the lifespan of HIV-infected individuals; (iv) reducing the risk of HIV transmission; and (v) improving the overall

quality of life of HIV patients (302). For treatment naïve patients- that is, HIV-infected individuals that have never received ART- a combination regimen is often recommended consisting of a backbone of 2 nucleotide reverse transcriptase inhibitors (NRTIs) in addition to a second class of drugs, such as a protease inhibitor (PI), a non-nucleoside reverse transcriptase inhibitor (NNRTI), an integrase inhibitor (INTI), or the CCR5 antagonist maraviroc. HIV-infected individuals placed on a cART regimen typically achieve undetectable plasma viremia by approximately 12 weeks of therapy (303, 304). Six different mechanistic classes are currently available in the US to suppress HIV replication and limit disease progression.

Reverse transcriptase inhibitors, which consists of both NRTIs and NNRTIs, were the first class of antiretrovirals approved in the US and are largely considered the backbone of most patient ART regimens due to their specificity for HIV (305). NRTIs, including tenofovir, emtricitabine, and abacavir, inhibit HIV reverse transcriptase (RT) by being phosphylated into di- or triphosphate metabolites that can then be incorporated into the nucleotide analogue being transcribed by RT. This incorporation is terminal for transcription, as it competes with viral substrates, which ultimately prevents the production of double stranded HIV DNA (306). NNRTIs are noncompetitive inhibitors of RT, changing the conformational shape of the enzyme to reduce its activity. Of the two RT inhibitors, NRTIs result in fewer toxicities and less drug interactions than NNRTIs, and thus, have been recently approved as pre-exposure prophylaxis (PrEP) for individuals at increased risk of HIV acquisition (307).

Protease inhibitors block HIV replication by binding to and preventing the activity of HIV proteases, so that virions remain immature and unable to initiate a new infection. This class of drugs is an appealing addition to ART regimens due to their low likelihood of developing resistances. Yet, the potency and tolerability of integrase inhibitors (INTIs) has recently surpassed the resistance benefits of PIs, so that the preferred ART regimen for treatment naïve patients

includes 2 NRTIs in combination with an INTI (308, 309). INTIs prevent the HIV integrase enzyme from incorporating viral DNA into the host genome, specifically by preventing integrase from forming covalent bonds between the two DNA strands. A new class of inhibitors, fusion inhibitors, can facilitate reductions in viral load in patients with multiple drug class resistances, by binding the viral envelope glycoprotein gp41 to prevent the fusion of the viral and cellular membranes, as opposed to targeting viral machinery (310). In addition to viral specific inhibitors, ART has more recently expanded to include entry inhibitors that bind cell surface proteins, including CCR5 (maraviroc) and CD4 (ibalizumab), that can then block virus-cell interaction. Yet, the impact of maraviroc is limited to individuals infected with CCR5-tropic viruses and must be routinely reassessed since cellular tropism can change over time.

Recent data has demonstrated that the timing of ART initiation can significantly impact the magnitude of HIV infection and its immunologic effects. For one, starting ART early is associated with lower viral dissemination throughout the body (311). Early initiation of ART can also promote more significant CD4+ T restoration as well as less perturbed HIV-specific T cell responses (93, 312-318). Perhaps the most important effect of early ART initiation, though, is the establishment of a smaller viral reservoir than those who begin ART during chronic infection (202, 284, 311, 319, 320). ART, while able to prevent viral infection of new CD4+ T cells, is unable to eliminate cells infected at the time of initiation; these persistently infected cells comprise the latent viral reservoir (321, 322). Even among patients who have been adherent to cART for several years, ART interruption results in viral rebound within weeks, demonstrating the longevity of the HIV reservoir (323). Therefore, initiating ART soon after HIV infection represents an appealing strategy for reducing the viral reservoir. For example, in one French cohort, adults treated within 2 months of their HIV infection were later able to immunologically control viremia following treatment interruption (324). However, the case of the Mississippi baby, who was treated with ART as early as 30 hours, demonstrates that even early initiation of

ART is unable to prevent the formation of the viral reservoir. This child discontinued ART after 18 months, but viremia rebounded after 27 months off treatment (325). Nevertheless, despite its inability to fully prevent reservoir seeding, early initiation of ART may be able to promote a faster reservoir decay in HIV-infected individuals (320, 326). Clinical trials aimed at investigating the effects of early ART have been difficult to perform, since serological tests are undetectable until 4 weeks into infection.

Limitations of ART

Despite the successes of ART in reducing HIV-associated morbidity and mortality, ART is unable to fully restore the health and immune status of HIV-infected individuals. For one, long-term ART is unable to reduce chronic immune activation in HIV-infected patients to pre-infection levels, which is associated with an increase in cardiovascular disease, cancer, and various other end-organ diseases, and significantly reduces the life span for ART-treated, HIV-infected persons compared to HIV-uninfected age-matched peers (327, 328). Ongoing viral replication is a controversial, yet potential, contributor to chronic immune activation during ART (329, 330). While ART reduces plasma viremia to below the clinical levels of detection (typically less than 50 copies/mL) in the majority of treated patients, more sensitive viral load assays have been able to detect residual viremia in these clinically "suppressed" patients (331-333). These trace levels of viremia, then, have the ability to constantly activate the host immune response, perpetuating increased levels of cell activation and inflammation, which invariably increases the number of susceptible HIV targets at a site of viral replication (334). As evidence of this ongoing viral replication, ART intensification demonstrated an increase in 2-LTR circles in PBMCs from HIV-infected patients (329, 330). Nevertheless, several studies have been unable to detect the presence of viral evolution in anatomic reservoirs during ART, which would be expected only if ongoing viral replication occurred (284, 335-339). In this case, residual viremia may simply reflect viral production, and not replication, whereby previously latent but now activated cells release trace

levels of virus that are unable to initiate new infection in the presence of ART. Regardless of the source of virus, residual viremia contributes to the prolonged presence of antigen in the host and consequently, ART cannot eliminate chronic immune activation in HIV-infected individuals.

ART also is unable to fully restore dysfunctional antiviral immune responses. Virus-specific CD8+ T cells are critical for the control of virus replication. Yet, HIV/SIV-specific CD8+ T cells have been shown to become "exhausted" during the chronic phase of HIV/SIV infection, as demonstrated through their loss of cytokine production (namely, IL-2 and IFN-G), cytotoxic activity, and ability to proliferate, which ultimately contribute to the inability of the host to eliminate HIV/SIV (340-343). Signaling through Co-IRs, which are upregulated on virus-specific CD8+ T cells during chronic infection, contribute to this CD8+ T cell dysfunction and exhaustion. Programmed Death-1 (PD-1) is upregulated on T cells following TCR activation and signals a negative feedback mechanism to inhibit further T cell activation and proliferation (344, 345). HIV/SIV-specific CD8+ T cells have increased levels of PD-1 expression, which correlate with impaired CD8+ T cell function and measures of disease progression, and are increasingly prone to apoptosis (126, 144, 346-348). Apart from PD-1, several other Co-IRs have been shown to not only regulate T cell responses, but become elevated on CD8+ T cells during chronic viral infections (143, 345, 349). Increased levels of TIM-3, LAG-3, CD160, 2B4, and TIGIT have been found on HIV-specific CD8+ T cells during chronic infection (126, 143, 346, 350-353). Moreover, greater co-expression of multiple Co-IRs has been associated with more advanced stages of T cell exhaustion (143, 351). ART is unable to fully reverse this T cell exhaustion and restore T cell functionality (106). Upon the initiation of ART, the frequency of HIV-specific CD8+ T cells decreases, as a result of the reduction in antigen load and infected targets (354, 355). Nevertheless, improvements in CD8+ T cell functionality are limited during ART, suggesting that the dysfunction is not directly controlled by viral replication but is an effect of programming (106). With an impaired ability to secrete cytokines, proliferate, and yield cytotoxic

activities, antiviral CD8+ T cells are unable to eliminate infected cells, thus contributing to viral persistence within HIV-infected individuals during ART.

The main limitation of ART, though, is its inability to eliminate a persistent reservoir of latently infected cells, known as the latent HIV reservoir (356).

HIV Reservoir

In 1995, Chun et al. were the first to demonstrate a latent form of HIV-1 infection *in vivo*, whereby a small pool of resting memory CD4+ T cells contained integrated provirus (357). This population of latently infected cells was then characterized in patients on ART, suggesting that current therapies are unable to eliminate this viral reservoir. In fact, despite their frequency being as low as 1 in 1 million resting CD4+ T cells, longitudinal analysis modeling the decay of latently infected cells in patients on ART have suggested that it would take over 60 years to eradicate the latent reservoir with ART alone (358, 359). Latency is believed to be established by two main mechanisms. First, as discussed above, HIV infects highly susceptible activated CD4+ T cells. While most CD4+ T cells productively infected with HIV die from virus-induced cytopathic effects, a small subset are able to persist and transition to a resting memory state. By reverting to a resting memory state, the transcriptional environment is altered to prevent viral gene expression in these CD4+ T cells; thus, this subset harbors an integrated provirus that is replication-competent but transcriptionally silent, which establishes latent infection (359-361). Latency can also be established as a result of the direct infection of resting CD4+ T cells (362-364). In this model, referred to as pre-activation latency, resting CD4+ T cells in the presence of chemokines (including CCL19 and CCL21) are increasingly permissive to viral entry and integration, without significant changes to their activation status (363). Nevertheless, this mechanism has been more difficult to demonstrate ex vivo.

As described above in *Antiretroviral therapy (ART) and the Treatment of HIV*, the latent reservoir is established early in infection (319, 365). Despite the difficulty in identifying recently infected humans, one study demonstrated that initiating ART as early as 10 days after symptom onset was unable to prevent the seeding of latently infected cells (365). In fact, the reservoir size has been shown to gradually increase throughout the first weeks of infection, supported by evidence in which individuals that start ART during Fiebig stage I have lower frequencies of infection than individuals who start at Fiebig stage II (311). Studies in NHPs have confirmed this early seeding, with a recent landmark study demonstrating that initiation of ART as early as 3 days post-SIVmac251 infection was unable to prevent the establishment of a viral reservoir and subsequent viral rebound following ART interruption (approximately 6 months ART)(366). However, it is unclear, from our current understanding, if NHPs establish reservoirs in the same manner as HIV-infected humans. For example, SAMHD1 is an HIV restriction factor that can block viral replication in myeloid cells, including macrophages and dendritic cells, as well as CD4+ T cells (367, 368). Yet, the SIV genome, but not HIV-1, contains the accessory protein vpx, which is able to counteract the restriction of SAMHD1 by inducing its degradation (369-371). As a result, resting CD4+ T cells and myeloid cells are more prone to viral infection and replication by SIV, thus illustrating a potential caveat when interpreting reservoir data from NHPs.

The latent viral reservoir is then maintained due to both cellular and molecular mechanisms. Memory CD4+ T cells are suited to harbor latent HIV/SIV infection due to their capacity to persist through homeostatic proliferation and self-renewal (202). Homeostatic proliferation promotes cell proliferation without activating or differentiating the cell, thus supporting a model of viral latency and a mechanism of reservoir maintenance (372). Moreover, homeostatic proliferation is regulated, predominantly, by IL-7, which is elevated during HIV/SIV infection (373-375). The long life spans of memory CD4+ T cells, particularly TCM and TSCM cells, also contribute to the maintenance and stability of the viral reservoir (188, 203). Recent evidence

suggests that while the overall size of the viral reservoir is reduced, the contribution of the less-differentiated memory CD4+ T cell subsets TCM and TSCM increase, which supports their longevity and the importance of their self-renewal to viral persistence (203). In addition to these cellular mechanisms, molecular mechanisms within the nucleus are also critical for maintaining transcriptional silence of HIV DNA. Integrated HIV proviruses utilize the same transcriptional machinery as the host. In a resting state, host transcription factors, including NFKb and NFAT, are excluded from the nucleus, thereby inhibiting transcription of both viral and host genomes (376, 377). Insufficient levels of pTEFb, a complex that associates with HIV-1 Tat to promote elongation, also prevent transcription of viral DNA (24, 378-380). Additionally, chromatin and epigenetic modifications, such as DNA methylation at the HIV-1 LTR, can reduce the access of host transcription factors and polymerase to initiate transcription (381, 382). Therefore, targeting these cellular and molecular pathways that maintain viral latency represent a key strategy in reducing and/or eliminating the viral reservoir in ART-treated, HIV-infected individuals.

As described above in *CD4+ T cell selection and depletion*, multiple subsets of CD4+ T cells contribute to the latent viral reservoir by harboring replication-competent cells during ART; these subsets have been characterized by their memory differentiation status, their functionality, as well as their anatomic location. The best-characterized cellular reservoirs of HIV during long-term ART are resting central, transitional, and stem cell memory CD4+ T cells (199, 202, 203). Functionally, Th17 and Th17/Th1 CD4+ T cells have also been demonstrated to contribute to viral persistence during ART, which likely results from their anatomic localization at a site of early viral replication (234, 235). More recent evidence from long-term ART-treated, HIV-infected patients demonstrated that TFH cells, localized in the GC of LNs, are a major source of persistent virus and account for approximately 46% of inducible replication competent virus (278). Importantly, these CD4+ T cell classifications are not mutually exclusive; for example, many Th17 cells share memory characteristics with TCM cells. Additionally, the heterogeneity of

these subsets has made therapeutically targeting these latently infected cells a significant challenge. Thus, there is an urgent need to identify additional phenotypic markers on memory CD4+ T cells that will differentiate cell subsets enriched for latent HIV. This particular challenge served as the rationale for my fourth chapter, *CTLA-4 and PD-1-expressing T-cells are key contributors to viral persistence in ART-suppressed SIV-infected rhesus macaques*, in which we investigated the use of Co-IRs to identify persistently infected cells. Although not further described here, non T-cell reservoirs have also been proposed to play a role in HIV persistence, including monocytes and macrophages, astrocytes, thymocytes, and hematopoetic progenitor cells (383-385).

Of critical value in determining contributors to viral persistence has been the identification of anatomic sites of viral persistence. In addition to the blood, where the majority of reservoir studies have been conducted due to human sampling limitations, the gut mucosa, lymph nodes, genital tract and central nervous system (CNS) have all been demonstrated to serve as anatomic reservoirs of HIV infection (384, 386, 387). These sites may harbor persistent virus due to the inability or reduced ability of ART to access these compartments, leaving biologically distinct virus in these sites compared to circulation; yet, this evidence has been conflicting and controversial (388-390). Nevertheless, additional anatomic sites have also been suggested to contribute to latent infection, including the spleen, liver, and bone marrow, as well as tissue compartments such as adipose tissue (391-393). The use of NHP models of SIV infection are and will be essential for addressing these research questions. First, utilizing the RM model of ART-treated SIV infection can minimize the variability in the composition of the viral reservoir, since investigators can control the timing, duration, and adherence of RMs to the ART regimen. Second, a previous limitation to the use of RMs in studies of latency and viral reservoirs had been the inability of ART regimens to suppress plasma viremia to clinically relevant levels. Importantly, several studies have recently demonstrated the efficacy of ART regimens in

suppressing plasma viremia to levels similar to those seen in ART-treated, HIV-infected humans (227, 394-396). Additionally, a chief advantage of using RMs is being able to access blood and other tissue compartments at multiple time points during viral suppression, thus allowing for an improved understanding of reservoir dynamics and anatomic differences. Thus, NHP studies of the viral reservoir will likely underline our understanding of anatomic sites of viral persistence.

Summary

CD4+ T cells are depleted progressively, particularly in the blood and mucosal tissues, during HIV/SIV infection, significantly perturbing CD4+ T cell homeostasis in untreated individuals. Previous HIV research has demonstrated that the preference of CD4+ T cell targets by the virus- whether classified by memory state, functionality, or migratory potential- influences the immunologic consequences incurred by the host. For one, the preferential infectivity and depletion of functional CD4+ T cell subsets alters the functional capacity of the host immune system. For example, the altered ratio of Th17/Treg cells in the mucosa following HIV/SIV infection impairs mucosal immunity and facilitates microbial translocation. A second immunologic consequence of this CD4+ T cell selection and depletion, though, is an increased CD4+ T cell output and activation state by the host, in an effort to off-balance the effects of CD4+ T cell depletion, thus leading to more viral targets and cell depletion in infected individuals. Therefore, the preferential infection and depletion of CD4+ T cells by HIV, through its immunologic consequences, ultimately promotes disease progression in the absence of ART. Understanding which CD4+ T cell subsets are preferentially targeted by HIV and how this impacts CD4+ T cell homeostasis will provide further insights into mechanisms of disease progression.

In this dissertation, I sought to assess how alterations in distinct CD4+ T cell subsets contribute to HIV/SIV disease progression. In the first study (Chapter 2), we investigated how preferential

infectivity of CD4+ TCM cells in RMs affects the stability of this memory compartment in SIV-infected hosts. Comparing the dynamics of CD4+ TCM cells between pathogenic and non-pathogenic SIV-infected RMs and SMs, respectively, allowed us to link the homeostasis of this cell subset to disease progression. In the second study (Chapter 3), we sought to determine whether the loss of CCR6+ and CD161+ CD4+ T cells from the blood of SIV-infected RMs was critical for disease progression. Although these cell subsets, which are associated with the Th17 cell lineage, had previously been shown to be depleted from the blood of HIV-infected individuals, their cellular dynamics in tissue sites was unknown. Thus, in this study, we examined the kinetics of CCR6+ and CD161+ CD4+ T cells across multiple tissue compartments to understand the mechanisms behind their depletion during progressive SIV infection of RMs, but also compared these levels to those seen in SIV-infected SMs to assess how their alterations impact disease progression. Determining which CD4+ T cell subsets are preferentially targeted and altered during progressive HIV infection, then, better elucidates therapeutic targets to slow disease progression and reverse immunologic impact in HIV-infected individuals.

As critical as discerning which CD4+ T cell subsets contribute to disease progression, understanding which CD4+ T cells persist during ART is also a priority for functionally curing HIV. Therefore, in the third study (Chapter 4), we sought to determine the immunophenotypic nature of persistently SIV-infected cells during ART *in vivo*. Given the previous evidence that PD-1+CD4+ T cells are enriched in latently infected cells within ART-treated, HIV-infected individuals and the known upregulation of Co-IRs during cellular deactivation, we focused our investigation on subsets of memory CD4+ T cells expressing the co-inhibitory receptors PD-1, CTLA-4, and TIM-3 to identify potential therapeutic targets for HIV cure strategies. The findings outlined in this dissertation, thus, not only illustrate how and which CD4+ T cells are critical for HIV pathogenesis and persistence, but also inform the design of future HIV therapeutics.

Chapter Two

Increased stability and limited proliferation of CD4+ central memory T cells differentiate non-progressive SIV-infection of sooty mangabeys from progressive SIV-infection of rhesus macaques*

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Abstract

Depletion of CD4+ central memory T (TCM) cells dictates the tempo of progression to AIDS in simian immunodeficiency virus (SIV)-infected rhesus macaques (RMs) both in the natural history of infection and in the context of vaccination. CD4+ TCM of sooty mangabeys (SMs), a natural host for SIV in which infection is non-pathogenic, are less susceptible to SIV infection than CD4+ TCM cells of RMs. Whether this relative protection from infection translates into increased stability of CD4+ TCM cells in natural versus non-natural hosts has not yet been determined. Here we compared, both cross-sectionally and longitudinally, the levels of CD4+ TCM cells in a large cohort of SMs and RMs, and the association between CD4+ TCM levels and the main virologic and immunologic markers of disease progression. Consistent with their lower susceptibility to infection, CD4+ TCM cells of SIV-infected SMs are lost with a kinetic 20 times slower than SIV-infected RMs. Remarkably, the estimated length of SIV infection needed for CD4+ TCM cells to fall to half of their initial levels is less than 16 months for RMs, but over 17 years for SMs. Furthermore, the fraction of proliferating CD4+ TCM cells is significantly lower in SIV-infected SMs than RMs, and the extent of CD4+ TCM cell proliferation associates positively with CD4+ T cell levels in SIV-infected SMs, but negatively in RMs. Collectively, these findings identify increased stability and maintenance of the pro-homeostatic role of CD4+ TCM cells as features distinguishing non-progressive from progressive SIV infections, and support the hypothesis of a direct mechanistic link between loss of CD4+ TCM cells and disease progression.

Introduction

The precise factors determining the rate of CD4+ T cell decline, and ultimately the rate of progression to AIDS, in HIV-infected humans remain poorly defined. Understanding this complex interplay between CD4+ T cell homeostasis and immune control of the virus has been complicated by the paradoxical nature of their relationship (397). CD4+ T cells are critical in enhancing both cellular and humoral immune responses that can effectively suppress virus replication, yet their activation makes these cells more susceptible to infection by HIV, thus creating more targets for virus replication (398, 399). In marked contrast to HIV-infected humans, and despite similar levels of viral loads, natural SIV hosts, such as sooty mangabeys (SMs) and African Green Monkeys (AGMs), generally maintain healthy CD4+ T cell levels and avoid chronic immune activation, thus remaining AIDS-free (133, 140, 400-405). Comparing and contrasting the mechanisms of CD4+ T cell homeostasis in natural hosts for SIV to those in experimentally SIV-infected rhesus macaques (RMs), which progress to AIDS, may provide important insight into the mechanisms of disease progression in HIV-infected humans.

The ability of natural hosts of SIV to maintain low levels of immune activation despite high viremia represents a key difference between these infections and the typical pathogenic course of infection observed in HIV-infected humans and SIV-infected RMs. However, the mechanisms responsible for the benign nature of SIV infection in SMs and other natural hosts remain poorly understood. Several non-mutually exclusive mechanisms have been proposed to contribute to this phenomenon (403), including: (i) preserved physical and immunological integrity of the mucosal barrier, with healthy levels of Th17 cells and absence of microbial translocation into systemic circulation (135, 138, 140); (ii) timely resolution of the innate immune response initiated during the acute phase of infection (167, 169, 170); (iii) preserved ability of the SIVsmm and SIVagm nef to down-modulate CD3/TCR expression (406); (iv) reduced expression of the dominant SIV coreceptor, CCR5, on CD4+ T cells (179); and (v) the ability of CD4+ T cells to down-modulate

the surface expression of CD4 during their differentiation into memory cells (in AGM), thus protecting this critical cell subset from SIV infection (407).

CD4+ T cells are composed of several subsets that differ by phenotype, function, and anatomic localization. Central memory CD4+ T cells (CD4+ TCM) express CD62L and CCR7, reside in lymph node (LN) and other inductive lymphoid tissues, and show limited effector functions but strong proliferation in response to antigenic re-stimulation (408). CD4+ TCM are of particular importance for immune function since they are longer-lived, self-renewing cells that maintain CD4+ T cell homeostasis by replenishing the pool of shorter-lived, non-self-renewing CD4+ effector memory cells (CD4+ TEM) (399). The importance of preserving CD4+ TCM cell homeostasis is clearly highlighted by *in vivo* studies showing that depletion of CD4+ TCM is the key factor dictating the tempo of progression to AIDS in SIV-infected RMs, both in the natural history of infection and in the context of vaccination (160, 201, 409, 410). Of note, circulating CD4+ TCM cells are infected at high frequencies in the majority of HIV-infected humans both *in vivo* and *in vitro* and represent the largest reservoir of latently infected CD4+ T cells in individuals treated with antiretroviral therapy (ART) (202, 411). In a recent study, we showed that the infection frequencies of CD4+ TCM cells of SMs are relatively lower than those of CD4+ TCM of RMs (161). However, this difference was not found in CD4+ TEM. This observation, originally made in blood (161), was recently confirmed also at the lymph node level (274). It is still unclear, however, whether and to what extent this protection from infection translates into increased stability and differential kinetics of depletion of CD4+ TCM in natural versus non-natural hosts.

To answer this question, we measured the number and proportion of CD4+ TCM cells in a large cohort of SMs naturally infected with SIV that are housed at the Yerkes National Primate Research Center (YNPRC), as well as their association with the main virologic and immunologic

markers of disease progression. These data were compared to those obtained in a cohort of experimentally SIV-infected RMs. We found that the levels of total and proliferating CD4+ TCM cells are not significantly affected by SIV infection in SMs and that, in these animals, CD4+ TCM are lost at a rate 20 times slower than in SIV-infected RMs. In addition, our results revealed that the extent of CD4+ TCM proliferation associates positively with the CD4+ T cell levels in SIV-infected SMs, but negatively with the levels of total and TEM CD4+ T cells in RMs. These data support the hypothesis of a direct mechanistic link between loss of CD4+ TCM homeostasis and disease progression, as well as suggest that proliferation of CD4+ TCM cells maintains its pro-homeostatic role in SIV-infected SMs but becomes inefficient, if not deleterious, in maintaining CD4+ T cell homeostasis in SIV-infected RMs.

Materials and Methods

Animals

Forty-six SIV-negative (11.2 ± 0.7 years old) and 94 naturally SIV-infected (16.9 ± 0.4 years old) SMs, and 17 SIV-negative (9.1 ± 0.6 years old) and 23 experimentally SIVmac239-infected (7.4 ± 0.7) RMs were included in the study. In the SIV-infected animals, the average duration of infection was 12.0 ± 0.5 years for SMs, as estimated by the date of the first SIV-positive test, and 0.8 ± 0.1 years for RMs. All animals were housed at the YNPRC, Atlanta, GA. Peripheral blood (PB) was collected from all animals at a single time point using EDTA-containing tubes. Blood samples were used for a complete blood count and flow cytometry analyses, and plasma was separated by centrifugation within 1hr of phlebotomy. Due to the known down-regulation of CD62L expression during the freeze-thaw process, all flow cytometry staining was performed on fresh blood.

Study approval

All animal experimentations were conducted following guidelines established by the Animal Welfare Act and the NIH for housing and care of laboratory animals and performed in accordance with Institutional regulations after review and approval by the Institutional Animal Care and Usage Committee at the YNPRC.

Flow cytometric analysis

Twelve-parameter flow cytometric analysis was performed on whole blood derived cells according to standard procedures using a panel of monoclonal antibodies that we and others have shown to be cross-reactive with SMs and RMs (161, 226, 412). Predetermined optimal concentrations were used of the following antibodies: anti-CD3-Alexa700 (clone SP34-2), anti-CD3-APC-Cy7 (clone SP34-2), anti-CD4-PerCp-Cy5.5 (clone L200), anti-CD8-PacBlue (clone

RPA-T8), anti-CD95-PE-Cy5 (clone DX2), anti-CD62L-FITC (clone SK11), anti-CD62L-PE (clone SK11), anti-Ki-67-FITC (clone B56), anti-Ki-67-Alexa700 (clone B56) (all from BD Bioscience); anti-CD28-PE-Cy7 (clone CD28.2) (from eBioscience); anti-CD28-PE Texas Red (clone CD28.2) (from Beckman Coulter); anti-CD4-PacBlue (clone OKT4) (from Biolegend); and anti-CD8-Qdot705 (clone 3B5) (from Invitrogen). Flow cytometric acquisition was performed on an LSRII cytometer driven by the FACS DiVa software. Analysis of the acquired data was performed using FlowJo software (TreeStar).

Viral load

SIVsmm and SIVmac loads were measured in plasma samples by real-time PCR as previously described (186, 413).

Statistical analysis

Based on sample distribution (normal or non-normal), T-tests or Mann Whitney tests were used to compare the differences of each parameter between SIV-negative and SIV-positive SMs, or between SMs and RMs. Statistical tests were two-sided. Pearson product-moment correlation coefficients were utilized to estimate linear associations for normally distributed data and Spearman rank correlation coefficients were used for skewed and other non-normal distributions. A P value ≤ 0.05 was considered statistically significant. The mean ± SEM were used for descriptive statistics for each parameter. In order to estimate the rate of loss of CD4+ TCM cells for each group of animals, we used linear regression on the time since infection versus percent TCM cells. The slopes of SMs and RMs shown in figure 2.2E were compared using ANCOVA.

Results

Virologic and immunologic features of the SIV-infected SMs and RMs

Forty-six SIV-uninfected and 94 naturally SIV-infected SMs as well as 17 SIV-uninfected and 23 experimentally SIV-infected RMs were included in this study. Within the natural host SMs, the average age of infected animals was older than for uninfected animals (16.9 ± 0.4 for SIV-infected SMs versus 11.2 ± 0.7 years old for SIV-uninfected; $p<0.0001$). On the other hand, the average age of infected RMs was slightly lower than for uninfected RMs (7.4 ± 0.7 for SIV-infected RMs versus 9.1 ± 0.6 for SIV-uninfected; $p=0.1125$), reflecting the fact that many studies involving experimental SIV infection of RMs preferentially involved younger animals. The average duration of infection in the SIV-infected SMs was 12.0 ± 0.5 years, as estimated by the date of the first SIV-positive test, while the average duration of infection for RMs was 0.8 ± 0.1 years. A well-documented feature that distinguishes natural SIV infection of SMs from progressive infection of RMs is the sustained preservation of circulating CD4+ T cell counts despite high levels of virus replication (133, 186, 414, 415). To validate this feature in our study, we first compared the relative (fraction of CD3+ T cells) and absolute (number of cells per mm^3 of blood) levels of CD4+ T cells between SIV-infected and uninfected animals. As expected, both the fraction (33.97±2.64 vs. 57.88±1.69%; $p<0.0001$; Figure 2.S1A) and absolute number (315.8±51.8 vs. 619.8±58.1 cells/mm^3; $p=0.0004$; Figure 2.S1B) of CD4+ T cells were significantly lower in SIV-infected than uninfected RMs. However, consistent with previous reports (133, 186, 414, 415), the large majority of SIV-infected SMs maintained their fraction and number of CD4+ T cells at levels similar to those found in uninfected animals (Figure 2.S1C,D). As previously described (416), plasma levels of viral RNA copies were significantly higher in chronically SIV-infected RMs than in SIV-infected SMs ($3.0x10^6$ ±$1.0x10^6$ copies/mL vs. $1.2x10^5$ ±$1.2x10^4$; $p<0.0001$; data not shown). However, significant loss of CD4+ T cells was still present in SIV-infected RMs when only animals with viral loads similar to the average of the SIV-

infected SMs were included, despite these animals being infected for less than eight months time (41.3±4.6 vs. 58.9±2.9%; p=0.0193; data not shown). Collectively, these data confirm in this study cohort the well-described phenomenon of preservation of CD4+ T cells in the vast majority of SMs naturally infected with SIV.

CD4+ TCM are preserved at healthy frequencies in SIV-infected SMs, but depleted in RMs

The infection frequencies of CD4+ TCM in the natural host SMs are lower relative to those of RMs in both the blood and lymph node, and SM CD4+ TCM are less permissive to SIV infection *in vitro* as compared to RM CD4+ TCM (161, 274). Whether this lower level of infection translates into an increased stability of CD4+ TCM cells in SIV-infected SMs compared to RMs has not yet been determined. To answer this question, we compared the relative (fraction of memory CD4+ CD95+ T cells) and absolute (number of cells per mm^3 of blood) levels of circulating CD4+ T cells with a central memory (CD4+ TCM) or effector memory (CD4+ TEM) phenotype in SIV-infected and uninfected SMs and RMs. Specifically, cells pre-gated on lymphocytes, CD3+, and CD4+CD8-, were defined as naïve (CD28+CD95-) or memory (CD95+) $CD4^+$ T cells. CD4+ CD95+ memory T cells were then defined as TCM or TEM based on the expression, or lack thereof, of CD62L. Importantly, this combination of surface markers has been extensively characterized in nonhuman primates (161, 186, 417) and represents the same gating strategy used to determine the reduced frequency of infection of circulating CD4+ TCM cells in SMs when compared to the same cells in RMs (161). An example of the gating strategy used to define CD4+ TCM or TEM cells is shown for an SIV-infected SM in Figure 2.1A.

Levels of CD4+ TCM were compared between age-matched SIV-infected and uninfected SMs. This correction is particularly important due to the significant inverse correlation found between age and levels of CD4+ TCM in SMs ($p<0.0001$; Figure 2.S2), and the fact that, within the YNPRC colony, the SIV-infected SMs are significantly older than SIV-uninfected animals

(16.9±0.4 vs. 11.2±0.7 years of age; p<0.0001). However, because the age of SIV-infected RMs was not significantly different from that of SIV-uninfected RMs (7.4±0.7 years vs. 9.1±0.6 years of age; p=0.1125), and the age range in RMs was narrower, we did not stratify these animals by age. We found that the fraction of CD4+ TCM was significantly lower in SIV-infected RMs when compared to SIV-uninfected RMs (24.6±2.3 vs. 36.6±1.6%; p=0.0003; Figure 2.1B). As a consequence, the fraction of CD4+ TEM was significantly higher in SIV-infected RMs compared to uninfected RMs (75.4±2.3 vs. 63.4±1.6%; p=0.0003; Figure 2.S3A). The loss of CD4+ TCM cells in SIV-infected RMs was particularly evident when expressed as absolute count, with a severe decrease from 144.2±14.6 to 36.2±8.5 cells/mm^3 (p< 0.0001; Figure 2.1C). Due to the overall depletion of CD4+ T cells, the absolute number of CD4$^+$ TEM cells was also significantly lower in SIV-infected than uninfected RMs (93.4±14.2 vs. 250.1±25.4 cells/mm^3; p<0.0001; Figure 2.S3B). In contrast to RMs, comparison of the levels of CD4+ TCM cells in age-matched SMs revealed that the fraction and number of CD4+ TCM cells were remarkably similar between SIV-infected and uninfected animals. For example, in animals age 10-15 years old, the proportion of TCM in SIV-infected and uninfected animals was 16.35±1.82% versus 17.04±2.34%, respectively, and the number of TCM was 58.5±10.4 versus 74.7±14.4 cells/mm^3, respectively. Likewise, for animals older than 15 years old, the proportion of TCM cells was 12.74±1.26% versus 20.05±6.84% for infected versus uninfected SMs (Figure 2.1D), and the number of TCM cells was 51.4±7.1 versus 93.0±41.7 cells/mm^3 respectively (Figure 2.1E). Similarly, and supporting the model in which preservation of CD4+ TCM cells results in maintenance of the whole CD4+ T cell compartment, the fraction and absolute number of CD4+ TEM cells were maintained in SIV-infected SMs when compared to age-matched SIV-uninfected SMs (Figure 2.S3C,D). Taken together, these results suggest that, differently from RMs, SMs are able to preserve their memory CD4+ T cell compartment despite SIV infection, a feature that is likely influenced by the reduced infectivity of CD4+ TCM cells in SMs compared to RMs.

Long-term maintenance of CD4+ TCM in SIV-infected SMs as compared to RMs

To account for the different duration of infection in natural host SMs versus AIDS-susceptible RMs, we further investigated the rate of loss of CD4+ TCM cells over time between the two species. We found that, in RMs, a significant loss of CD4+ TCM cells was already present in animals infected with SIV for less than 1.5 years compared to uninfected animals (20.32±2.41% vs. 36.57±1.58%; $p<0.0001$; Figure 2.2A). In contrast, SIV-infected SMs maintained a frequency of CD4+ TCM cells comparable to those found in uninfected animals for up to 10 years post infection. In fact, the fraction of CD4+ TCM cells was significantly lower compared to uninfected SMs only in animals infected for more than ten years (12.66±1.32% vs. 22.86±2.19%; $p<0.0001$; Figure 2.2B). The trend is upheld when comparing absolute numbers of CD4+ TCM cells between natural and non-natural hosts, as CD4+ TCM cells were significantly depleted within a half year of infection in RMs (25.1±5.5 vs. 144.2±14.6 cells/mm^3, $p<0.0001$; Figure 2.2C). This trend was not observed in the natural host SMs, in which CD4+ TCM cells were only depleted at significant levels after 10 or more years of SIV infection (47.9±6.2 vs. 84.0±10.9 cells/mm^3, $p=0.0004$; Figure 2.2D).

We then used the data on the frequency of CD4+ TCM cells in SIV-infected RMs and SMs with different lengths of infection to model the kinetics of CD4+ TCM loss in the two species. CD4+ TCM cells declined at a rate of 14.2% per year in SIV-infected RMs. However, CD4+ TCM cells in SIV-infected SMs declined at a rate of only 0.65% per year (slopes estimated by linear regression, and significant $p<0.0001$ for both groups; Figure 2.2E). The slower loss of CD4+ TCM cells in SMs was not simply due to a slowing of decay after prolonged infection, since when we analyzed the slopes in animals infected for <10 years versus animals infected for >10 years, there was no significant difference ($p= 0.74$, ANCOVA). Therefore, it is estimated that

CD4+ TCM are lost at a rate approximately 20 times slower in SIV-infected SMs than in RMs ($p<0.0001$ for differences in slopes; ANCOVA). Consistent with the different susceptibility to SIV infection in the two species, these data and those presented in Figure 2.1 indicate that prolonged preservation of CD4+ TCM cells is a critical feature distinguishing non-pathogenic SIV infection of SMs from pathogenic SIV infection of RMs.

Limited proliferation of CD4+ TCM in SIV-infected SMs as compared to RMs

CD4+ TCM cells are self-renewing cells that proliferate to maintain long-term stability of the CD4+ T cell compartment (402). In the context of SIV infection in RMs, CD4+ TCM cells undergo a striking increase in proliferation that is thought to represent, at least in part, an attempt to maintain CD4+ T cell homeostasis by compensating for the cell loss due to both direct virus infection and chronic immune activation (160, 410). We next examined the proliferation levels of CD4+ TCM cells in the SIV-infected and uninfected SMs and RMs included in our study by staining for the proliferative marker Ki-67. The fractions of proliferating CD4+ TCM cells (16.69±1.91% vs. 4.82±0.73%; $p<0.0001$; Figure 2.3A) and TEM cells (25.99±2.74% vs. 9.54±1.09; $p<0.0001$; Figure 2.S4A) were significantly higher in SIV-infected RMs than in uninfected RMs. Despite the significant increase in the fraction of these cells that are proliferating, the absolute number of CD4+ Ki-67+ TCM cells remained similar between SIV-infected and uninfected RMs due to the overall loss of CD4+ TCM cells (4.58±0.99 vs. 6.27±0.80 cells/mm^3; Figure 2.3B). This effect was also seen in CD4+ Ki-67+ TEM cells, in which SIV-infected RMs maintained numbers near to uninfected (22.07±4.20 vs. 22.31±2.99 cells/mm^3; Figure 2.S4B). These data clearly contrast with those found in SIV-infected SMs, in which SIV infection had little impact on the levels of proliferating CD4+ Ki-67+ TCM cells in age-matched SMs when measured as both fraction (10-15 yrs: 3.18±0.21% vs. 2.96±0.48%; 15+ yrs: 2.61±0.20% vs. 2.68±0.46%; Figure 2.3C) and absolute number (10-15 yrs: 2.00±0.51 vs.

2.06±0.46 cells/mm^3; 15+ yrs: 1.39±0.24 vs. 2.56±1.26 cells/mm^3; Figure 2.3D). Similarly, there were no differences in the fraction and number of CD4+ Ki-67+ TEM cells between age-matched SIV-infected and uninfected SMs (Figure 2.S4C,D). Overall, these data indicate that limited proliferation of the CD4+ TCM compartment distinguishes non-progressive SIV infection in SMs from progressive SIV infection in RMs. Furthermore, they suggest that different levels of proliferation in chronic infection may account, at least in part, for the differential susceptibility of CD4+ TCM cells from SMs and RMs to SIV infection (161).

Proliferation of CD4+ TCM maintains its pro-homeostatic role in SIV-infected SMs but becomes detrimental in SIV-infected RMs

We then sought to determine whether the levels of total and proliferating CD4+ TCM cells were correlated with the main virologic and immunologic markers of SIV infection. We first found that, in SIV-infected RMs, the level of CD4+ TCM cells correlates negatively with plasma viral load (r= -0.7131; p=0.0028; Figure 2.4A) and positively with the fraction of CD4+ T cells (r= 0.4196; p=0.0463; Figure 2.4B). Thus, in SIV-infected RMs, loss of CD4+ TCM cells associates with the main features of pathogenic infection, including higher viral load and depletion of CD4+ T cells. Unlike RMs, the levels of CD4+ TCM did not correlate with viral load in SIV-infected SMs (r= -0.0656; p=0.539; Figure 2.4C). Additionally, there was a weak negative correlation between the percentage of CD4+ TCM cells and the fraction of CD4+ T cells (r= -0.1991; p=0.0557; Figure 2.4D), unlike the positive correlation found in RMs.

We then focused on the levels of proliferating CD4+ TCM cells, and found peculiar differences between progressive and non-progressive SIV infection. Indeed, in SIV-infected RMs, the percentage of CD4+ Ki-67+ TCM cells correlates negatively with the percentage (r = -0.5353; p=0.0085; Figure 2.5A) and number (r= -0.5808; p=0.0037; Figure 2.5B) of CD4+ T cells, as well as negatively with the number of CD4+ TEM cells (p=0.0174; data not shown). Intriguingly,

the association is exactly the opposite in SIV-infected SMs, where the percentage of CD4+ Ki-67+ TCM cells correlates positively with the percentage (r= 0.2916; p=0.0044; Figure 2.5D) and number (r= 0.3168; p=0.0028; Figure 2.5E) of CD4+ T cells. Consistent with the different frequencies of CD4+ TCM infection previously described between the two species (161, 274), we found a positive correlation between the percentage of proliferating CD4+ TCM cells and viral load in SIV-infected RMs (r=0.6023; p=0.0175; Figure 2.5C), but not in SIV-infected SMs (r=0.0208; p=0.8456; Figure 2.5F). Overall, these findings support the working hypothesis that proliferation of CD4+ TCM cells maintains its beneficial, pro-homeostatic role in SIV-infected SMs, but becomes inefficient, if not deleterious, in maintaining CD4+ T cell homeostasis in SIV-infected RMs.

Discussion

We recently described that CD4+ TCM cells are infected at lower levels in SIV-infected SMs as compared to RMs (161, 274). Based on these findings, we proposed that protection of CD4+ TCM cells is a key factor used by SMs to remain AIDS-free (403). In this view, we hypothesized that in SIV-infected SMs, reduced infection of CD4+ TCM cells will result in a better preservation of this important CD4+ T cell compartment, and that preservation of CD4+ TCM cells is the proximal event that promotes the other key features of non-progressive SIV infection, including preservation of overall CD4+ T cell homeostasis and low levels of chronic immune activation. Indeed, CD4+ TCM cells may contribute more than any other CD4+ T cell subsets to long-term stability of the whole CD4+ T cell compartment, given the elevated clonogenic potential and longer lifespan of these cells (408, 418). In turn, a preserved CD4+ T cell compartment will reduce the homeostatic pressure on the immune system and contribute to limiting the chronic immune activation that is typical of pathogenic HIV and SIV infection in humans and RMs (133, 187, 403, 419, 420).

To test this hypothesis, in this study we compared the levels of total and proliferating CD4+ TCM cells between non-progressive SIV-infection of SMs and progressive SIV-infection of RMs. Furthermore, we investigated the association between the levels of CD4+ TCM cells, maintenance of the overall CD4+ T cell compartment, and the levels of T cell proliferation and viral replication. Importantly, these analyses were performed in the large cohort of SMs housed at the YNPRC, with 94 SIV-infected and 46 SIV-uninfected SMs included in this study. The main findings generated here are the following: (i) the CD4+ TCM cell compartment is remarkably more stable in SIV-infected SMs than RMs; (ii) SIV infection of RMs, but not of SMs, is associated with a significant increase in the level of proliferating CD4+ TCM cells; (iii) the increased proliferation of CD4+ TCM cells in RMs is not sufficient to maintain overall CD4+ T cell homeostasis, and in fact associates with virologic and immunologic parameters of

progression to AIDS; and (iv) proliferation of CD4+ TCM cells in SMs positively contributes to CD4+ T cell homeostasis, and is maintained throughout SIV infection.

In contrast to SIV-infected RMs, SIV-infected SMs were able to maintain levels of CD4+ TCM cells similar to those found in age-matched uninfected SMs. We also evaluated the stability of the CD4+ TCM compartment in both species by determining the levels of CD4+ TCM cells longitudinally in both experimentally SIV-infected RMs and naturally SIV-infected SMs. This approach allowed us, for the first time, to effectively model the kinetics of CD4+ TCM cell loss as a result of SIV infection in both species. Consistent with our cross-sectional data, we found that the depletion of CD4+ TCM cells is approximately 20 times slower in SIV-infected SMs than in RMs. In fact, this analysis revealed that it is estimated to take just over 15 months of infection for RMs to fall to half of their initial percentage of CD4+ TCM cells, whereas it would take SMs over 17 years of SIV infection to reach half of their starting level of CD4+ TCM cells. The loss of CD4+ TCM cells that we do see in the SMs with more than 10 years of SIV infection is likely age-related, since we found a significant inverse correlation between the age and levels of CD4+ TCM cells in SMs, yet no significant correlation in RMs. These findings support the hypothesis that preservation of the CD4+ TCM compartment is a critical feature that distinguishes non-progressive SIV infection in SMs from progressive SIV infection in RMs. Furthermore, they are consistent with our previous finding of reduced CD4+ TCM cell infection in SMs (161, 274), as well as with the hypothesis that more differentiated, short lived CD4+ T cell subsets are the main cells supporting viral replication in SMs (403). An alternative explanation is that non CD4+ T cells, for example myeloid cells, critically contribute to viral replication in SIV-infected SMs. However, an important contribution of non CD4+ T cells is not supported by our previous data showing that, in chronically SIV-infected SMs, experimental depletion of CD4+ T cells associates with a rapid, significant decline in viral load, as well as the existence of a strong association between availability of activated CD4+ T cells and level of viremia (178).

A second key finding is that the percentage of proliferating CD4+ Ki-67+ TCM cells is significantly increased in SIV-infected RMs but not in SIV-infected SMs. Proliferation of CD4+ TCM cells is critical for their self-renewal capacity and the generation of more differentiated CD4+ TEM cells (421-424). This CD4+ TCM cell proliferative response is particularly important when the homeostasis of the CD4+ T cell compartment is compromised, as it is during pathogenic HIV/SIV infection. On the other hand, HIV and SIV preferentially infect proliferating, antigen-experienced memory CD4+ T cells (72, 398, 425, 426). Hence, in the context of HIV/SIV infection, the "pro-homeostatic" role of CD4+ TCM proliferation may be overruled by its effect on the expansion of the major target cell population for the virus (activated CD4+ T cells). Remarkably, our data show that in chronically SIV-infected RMs, the percentage of CD4+ Ki-67+ TCM cells positively correlates with viral load and negatively correlates with the number of total CD4+ and CD4+ TEM cells. These data thus suggest that, in pathogenic SIV infection, increased proliferation of CD4+ TCM fails to maintain the homeostasis of the overall $CD4^+$ T cell compartment. Of note, this finding lends support to a previously described model that a heightened level of CD4+ T cell proliferation can result in decreased numbers of uninfected CD4+ T cells (414). The situation is exactly the opposite in SIV-infected SMs, in which the fraction of CD4+ Ki-67+ TCM cells is not increased compared to uninfected animals and, in fact, correlates positively with the level of CD4+ T cells. These data suggest that the limited proliferation of CD4+ TCM cells in SMs contributes to the preservation of CD4+ T cell homeostasis during this model of non-pathogenic SIV infection. Collectively, these data identify proliferation of CD4+ TCM cells as a feature distinguishing progressive from non-progressive SIV infection in nonhuman primates, and indicate that, during pathogenic SIV infection of RMs, increased proliferation of CD4+ TCM is inefficient, if not deleterious, in maintaining the homeostasis of the overall CD4+ T cell compartment.

A series of previous studies identified the progressive demise of CD4+ TCM cells as a key determinant of the timing of disease progression in SIV-infected RMs (160, 410). Our current set of data adds to these previous studies by suggesting that, in SIV-infected RMs, failure in the regenerative capacity of CD4+ TCM cells can arise also in the presence of CD4+ TCM cells that proliferate at high levels, due to the increased susceptibility of these activated and proliferating cells to virus-induced and/or activation-induced cell death. Consistent with this model, the absolute number of CD4+ Ki-67+ TCM cells remained similar between SIV-infected and uninfected RMs, despite the significant increase in proliferating CD4+ TCM cells when measured as fraction of the total CD4+ T cells.

Unfortunately, tissues were not collected during this survey of the SMs housed at YNPRC, thus we were unable to determine, and compare with RMs, the levels of CD4+ TCM cells in key anatomic sites such as lymph nodes and intestinal tissues. A second possible limitation of this study is that the SIV-infected SMs were, on average, significantly older than the SIV-infected RMs. This is important as we found a significant inverse correlation between the fraction of CD4+ TCM and age in SMs. Interestingly, it seems that the slow decay of CD4+ TCM cells seen in SIV-infected SMs may be more attributable to aging than to the SIV infection, since the decay of CD4+ TCM cells with age was not significantly different between SIV-infected and uninfected animals ($p=0.81$; ANCOVA). By evaluating the levels of CD4+ TCM cells between age-matched SMs, we believe that we compensated for any additive effects of aging on the percentages of CD4+ TCM cells, although it may be possible that we are underestimating the stability of the CD4+ TCM compartment in SIV-infected SMs compared to SIV-infected RMs.

Overall, these findings identify increased stability of CD4+ TCM cells as a key feature distinguishing non-progressive from progressive SIV infections, and support the working hypothesis that proliferation of CD4+ TCM cells maintains its beneficial, pro-homeostatic role in

SIV-infected SMs, but becomes inefficient, if not deleterious, in maintaining CD4+ T cell homeostasis in SIV-infected RMs. Therefore, the results of this study highlight the importance of investigating immunomodulatory interventions that are able to improve the homeostasis of CD4+ TCM cells in HIV-infected humans.

Figures

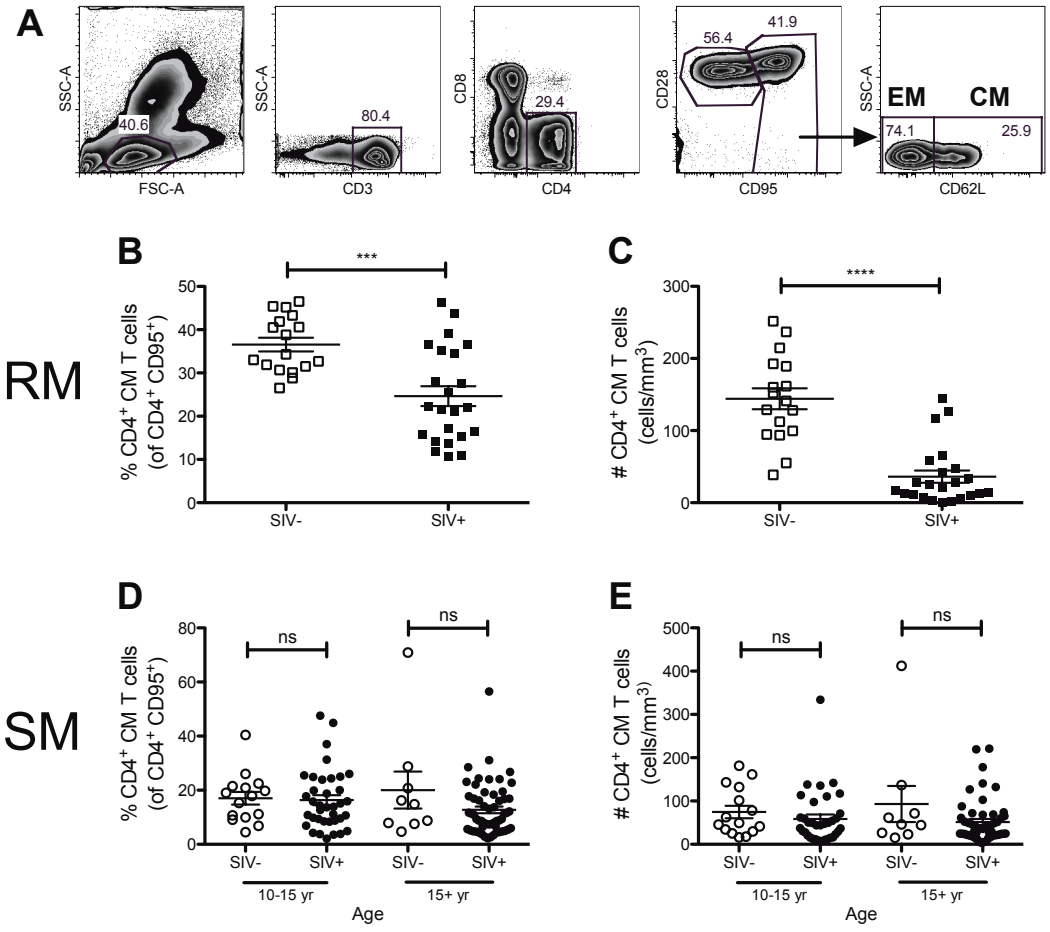

Figure 2.1. Maintenance of CD4+ central memory T cell levels in naturally SIV-infected SMs. Representative plots of the flow cytometry gating strategy for measurement of CD4+ TCM and TEM levels by CD62L staining are shown for an SIV-infected SM (A). Frequencies (of CD4+CD95+ cells; B) and cell counts (C) of CD4+ TCM were quantified in the whole blood of uninfected (□) and SIV-infected (n) RMs (***, $p< 0.001$; ****, $p< 0.0001$; as determined by *t* tests). The percentages of CD4+ TCM cells (of $CD4^+CD95^+$ cells) in whole blood were compared between age-matched uninfected (o) and SIV-infected (•) SMs (D). Numbers of CD4+ TCM cells (cells/mm^3) were measured in the same cohort of SMs (E) (p = ns; as determined by the Mann-Whitney *U* test).

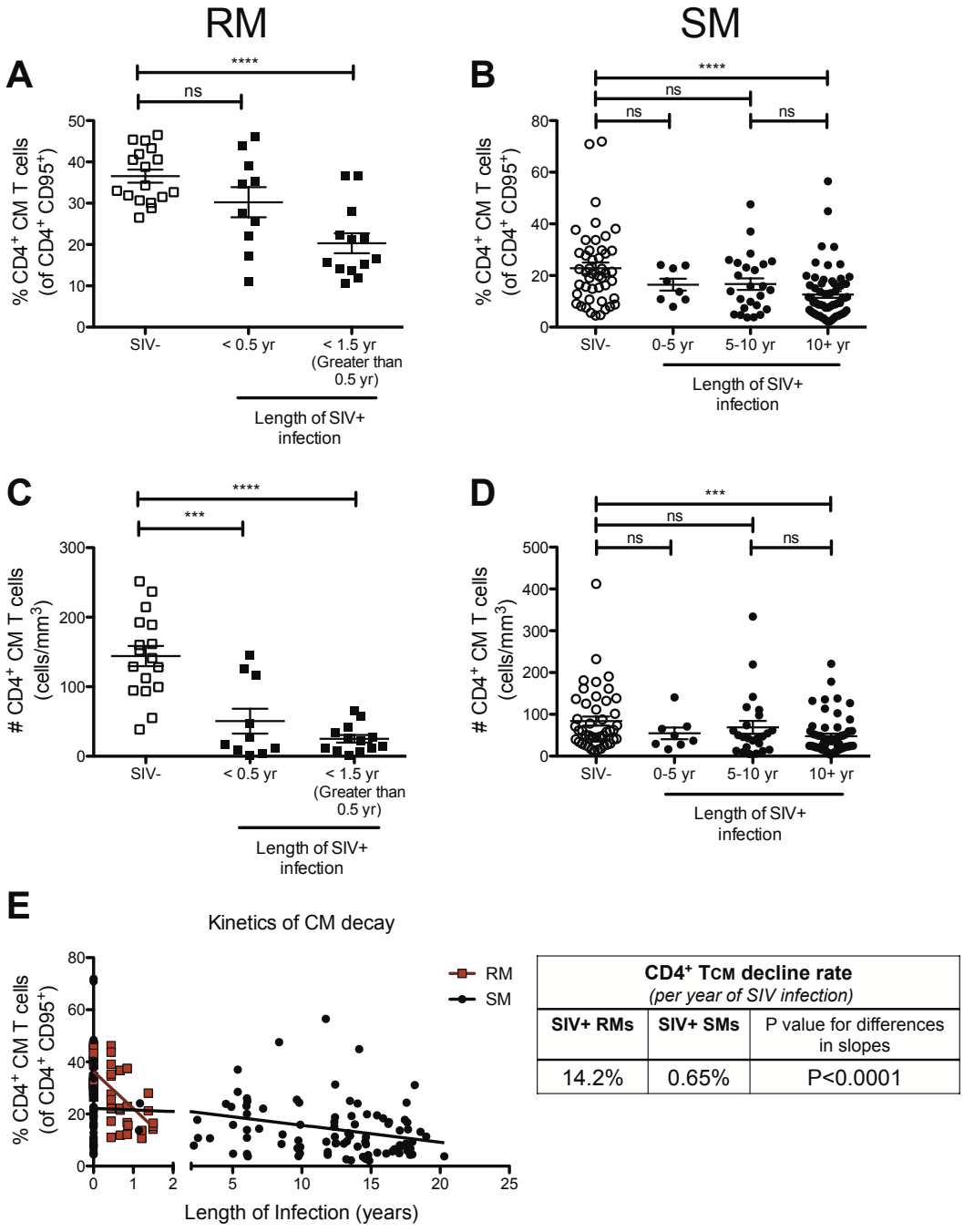

Figure 2.2. CD4+ TCM cells have increased stability in naturally SIV-infected SMs compared to SIV-infected RMs. The percentages (of CD4+CD95+ cells; panels A,B) and counts (cells/mm^3; panels C,D) of CD4+ TCM cells were compared between uninfected and SIV-infected RMs (A,C; *left panels*) and SMs (B,D; *right panels*) based on their length of SIV

infection (years) (***, p< 0.001; ****, p< 0.0001; p = ns; as determined by *t* test (A, C) or Mann-Whitney *U* test (B,D)). (E) Scatterplots depict the relationship between the fraction of CD4+ TCM cells and the length of infection at the time of sampling for RMs (□) and SMs (•) in order to estimate a linear decay rate of central memory cells (p<0.0001 for differences in slopes; ANCOVA).

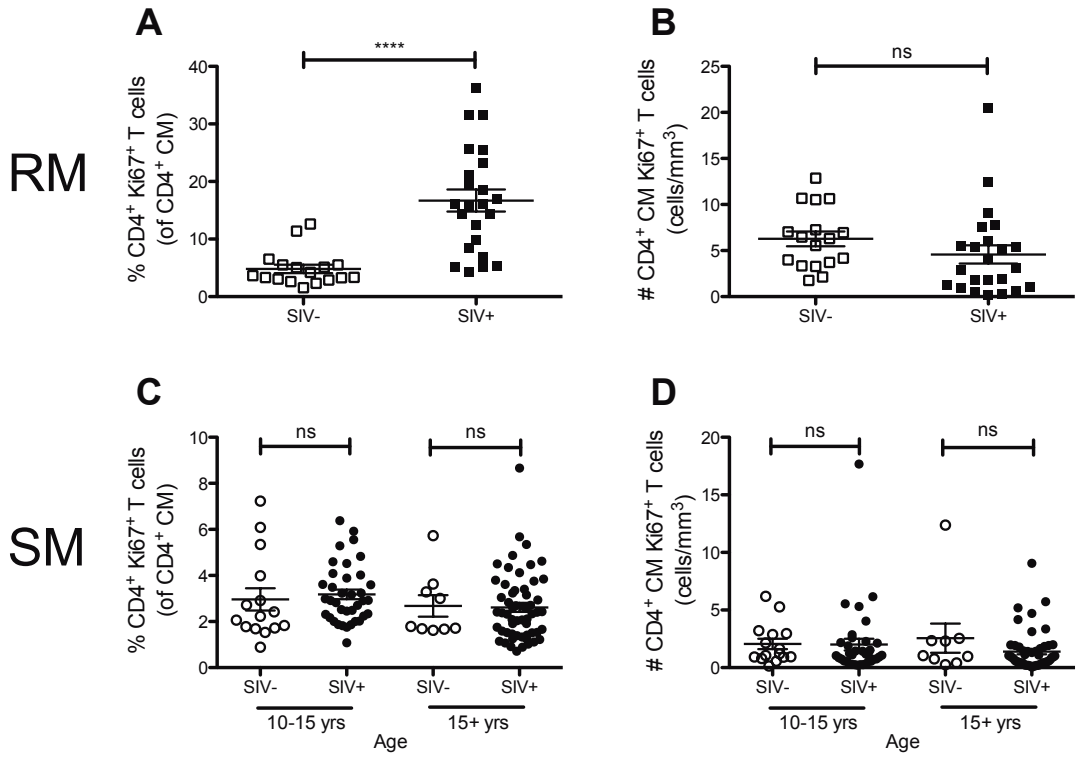

Figure 2.3. SIV infection of RMs, but not of SMs, is associated with a significant increase in the level of proliferating CD4+ TCM cells. The percentages (A) and numbers (B) of proliferating (Ki-67+) CD4+ TCM cells were compared in uninfected (□) and SIV-infected RMs (n). The levels of proliferating $CD4^+$ TCM cells were compared between age-matched uninfected (o) and SIV-infected SMs (•), by both frequencies (C) and cell counts (D) (****, $p < 0.0001$; p = ns; as determined by Mann-Whitney *U* tests).

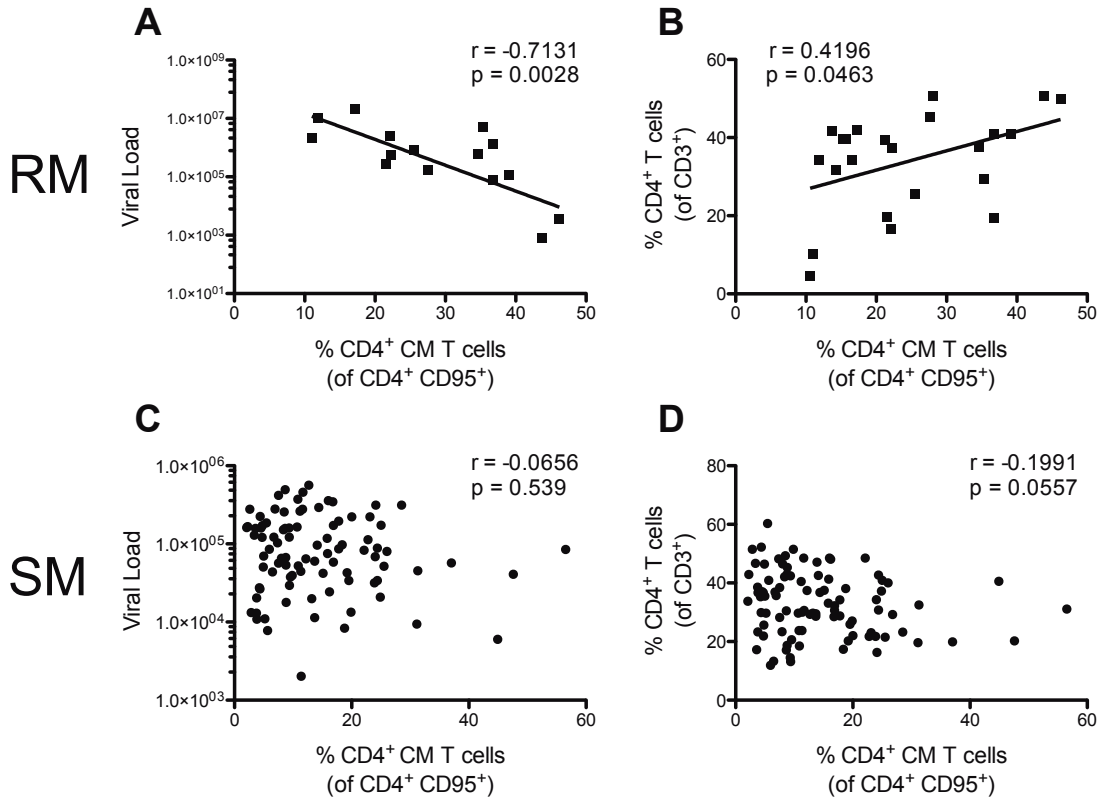

Figure 2.4. The level of central memory CD4+ T cells correlates with viral load and CD4+ T cell levels in SIV infection of RMs. Shown are the correlations between the percentages of CD4+ TCM cells (of CD4+CD95+ cells) and the viral load (A,C), and the percentages of CD4+ T cells (of CD3+ T cells) (B, D), for SIV-infected RMs (n; *top row*; A-B) and naturally SIV-infected SMs (•; *bottom row*; C-D). All statistical analyses were determined by Spearman rank correlation tests.

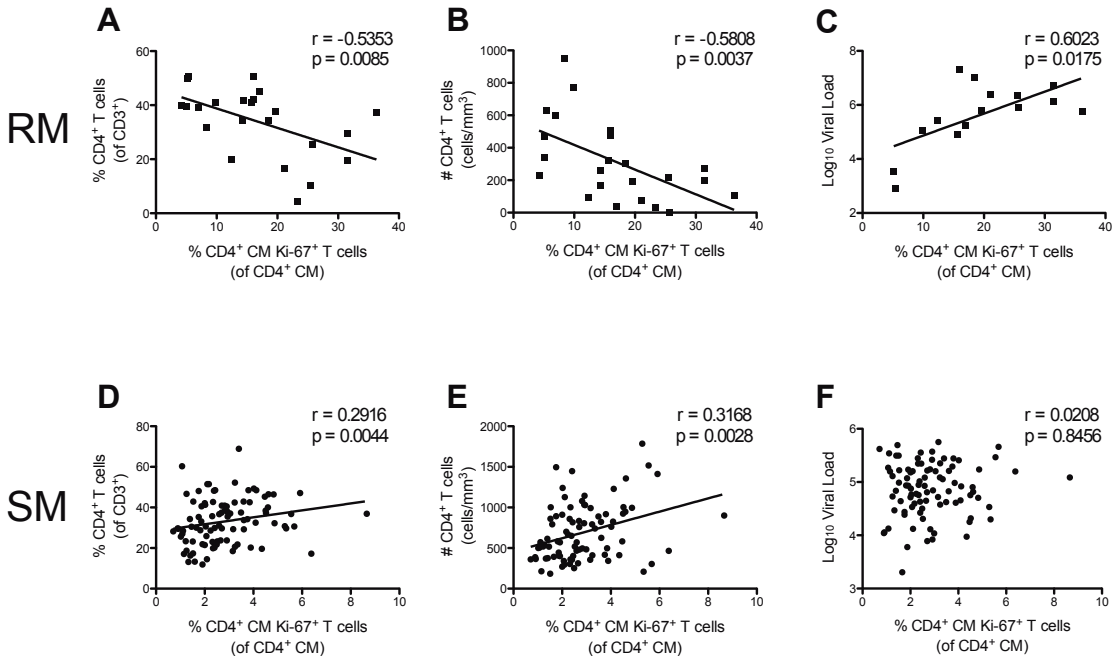

Figure 2.5. Increased levels of proliferating CD4+ TCM cells are unable to maintain CD4+ T cell levels in pathogenic SIV infection of RMs. The correlations between the percentages of CD4+ TCM Ki-67+ cells (of CD4+ TCM cells) and the percentages of CD4+ T cells (of CD3+ T cells) (A,D), and CD4+ T cell counts (B, E), are shown for SIV-infected RMs (n; *top row*; A-B) and naturally SIV-infected SMs (•; *bottom row*; D-E). The fractions of proliferating CD4+ TCM cells were positively correlated with the viral load for SIV-infected RMs (C), but not in naturally SIV-infected SMs (F). Viral load measurements were log transformed prior to graphically plotting them against frequencies of proliferating CD4+ TCM cells; linear regression was then used to model the line of best fit between the two variables. All statistical analyses were determined by Spearman rank correlation tests.

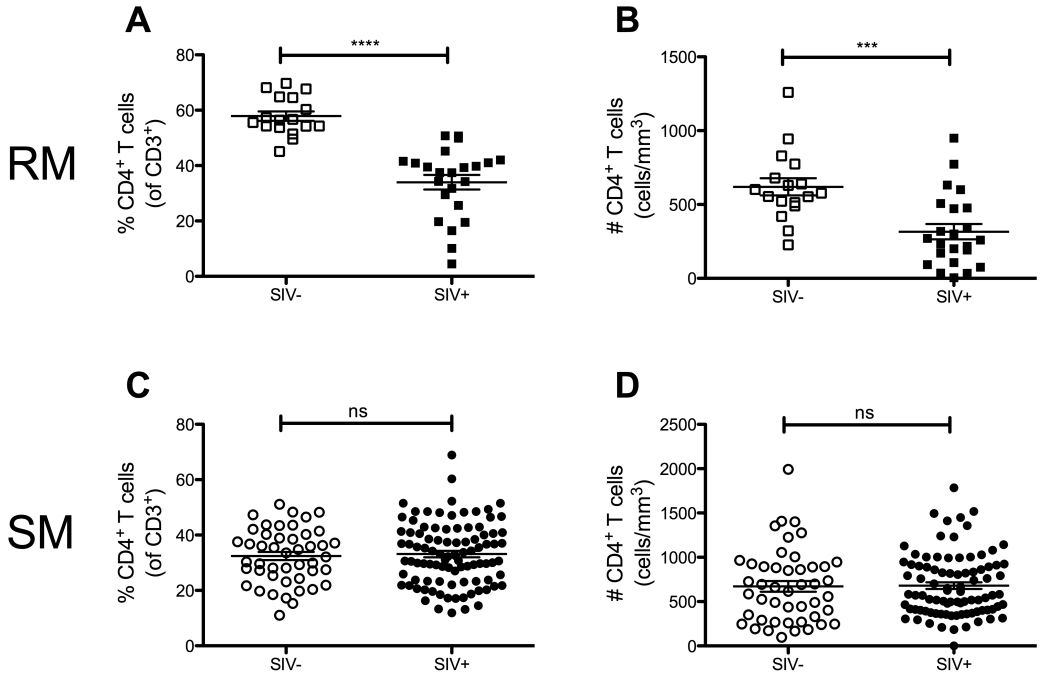

Figure 2.S1. CD4+ T cells are depleted in SIV-infected RMs, but are stable in SIV-infected SMs. The percentages (A) and absolute counts (B) of CD4+ T cells were compared between 17 uninfected (□) and 23 SIVmac239-infected (n) RMs. Similar analyses were performed between 46 uninfected (o) and 94 naturally SIV-infected (•) SMs in (C) and (D) (***, $p< 0.001$; ****, $p< 0.0001$; p=ns; as determined by *t* tests).

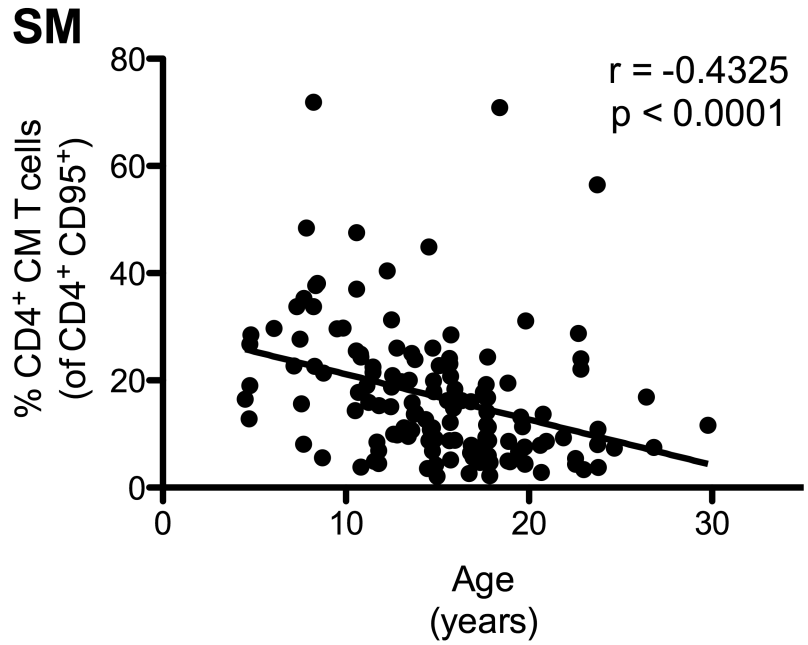

Figure 2.S2. The percentage of CD4+ TCM cells in SMs is negatively correlated with the age of the animals. A scatterplot depicts the significant relationship between the fraction of CD4+ TCM cells (of CD4+CD95+ T cells) and the age of SMs in SIV-infected and uninfected SMs at the time of sampling ($p<0.0001$, as determined by the Spearman rank correlation test).

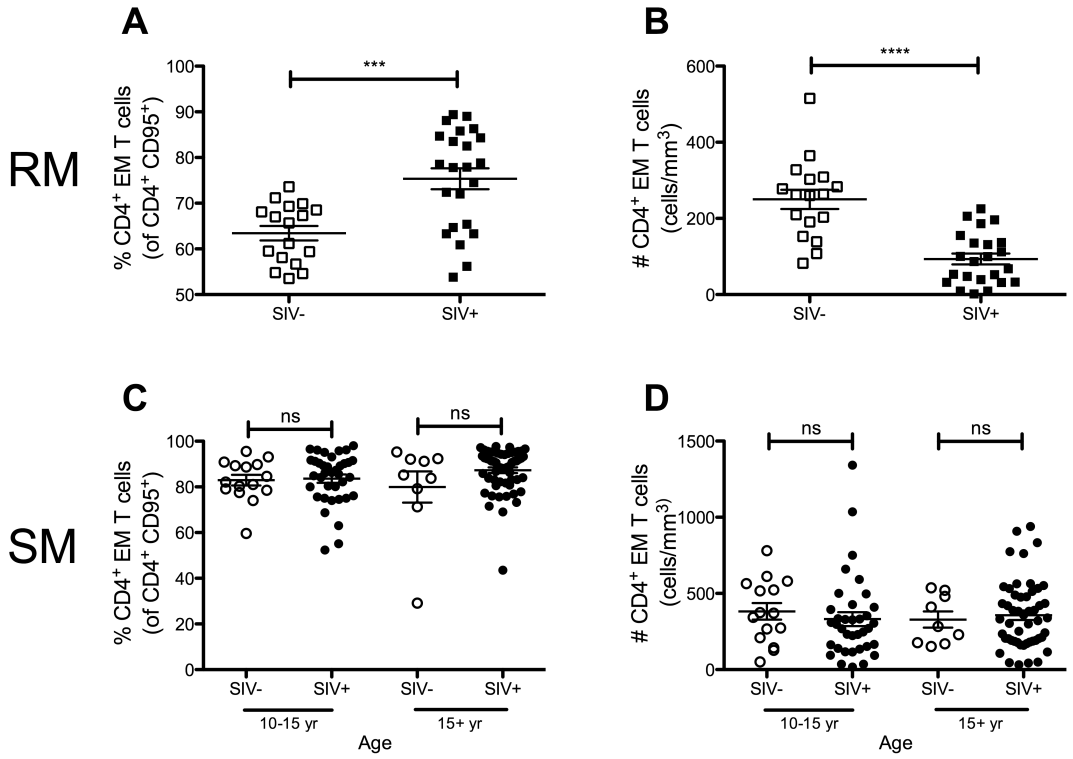

Figure 2.S3. CD4+ TEM cells are depleted as a result of pathogenic SIV infection in RMs, but are maintained in the natural SIV infection of SMs. Frequencies (of CD4+CD95+ cells; A) and cell counts (B) of CD4+ TEM in whole blood were compared between uninfected (□) and SIV-infected (n) RMs (***, $p < 0.001$; ****, $p < 0.0001$; as determined by *t* test). The percentages (C) of CD4+ TEM cells (of CD4+CD95+ cells) in whole blood were compared between age-matched uninfected (o) and SIV-infected (•) SMs. Numbers of CD4+ TEM cells (cells/mm^3) were measured in the same cohort of SMs (D) (p = ns; as determined by the Mann-Whitney *U* test).

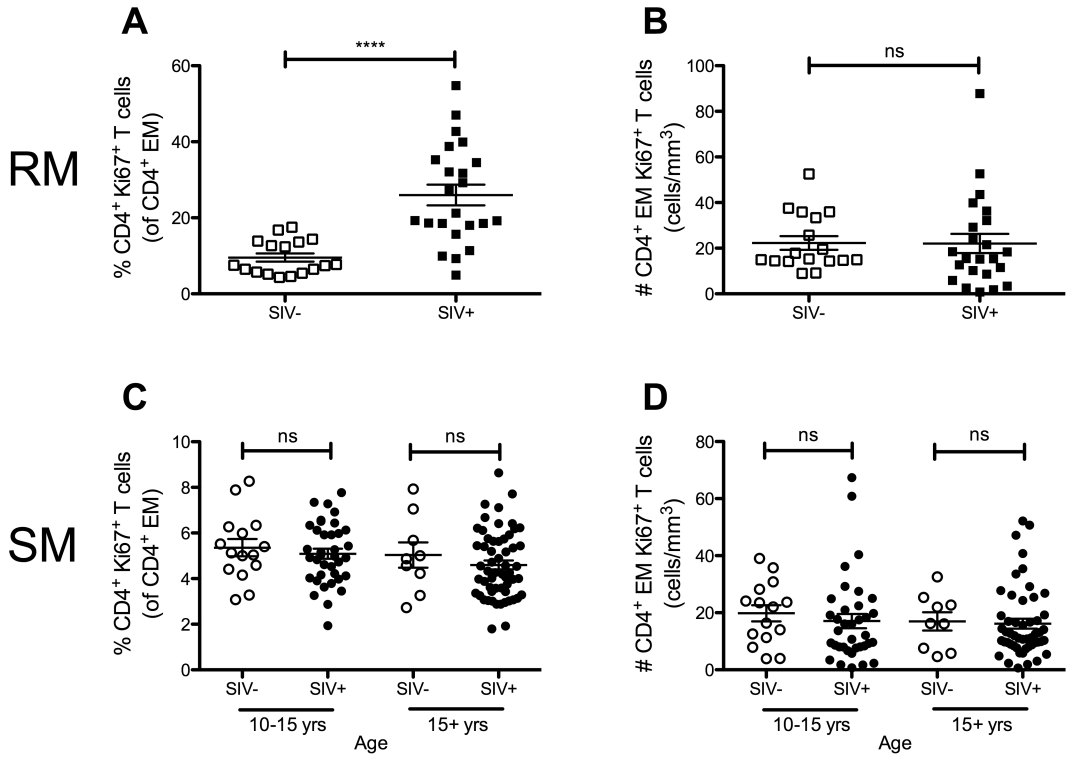

Figure 2.S4. Pathogenic SIV infection of RMs results in increased levels of proliferating CD4+ TEM cells, which is unseen in the natural SIV infection of SMs. Comparison of the percentages (A) and numbers (B) of proliferating CD4+ Ki-67+ TEM cells between uninfected (□) and SIV-infected (n) RMs. The levels of proliferating CD4+ TEM cells were compared between age-matched uninfected (o) and SIV-infected SMs (•), by both frequencies (C) and cell counts (D) (****, $p < 0.0001$; p = ns; as determined by Mann-Whitney *U* tests).

Chapter Three

The loss of CCR6+ and CD161+ CD4+ T cell homeostasis contributes to disease progression in SIV-infected rhesus macaques*

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Abstract

While previous studies have shown that CD4+ T cells expressing CCR6 and CD161 are depleted from blood during HIV infection, the mechanisms underlying their loss remain unclear. In this study, we investigated how the homeostasis of CCR6+ and CD161+ CD4+ T cells contributes to SIV disease progression and the mechanisms responsible for their loss from circulation. By comparing SIV infection in rhesus macaques (RMs) and natural host sooty mangabeys (SMs), we found that the loss of CCR6+ and CD161+ CD4+ T cells from circulation is a distinguishing feature of progressive SIV infection in RMs. Furthermore, while viral infection critically contributes to the loss of CD161+CCR6-CD4+ T cells, a redistribution of CCR6+CD161- and CCR6+CD161+CD4+ T cells from the blood to the rectal mucosa is a chief mechanism for their loss during SIV infection. Finally, we provide evidence that the accumulation of CCR6+CD4+ T cells in the mucosa is damaging to the host by demonstrating their reduction from this site following initiation of antiretroviral therapy in SIV-infected RMs and their lack of accumulation in SIV-infected SMs. These data emphasize the importance of maintaining CCR6+ and CD161+ CD4+ T cell homeostasis, particularly in the mucosa, to prevent disease progression during pathogenic HIV/SIV infection.

Introduction

Progressive HIV-1 infection results in both a disruption of mucosal barrier integrity (138, 140, 427) as well as a massive depletion of mucosal CD4+ T cells (67, 70, 74). The impact of mucosal CD4+ T cell depletion on HIV disease progression was initially unclear, as CD4+ T cells were found to be lost during the acute phase of both pathogenic SIV infection of rhesus macaques (RMs) as well as in nonpathogenic SIV infection of African green monkeys (AGMs) and sooty mangabeys (SMs) (69, 152). Yet, further investigation revealed the preferential loss of T helper 17 cells (Th17) in pathogenic HIV/SIV infection of humans and RMs, respectively (135-137, 180, 220). Th17 cells, a subset of CD4+ T cells found predominantly in mucosal tissues (135, 428), critically contribute to mucosal defenses through their secretion of IL-17 and IL-22 (429), which cause the production of antimicrobial molecules and strengthen the intestinal barrier through the production of enterocytes and claudins (215, 216, 218, 225). As a result, the preferential depletion of Th17 cells during HIV/SIV infection is associated with a reduction of mucosal barrier integrity and an increase in microbial translocation from the intestinal lumen into circulation, thereby leading to systemic immune activation in HIV-infected individuals (136, 137, 180, 430, 431). Thus, the maintenance of Th17 cells has been a major goal for reducing immune activation and subsequent disease progression in HIV-infected individuals.

Several mechanisms have been proposed to contribute to the loss of Th17 cells from the mucosa during pathogenic HIV/SIV infection. First, numerous groups have demonstrated that Th17 cells are highly permissive to HIV/SIV infection (135, 221-223, 432, 433) and are primary targets of SIV infection (434), which supports their early and sustained depletion. Second, CD103+ DCs, a subset of dendritic cells that promote Th17 differentiation (435), are similarly depleted from the mucosa of SIV-infected RMs (225). Third, HIV and SIV infection results in decreased levels of IL-21-producing cells and serum IL-21, a pleiotropic cytokine whose functions include the maintenance of Th17 cells (226, 436-439). Indeed, the administration of IL-21 to SIV-infected

RMs transiently increased intestinal Th17 cell frequencies and decreased systemic immune activation, even in the absence of ART, thus supporting the molecular link between IL-21 availability and Th17 cell homeostasis (136, 227). Additionally, alterations in the recruitment of CD4+ T cells have been suggested to contribute to Th17 cell loss during HIV/SIV infection (440).

CCR6 is a chemokine receptor expressed on Th17 cells (although not exclusively) that governs their migration to the small intestine as a result of CCL20 (MIP-3α) production, the exclusive chemokine for CCR6 (441-444). Th17 cells and their precursors also express the C-type lectin receptor CD161 (445, 446). CD161 is expressed on NK cells, as well as on CD8+ and CD4+ T cells, where its expression is tightly associated with the expression of other Th17 markers, including CCR6, thus imprinting a gut homing potential (446-448). In HIV-infected individuals, circulating CCR6+ and CD161+ CD4+ T cells are lost from the blood and are unable to be restored to near-normal levels with ART (157, 223, 449, 450). However, whether the loss of these CCR6 and CD161-expressing cells from the blood reflects a true depletion of this subset from HIV-infected individuals is uncertain, since few studies have been able to examine their dynamics in different anatomic tissues. CCL20 is secreted by mucosal epithelial cells in response to inflammatory stimuli, including cytokines (IL-1α and TNFα) and bacteria (*Salmonella* and Segmented filamentous bacteria) (451-453). Therefore, progressive HIV infection, which is characterized by loss of mucosal barrier integrity and increased interaction with pro-inflammatory mediators, may lead to increased CCL20 production and consequently, increased recruitment of CCR6-expressing cells to the gut mucosa (138, 140). It is unclear, then, what drives the loss of CCR6+ and CD161+ CD4+ T cells from the blood and how these dynamics contribute to the preferential depletion of mucosal Th17 cells during pathogenic HIV/SIV infection.

In this study, we sought to determine how the homeostasis of CCR6+ and CD161+ CD4+ T cells contributes to SIV disease progression by comparing the dynamics of these populations between a cohort of SIV-infected RMs and a cohort of naturally SIV-infected SMs. In addition, we investigated the mechanisms responsible for the loss of these cell subsets from circulation during pathogenic SIV infection. Our results indicate that CCR6+ and CD161+ CD4+ T cells are only depleted from the blood during progressive SIV infection of RMs, but not during SIV infection of SMs, which supports a link between the loss of these cells and SIV disease progression. Our results also revealed that, while CD161+CCR6- CD4+ T cells are depleted by viral infection across all tissues sampled, CCR6+CD161- and CCR6+CD161+ CD4+ T cells are redistributed to the gut. Altogether, these data underline the importance of maintaining CCR6+ and CD161+ CD4+ T cell homeostasis, particularly in the mucosa, to prevent HIV/SIV disease progression.

Materials and Methods

Animals and SIV infection

For cross-sectional analysis, 40 SIV-negative (13.3±0.8 years old) and 42 naturally SIV-infected (16.6±0.8 years old) SMs and 47 SIV-negative (8.3±0.9 years old) and 31 SIVmac239-infected (6.8±0.7 years old) RMs were included. In the SIV-infected animals, the average durations of infection were 8.91 ± 0.90 years for SMs, as estimated by the date of the first SIV-positive test, and 1.02 ± 0.12 years for RMs. For longitudinal analysis, six female RMs (8.6 ± 0.6 years old) were included, where all animals were *Mamu*-B*08 and B*17 negative. All 6 animals were infected intravenously (i.v.) with 300 TCID50 SIVmac239 (day 0). For cell sorting and analysis of cell-associated SIV DNA and RNA, ten chronically SIV-infected RMs were included, all of which had been infected with SIVmac239 i.v. (7 at a dose of 200 TCID50; 1 at 100 TCID50; and 2 at 3000 TCID50). The average duration of infection at the time of cell sorting (necropsy) was 517.0±34.4 days. For longitudinal analysis of CCR6+ and CD161+ CD4+ T cell frequencies during ART, eight RMs (4.2 ± 0.2 years old) were included. These RMs had been SIV-infected between 23 and 42 weeks (average of 31.5 weeks) before initiating a daily combination ART regimen consisting of a coformulated three-drug cocktail of tenofovir (PMPA), emtricitabine (FTC), and dolutegravir, administered with a daily subcutaneous injection. Two RMs were sacrificed within the first 4 weeks of ART due to rapid disease progression and weight loss.

Sample collection and processing

Blood, lymph node (LN) and rectal (RB) biopsies were performed longitudinally from RMs as previously described (136). Blood samples were taken for a complete blood count and routine chemical analysis, and centrifuged within 1 hour of phlebotomy for plasma separation. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density gradient centrifugation for both RMs and SMs. To obtain rectal biopsies, an anoscope was placed a short distance into the rectum for the collection of up to 20 pinch biopsies with biopsy forceps. RB-

derived lymphocytes were digested with 1 mg/mL collagenase for 2 hours at 37°C, and then passed through a 70-um cell strainer to remove residual tissue fragments. For LN biopsies, the skin over the axillary or inguinal region was clipped and surgically prepped before making an incision in the skin over the LN, which was exposed by blunt dissection and excised over clamps. LNs were then homogenized and passed through a 70-um cell strainer to isolate lymphocytes. All samples were processed, fixed (1% paraformaldehyde), and analyzed within 24 hours of collection. Chronically SIV-infected RMs were electively sacrificed and lymph nodes were collected from 3 sites- axillary, inguinal, and mesenteric, whose lymphocytes were pooled for FACS cell sorting.

Flow cytometric analysis

Fourteen-parameter flow cytometric analysis was performed on peripheral blood, LN, and RB derived cells according to standard procedures using a panel of monoclonal antibodies that we and others have shown to be cross-reactive with SMs and RMs.(136) The following antibodies were used at predetermined optimal concentrations: anti-CCR6-PE-Cy7 (clone 11A9), anti-CCR5-PE and -APC (clone 3A9), anti-CD3-APC-Cy7 (clone SP34-2), anti-CD62L-FITC (clone SK11), anti-CD95-PE-Cy5 (clone DX2), anti-Ki-67-Alexa700 (clone B56), anti-IFN-γ-Alexa700 (clone B27), and anti-CXCR3-AlexaFluor 488 (clone 1C6/CXCR3) from BD Biosciences; anti-CD161-PE (clone HP-3G10) and anti-IL-17-Alexa488 (clone eBio64DEC17) from eBioscience; anti-CD28-ECD (clone CD28.2) from Beckman Coulter; anti-CD4-BV421 (clone OKT4), anti-CD4-BV605 (clone OKT4), and anti-CD161-BV421 (clone HP-3G10) from Biolegend; anti-CD8-Qdot705 (clone 3B5) and Aqua Live/Dead amine dye-AmCyan from Invitrogen. Flow cytometric acquisition was performed on at least 100,000 CD3+ T cells on an LSRII cytometer driven by the FACS DiVa software, or at least 10,000 CD3+ T cells for RB-derived cells. The data acquired were analyzed using FlowJo software (version 9.8.5; TreeStar).

Intracellular cytokine staining

Freshly isolated PBMCs and RBMCs were resuspended at a concentration of $3x10^6$ cells/mL in RPMI 1640 medium supplemented with 10%FBS, 100 IU/mL penicillin, and 100 ug/mL streptomycin. Stimulations were conducted for 4 hours at 37°C in the presence of phorbol myristate acetate (PMA; 80 ng/mL), ionomycin (500 ng/mL), brefeldin A, and GolgiStop. After 4 hours, cells were washed once with PBS to remove stimuli and stained with surface markers for CD3, CD4, CD8, CCR6, CD161, CD28, CD95, and CCR5 for 30 minutes at room temperature. Cells were then fixed with cytofix/cytoperm (BD Pharmingen), washed, and stained intracellularly with antibodies specific for IL-17 and IFN-γ for 1 hour at room temperature. Following staining, cells were washed and fixed with PBS containing 1% paraformaldehyde prior to acquiring on an LSRII cytometer.

CCL20 in situ *hybridization and quantitative image analysis*

In situ hybridization (ISH) was performed on formalin-fixed, paraffin-embedded rectal biopsy tissues from the 6 RMs monitored longitudinally. First, tissue sections were deparaffinized. Antigen retrieval was then performed by heating slides with alkaline antigen retrieval (Vector) for 20 min in a microwave oven, transferring slides to 10 mM citrate buffer, pH 6.0 until cool, and then washing twice in 2 x SSC. A LAN probe was designed against CCL20 (Exiqon) and doubly labeled with Digoxigenin (dig) at each end (CCL20 probe: /5digN/TGTGAAAGACGACAGCATTGA/3DIG_N/). The protocol was optimized for the detection of CCL20 and beta-actin (control RNA) following common protocols. Briefly, tissue sections were covered with the hybridization buffer (50% deionized formamide, 10% dextran sulfate, 1x Denhardt medium, 2 mM EDTA) containing 20 ng/ml CCL20 probes and hybridized for 2 hours at 30°C. Slides were washed following hybridization: twice in 5 x SSC for 5 min, twice in 1 x SSC for 5 min, twice in 0.1 x SSC for 5 min- all at 37°C- and then once in 1x Tris-buffered saline (TBS) at room temperature for 5 min. Slides were blocked (Vector blocking

solution) for 30 min, before incubating with anti-DIG antibody (1:200, Roche) for 1 hour at room temperature. After washing slides twice with 1x TBS for 10 min, BICP/NBT was added to the slides and incubated for 20 minutes to allow for detection of the hybridized probe. DAPI (Sigma) was used as a counter stain for detecting nuclei. Quantification of CCL20 mRNA ISH positive cells was performed on SIV-infected rectal biopsies at different time points during the course of infection and compared to pre-infection control samples from the same animal. CCL20 RNA ISH positive cells were counted in ten high power fields (20x) for each biopsy. Images were collected using liquid crystal tunable filter multispectral imaging (Nuance Multispectral imaging system; Perkin-Elmer). Using the image analysis software Inform (Perkin-Elmer), we counted CCL20 positive cells and expressed this data as cells per nuclei counted (DAPI stained).

FACS cell sorting

Mononuclear cells isolated from the blood and pooled LNs (axillary, inguinal and mesenteric) of chronically SIV-infected RMs were stained with anti-CD3, anti-CD4 (clone OKT4; BV650, Biolegend), anti-CD28, anti-CD95, anti-CCR6, and anti-CD161 for 30 minutes at room temperature. Memory CD4+ T cells were then sorted based on their expression, or lack of expression, of CCR6 and CD161 using a FACS AriaII (BD Biosciences).

Quantitation of cell-associated SIV DNA and RNA

Cell-associated SIV DNA and RNA were quantified for CCR6 and CD161-expressing memory CD4+ T cells. Briefly, cellular DNA and RNA were extracted from sorted memory CD4+ T cells lysed in RLT Plus buffer (Qiagen) and isolated using the AllPrep DNA/RNA Mini Kit (Qiagen) per the manufacturer's instructions. Cell input ranged between 15,000 and 550,000 sorted cells from the PBMCs, and between 20,000 and 600,000 for LN. cDNA was synthesized from extracted RNA, and quantification of SIVmac *gag* DNA and cDNA was performed on samples using the QX100^{TM} Droplet Digital^{TM} PCR system (Bio-rad). Total SIV DNA and RNA were

quantified for these samples using SIVmac gag primers and probes, and normalized to the GAPDH gene. Data was analyzed using the Quantasoft analysis software 1.3.2.0 (Bio-rad).

Apoptosis analysis

Freshly isolated PBMCs from 8 healthy RMs ($2x10^6$ cells) were washed once with Annexin V buffer (BD Biosciences). PBMCs were then stained ex vivo with surface markers for CD3, CD4, CD8, CCR6, CD161, CD28, and CD95, as well as with Annexin V (APC; BD Biosciences) and Aqua Live/Dead amine dye-AmCyan (Invitrogen) for 30 min at room temperature. Following staining, cells were washed with Annexin V buffer and immediately acquired on an LSRII cytometer. 4×10^6 freshly isolated PBMCs from the same animals were also cultured for 24 hours in complete RPMI media prior to performing Annexin V staining, as described above.

Statistics

Prior to implementation of any specific statistical analysis for each cross-sectional outcome, assumptions were assessed (i.e., normality and homogeneity of variance). If the underlying assumptions were met, a two-sided two-sample equal-variance t-test was performed to compare the differences in immunologic parameters between uninfected and SIV-infected animals, as well as between different CD4+ T cell subsets. If the assumptions were violated, the two-sample Mann-Whitney U-test was used to compare differences in outcomes between uninfected and SIV-infected animals. DNA and RNA measurements were excluded for samples in which <15,000 cells were sorted, as the assay was not sensitive enough to detect viral measurements consistently outside of this range. Two additional data points were excluded due to technical error. Cross-sectional analyses and comparisons of SIV DNA were conducted using GraphPad Prism 6.0.

For our longitudinal investigations, repeated-measures analyses were performed for CD4+ and CD8+ T-cell subsets with a means model via the SAS MIXED Procedure (version 9.4; SAS

Institute, Cary, NC), which provides separate estimates of the means at each time point of the study. The model examined one predictor (time on study) with up to 13 categorical levels, and was fit separately for each anatomic location. A compound-symmetric variance-covariance form in repeated measurements was assumed for each outcome and robust estimates of the standard errors of parameters were used for performing statistical tests and constructing 95% confidence intervals. The model-based means are unbiased with unbalanced and missing data, so long as the missing data was non-informative (missing at random, MAR). Four statistical tests were done within the framework of the mixed effects linear model for blood and rectal biopsy comparisons, and three for lymph node comparisons. All statistical tests were two-sided and unadjusted for multiple comparisons. A p-value ≤ 0.05 was considered statistically significant for every statistical comparison.

To relate the decline of CCR6+CD4+ T cell frequencies from the blood to their increase in RB, subset frequencies were ln transformed (log base e) prior to analysis by a mixed-effects model. The model specified that ln(%CCR6+ of CD4) in RB followed a linear regression over ln(%CCR6+ of CD4) in blood, with a random intercept for each animal. The variance components included estimates of inter-animal and intra-animal variance.

Study Approval

All animal experimentations were conducted according to guidelines established by the Animal Welfare Act and the NIH for housing and care of laboratory animals and performed in accordance with Institutional regulations after review and approval by the Institutional Animal Care and Usage Committees (IACUC; Permit number: 2001973) at the Yerkes National Primate Research Center (YNPRC). Anesthesia was administered prior to performing any procedure, and proper steps were taken to minimize the suffering of the animals in this study.

Results

Characterization of CCR6+ and CD161+ CD4+ T cells in rhesus macaques

CCR6+ and CD161+ CD4+ T cells distinguish Th17 lineage polarization and Th17 precursor cells, respectively, in humans (441, 442, 445, 446). Therefore, we first sought to characterize the phenotypic and functional properties of circulating CCR6 and CD161-expressing CD4+ T cells in healthy RMs. CCR6 and CD161 was expressed on approximately 10.6±0.5% and 5.5±0.5% of CD4+ T cells in the blood, respectively (Figure 3.1A). Unlike previous human studies where the majority of CD161+ cells co-express CCR6 (445), less than 15% of CD161+ CD4+ T cells co-expressed CCR6 in RMs, suggesting a differential expression pattern between humans and RMs (Figure 3.1A). However, in agreement with human literature (441, 445), the majority of CCR6+CD161-, CD161+CCR6-, and CCR6+CD161+ CD4+ T cells fell within the memory (CD95+) compartment, where they were predominantly effector memory cells, defined here by the absence of CD62L expression (EM, CD95+CD62L-; Figure 3.1B).

The co-expression of CCR6 and CD161 has been used with other surface markers in humans to phenotypically distinguish IL-17-producing cells (Th17) ex vivo in lieu of intracellular staining for cytokine production or the master regulator RORgt (441, 442, 445). Thus, we investigated the ability of these subsets to produce IL-17 following stimulation with PMA and ionomycin. Within the blood, CCR6+CD161- CD4+ T cells were the CD4+ T cell subset contributing the most to IL-17 production, comprising 70.8±3.0% of IL-17-producing cells (Figure 3.1C). However, the contribution of CCR6+CD161- CD4+ T cells to IL-17 production was substantially lower in the rectal mucosa (RB), where they comprised only 1.05±0.13% of IL-17 producing cells (Figure 3.1C). This difference likely results from a combination of factors: (i) the lower frequency of CCR6+CD161- T cells within the CD4+ T cell population in the rectal mucosa; and (ii) a reduced ability of mucosal CCR6+CD161- CD4+ T cells to produce IL-17 (RB: 10.9±2.3% vs. PBMCs: 34.7±1.5%; Figure 3.1D). CCR6+CD161+ (DP) cells contained the highest frequency of IL-17

producers, both in PBMCs (41.5±3.1%) and in RB (22.7±3.7%; Figure 3.1D). Interestingly, only 5.4±1.9% of CD161+CCR6- CD4+ T cells produced IL-17 in the blood, as compared to 16.8±2.3% in the rectal mucosa (Figure 3.1D). Instead, CD161+CCR6- CD4+ T cells produced high levels of IFN-γ, in both the blood and rectal mucosa (Figure 3.1D). Moreover, CD161+CCR6- CD4+ T cells contributed significantly more to IL-17+IFN-γ+ production in the rectal mucosa than the other subsets (Figure 3.S1). Thus, in RMs, the small subset of CCR6+CD161+ behaves similarly to IL-17-producing CCR6+CD161+ CD4+ T cells in humans, while CD161+CCR6- CD4+ T cells contribute minimally to IL-17 production, particularly in the blood.

We then examined the potential susceptibility of these CCR6 and CD161-expressing subsets to SIV infection by investigating their expression of the SIV co-receptor CCR5 and Ki-67 as a marker of T cell activation and proliferation. Interestingly, we found that while few CCR6+CD161- CD4+ T cells expressed CCR5 (2.66±0.36%), CD161-expressing CD4+ T cells contained significantly higher frequencies of CCR5+ cells, particularly among the CD161+CCR6- subset (17.0%; Figure 3.2A). Furthermore, CCR6+CD161+ CD4+ T cells, which had high levels of CCR5+ cells similar to CD161+CCR6- cells (17.7%), contained the highest fraction of proliferating cells (20.1%; Figure 3.2B). In fact, the frequencies of proliferating CD4+ T cells were significantly higher in all CCR6 and CD161-expressing T cell subsets compared to non-expressing subsets, which is consistent with their status as effector memory cells. Taken together, the increased levels of CCR5, particularly among CD161-expressing cells, combined with the higher baseline levels of proliferating cells among all CCR6 and CD161-expressing CD4+ T cells would suggest a heightened permissivity of these CD4+ T cell subsets to SIV infection when compared to non-expressing cells.

Depletion of CCR6+ and CD161+ CD4+ T cells is specific to pathogenic SIV infection

Previous studies in humans have shown that CCR6+ and CD161+ CD4+ T cells are depleted in the blood of HIV-infected individuals, even upon ART initiation (157, 223, 449, 450). Whether this loss of CCR6+ and CD161+ CD4+ T cells contributes to disease progression, though, has not yet been determined. To answer this question, we compared the levels of CCR6 and CD161-expressing CD4+ T cell subsets between SIV-infected and uninfected RMs, in which SIV infection invariably progresses to AIDS, to those levels found in SIV-infected and uninfected SMs, a natural host for SIV infection that generally remain AIDS free. In the SIV-infected animals, the average durations of infection were 8.91 ± 0.90 years for SMs, as estimated by the date of the first SIV-positive test, and 1.02 ± 0.12 years for RMs. We found that CCR6+CD161-, CCR6+CD161+, and CD161+CCR6- CD4+ T cells were all significantly depleted from the blood of SIV-infected RMs when compared to their levels in uninfected animals (CCR6+CD161-: 5.5% vs. 9.1%, $p=0.0002$; CCR6+CD161+: 0.06% vs. 0.45%, $p<0.0001$; CD161+CCR6-: 1.18% vs. 2.35%, $p<0.0001$; Figure 3.3A). However, in the natural host SMs, we found that SIV infection did not alter the levels of these CCR6 and CD161-expressing CD4+ T cells, suggesting that the loss of these subsets from the blood may contribute to disease progression during pathogenic infection. Consistently, when we related the frequencies of CCR6+CD161- CD4+ T cells in SIV-infected RMs and SMs with their different durations of SIV infection, we confirmed that CCR6+CD161- CD4+ T cells decay at an approximate rate of 2.97% per year of SIV infection in RMs, while they are maintained over the duration of infection in SIV-infected SMs (Table 3.S1, slopes estimated by linear regression). Importantly, we found that the functional profiles of CCR6 and CD161-expressing CD4+ T cells, as determined by cytokine production, were comparable between SMs and RMs, with CCR6+CD161- CD4+ T cells also comprising the highest fraction of IL-17-producing cells in the blood of SIV-uninfected SMs (74.0±4.0%; Figure 3.S2A). In addition, CD161+CCR6- CD4+ T cells produced the highest levels of IFN-γ among the subsets, with 26.2±3.2% of CD161+CCR6- cells secreting IFN-γ in the blood, on average (Figure 3.S2B), which together, suggests that these subsets behave similarly between the two species.

Interestingly, we found that the loss of CCR6+ cells was specific to CD4+ T cells, as the levels of CD8+CCR6+ T cells were comparable between SIV-infected and uninfected animals in both SMs and RMs (Figure 3.3B). Nevertheless, SIV infection was associated with a loss of CD8+CD161+ T cells in RMs (Figure 3.3C), a finding that agrees with the well-documented loss of mucosal-associated invariant T (MAIT) cells in HIV-infected individuals (454). These results demonstrate for the first time that the loss of CCR6+ and CD161+ CD4+ T cells from the blood is a feature which distinguishes pathogenic from nonpathogenic SIV infection, and thus, may be a contributing factor to disease progression in SIV-infected RMs and HIV-infected individuals.

CCR6+CD161- and CCR6+CD161+ CD4+ T cells accumulate in the rectal mucosa as a result of SIV infection, while CD161+CCR6- CD4+ cells are depleted across all tissues

While CCR6+ and CD161+ CD4+ T cells are lost from the blood of HIV/SIV-infected humans/RMs (157, 223, 449, 450), previous studies have been unable to determine if this is due to a true depletion or if it reflects redistribution of these subsets into lymphoid tissues. Therefore, we performed a longitudinal analysis of the frequencies of CCR6 and CD161-expressing cells within CD4+ T cells in the blood, RB, and lymph nodes (LN) of 6 SIV-infected RMs to better understand the kinetics of these cell subsets. CCR6+CD161- CD4+ T cells were rapidly lost from the blood and LN following SIV infection, with their levels significantly lower than pre-infection levels by 6 and 2 weeks post-infection ($p=0.0018$ and 0.0006) and remaining diminished throughout chronic SIV infection (week 23, Figure 3.4A). Surprisingly, we found that both CCR6+CD161- and CCR6+CD161+ CD4+ T cells accumulated in the rectal mucosa as early as 4 and 6 weeks post-SIV infection, respectively, ($p=0.0280$ and $p=0.0055$) and were maintained at significantly elevated levels as late as 23 weeks post-SIV infection ($p=0.0120$ and $p<0.0001$). The kinetics of this increase was highly variable between animals. Nevertheless, by week 23, 5 of 6 RMs had heightened frequencies of CCR6+CD161- CD4+ T cells in the mucosa as compared to

pre-infection, which was unique to this anatomic location, since the frequencies of those cells were reduced 1.9 and 4.2-fold at the same time point in blood and LN, respectively (Figure 3.4B). Consistent with their CCR6 expression, CCR6+CD161+ CD4+ T cell frequencies were also increased 2.2-fold in the mucosa at 23 weeks post-SIV infection when compared to their pre-infection frequencies, but decreased 3.1 and 4.3-fold in the blood and LN, respectively (Figure 3.4B). This accumulation was distinct to CCR6-expressing cells, though, and was not seen in CD161+CCR6- CD4+ T cells. Instead, CD161+CCR6- CD4+ T cells were depleted across all compartments, where their decline was progressive and significant in all tissues (WB: $p<0.0001$; RB: $p=0.0016$; LN: $p=0.0026$) by week 23 post-SIV infection (Figure 3.4A,B). Of note, in agreement with the loss of CCR6 and CD161-expressing CD4+ T cells from blood and LN, there were corresponding increases in the frequencies of CCR6-CD161- (DN) CD4+ T cells during SIV infection in these sites (Figure 3.4A, B). These results suggest a different pattern of regulation between CCR6+ and CD161+CD4+ T cells during SIV infection, whereby the depletion of CCR6-expressing cells from the blood may result, at least in part, from their redistribution to the gut mucosa while the depletion of CD161+CCR6- CD4+ T cells from the blood and tissues may result more specifically from viral infection. Indeed, this scenario is consistent with the high levels of CCR5 expression found on CD161+CCR6-CD4+ T cells in Figure 3.2A.

Redistribution of CCR6+CD4+ T cells is concurrent with an increase in intestinal CCL20 levels

To investigate if an early increase in CCL20 (MIP-3α) production during acute SIV infection could be responsible for the observed accumulation of CCR6-expressing CD4+ T cells (both CCR6+CD161- and CCR6+CD161+) in the gut mucosa (444, 455, 456), we performed in situ hybridization analysis for CCL20 longitudinally in rectal biopsy tissues of the same 6 SIV-infected RMs (Figure 3.5A). Levels of CCL20 in RB significantly increased during acute SIV infection (4 wk p.i.; $p=0.019$; Figure 3.5B), which corresponded to the accumulation of CCR6-

expressing CD4+ T cells in the gut mucosa. This increase was transient, as the levels of mucosal CCL20 returned to pre-SIV baseline levels by 6 weeks post-infection and chronic SIV infection (Figure 3.5B). Importantly, when we related the rate of bulk CCR6+CD4+ T cell decline from the blood to the increasing rate of bulk CCR6+CD4+ T cell levels in RB, we found a statistically significant inverse relationship between the two populations, suggesting that the accumulation of CCR6-expressing CD4+ T cells in the mucosa is partially related to their loss from the blood (Figure 3.5C; $p=0.0150$). Furthermore, the effect of increased CCL20 production was not restricted to CD4+ T cells but extended to CCR6+CD8+ T cells, which similarly increased in the gut mucosa by 6 weeks of SIV infection (although not significantly, $p=0.098$; Figure 3.5D). Therefore, our data show that the redistribution of CCR6+CD4+ T cells from the blood to the gut mucosa is directed by the increased production of CCL20 during acute infection and is partially responsible for their depletion from circulation during SIV infection of RMs.

An additional chemotactic axis responsible for the migration of CD4+ T cells to the mucosal tract is the α4β7-MAdCAM-1 (mucosal addressin cell adhesion molecule-1) axis (285). While MAdCAM-1 is constitutively expressed on intestinal epithelial cells, previous studies have demonstrated increased MAdCAM-1 expression during HIV infection, particularly in patients with opportunistic infections (457). To evaluate if MAdCAM-1 may additionally facilitate the recruitment of CCR6-expressing CD4+ T cells to the mucosa of SIV-infected RMs, we measured the expression levels of α4β7 integrin on CCR6 and CD161-expressing CD4+ T cell subsets from the blood of healthy RMs. We found that both CCR6+CD161- and CCR6+CD161+ CD4+ T cells contained significantly higher levels of α4β7+ cells when compared to CD4+ T cells subsets lacking CCR6 expression (31.8±1.3% and 33.6 ±4.6%, respectively, $p<0.0001$; Figure 3.6). Altogether, these results implicate both the CCR6-CCL20 and the α4β7-MAdCAM-1 axes in coordinating the migration of CCR6-expressing CD4+ T cells to the gut mucosa of SIV-infected RMs.

CD161+CCR6- CD4+ T cells harbor higher levels of SIV RNA in lymphoid tissues

While the migration of CCR6-expressing CD4+ T cells to the gut mucosa affects the levels seen in circulation after SIV infection, additional mechanisms such as increased infectivity may be contributing to the loss of circulating CCR6+ and CD161+ CD4+ T cells during chronic SIV/HIV infection. As such, we sorted memory CD4+ T cells from the PBMCs and LN of 10 chronically SIV-infected, ART-naive RMs (average length of SIV infection: 517.0± 34.4 days) based on their expression of CCR6 or CD161 and quantified the levels of cell-associated SIV DNA and RNA within each subset (Figure 3.S3). While CCR6+CD161- cells contained the highest frequencies of infected cells in both PBMCs (median: 4560 copies SIV per million cells) and LN (median: 8000 copies of SIV per million cells), neither CCR6+CD161- nor CD161+CCR6- memory CD4+ T cells were enriched in SIV DNA when compared to CCR6-CD161- (DN) memory CD4+ T cells in both the blood and LN (Figure 3.7A).

We also measured cell-associated SIV RNA to determine the contribution of each subset to viral production. In the PBMCs, there was a trend towards CCR6+CD161- CD4+ T cells expressing higher levels of SIV RNA, as demonstrated by the three representative animals shown in Figure 3.7B; however, the RNA expression was not significantly different among any of the sorted subsets. Nevertheless, in the LN, CD161+CCR6- memory CD4+ T cells harbored significantly higher levels of SIV RNA on a per cell basis than DN memory CD4+ T cells (Figure 3.7C; $p=0.0078$). Together, these data suggest that in lymphoid tissues, CD161+CCR6- CD4+ T cells are highly susceptible to both viral infection and subsequent viral production. Consistent with their higher levels of cell-associated RNA, we found that CD161+CCR6- CD4+ T cells contained significantly higher frequencies of cells expressing the SIV co-receptor CCR5 than the other CD4+ T cell subsets in LN and RB (Figure 3.S4A). CD161+CCR6- CD4+ T cells also contained the highest levels of CXCR3+ cells, which is agreement with a recent report that found CXCR3+

T follicular helper (TFH) cells harbor higher levels of virus in the LN (Figure 3.S4B) (458). Therefore, while the redistribution of CCR6+CD161- and CCR6+CD161+ CD4+ T cells from circulation to the gut mucosa appears to be a predominant mechanism for their depletion during SIV infection, the depletion of CD161+CCR6- CD4+ T cells results more from their heightened susceptibility to SIV infection in lymphoid tissues, thus supporting their depletion from all tissue compartments following infection.

CCR6+CD161- and CCR6+CD161+ CD4+ T cells are more prone to apoptosis

Since there was no clear preference for viral infection in CCR6+CD161- CD4+ T cells, we also wanted to investigate if an increased susceptibility to apoptosis may contribute to the loss of these CD4+ T cells from the blood during SIV infection. To do this, we evaluated apoptosis by measuring Annexin V binding in combination with Live/Dead free amine staining in PBMCs from eight healthy RMs under two different experimental conditions: (1) ex vivo, or immediately following PBMC isolation; and (2) following a 24 hour incubation in the absence of additional stimulation (spontaneous apoptosis). We found that, at baseline, only CCR6+CD161+ CD4+ T cells had significantly elevated apoptosis levels when compared to the other CD4+ T cell subsets ($p<0.0001$, Figure 3.8A). However, following 24 hours of culture, the levels of spontaneous apoptosis significantly increased in the CCR6+CD161- CD4+ T cell subset, with 28.6±1.8% positive for both Annexin V and Live/Dead staining (Figure 3.8B) compared to only 3.20±0.53% at baseline (Figure 3.8A). Levels of spontaneous apoptosis in these CCR6+CD161- CD4+ T cells were comparable to DP levels, suggesting that CCR6-expressing CD4+ T cells are more prone to apoptosis. Thus, in addition to migrating to the mucosa during SIV infection, increased susceptibility to apoptosis may contribute to the preferential loss of CCR6+ CD4+ T cells from circulation during chronic HIV/SIV infection.

CCR6+CD161- CD4+ T cell relocalization is damaging to the host

Previous studies have reported a loss of Th17 cells from the mucosa following pathogenic HIV/SIV infection (135, 136). Whether the increase in CCR6-expressing CD4+ T cells in RB, then, is beneficial to the host in promoting Th17 reconstitution or detrimental by recruiting potential viral targets has yet to be determined. To assess this, we investigated if suppressive ART restored CCR6+CD161- CD4+ T cell levels in the mucosa to pre-infection levels in 6 ART-treated, SIV-infected RMs. Even when started during chronic SIV infection (ranging from 23 to 42 weeks of infection), ART was able to reduce CCR6+CD161- CD4+ T cell frequencies in RB within 4 weeks of therapy ($p=0.0047$, Figure 3.9A), and to levels comparable to those found in uninfected RMs (Figure 3.9B). In contrast, the effect of this short ART treatment on CD161+CCR6- CD4+ T cell frequencies was less pronounced, as two months of ART was unable to significantly increase the circulating levels of this subset (Figure 3.S5A, 5B). However, ART was able to restore mucosal CD161+CCR6- levels to pre-infection frequencies (Figure 3.S5A, 5B). These results suggest that the migration of CCR6+CD161- CD4+ T cells to the mucosa is a feature of disease progression.

Given these results, we hypothesized that CCR6+CD161- CD4+ T cells would not accumulate in the RB of naturally SIV-infected SMs. Therefore, we compared the levels of CCR6 and CD161-expressing CD4+ T cell subsets in RB between SIV-infected and uninfected SMs. In contrast to RMs, the frequencies of CCR6+CD161- CD4+ T cells in RB were remarkably similar between SIV-infected and uninfected SMs (Figure 3.9C). Additionally, the fraction of CCR6+CD161+ CD4+ T cells significantly decreased in RB of SIV-infected SMs compared to uninfected animals ($p=0.0049$, Figure 3.9C). To further confirm the association of CCR6+CD161- CD4+ T cell accumulation in RB with damage to the host, we examined the relationship between frequencies of CCR6+CD161- CD4+ T cells and immunologic and virologic measures of disease progression in SIV-infected RMs. We found that the levels of CCR6+CD161- CD4+ T cells in RB at week 23 p.i. were inversely related to the absolute levels of CD4+ T cells in the blood of untreated RMs at

the same time point (r= -0.8286, p=0.0583; Figure 3.9D). Similarly, the frequencies of CCR6+CD161- CD4+ T cells in the blood at week 6 p.i. were negatively associated with plasma viral load at the same time point (r= -1.000, p=0.0167; Figure 3.9E). Thus, these findings suggest that the migration of CCR6+CD161- CD4+ T cells from circulation into the mucosal tract promotes immune dysfunction, may contribute to damage to the host, and ultimately advances disease progression.

Discussion

The loss of mucosal CD4+ T cell homeostasis is one of the earliest manifestations of progressive HIV infection and has been linked to microbial translocation, systemic immune activation, and ultimately, disease progression in untreated, HIV-infected individuals (140, 427, 459). Concomitant with this disruption of mucosal immunity is the loss of gut homing CCR6+ and CD161+ CD4+ T cells from the periphery of untreated HIV-infected individuals (157, 223, 449, 450). These cell subsets are known to migrate toward the gut mucosa in response to CCL20 production, which is elevated in the presence of pro-inflammatory stimuli and bacteria (451-453, 460). Our findings revealed that the redistribution of CCR6+CD161- and CCR6+CD161+ CD4+ T cells to the gut mucosa, largely driven by the acute production of CCL20, is a key mechanism of this cell subset's depletion from the blood and LN. Additionally, our data demonstrate that the relocalization of CCR6+CD161- CD4+ T cells to the gut is damaging to the host, as accumulation is not seen in SIV-infected SMs (Figure 3.9C) and is reversed in RMs by effective ART (Figure 3.9A). In contrast, CD161+CCR6- CD4+ T cells were depleted from all anatomic sites accessed (blood, RB, and LN), and this subset harbored high levels of SIV RNA, particularly in the LN; therefore, we hypothesize that these cells are depleted from multiple tissue sites as a result of viral infection. Despite these distinct mechanisms, the differential loss of both CCR6+ and CD161+ CD4+ T cells between pathogenic and non-pathogenic SIV infections supports their depletion from the blood as a distinguishing feature of HIV/SIV disease progression.

While previous studies have reported the loss of CCR6+CD4+ T cells from the blood of HIV-infected individuals and their incomplete restoration during ART, few studies have been able to monitor their kinetics longitudinally to define the impact of HIV infection on subset frequencies (223, 449). Using the RM model of SIV infection, we were able to relate the loss of CCR6+CD161- and CCR6+CD161+ CD4+ T cells from the blood with their accumulation in gut mucosal tissues. The recruitment of CCR6-expressing CD4+ T cells to the gut mucosa during

untreated SIV infection is not unexpected, since breaches in the intestinal epithelial layer and microbial translocation are well-documented consequences of pathogenic HIV/SIV infection and previous studies have found increased CCL20 levels both in HIV-infected individuals and SIV-infected RMs (455, 461). Consistent with our findings, Loiseau et al. recently described an inverse relationship between CCR6+CD4+ T cell frequencies in the blood and mucosal tissues of ART-treated, HIV-infected individuals, confirming that the trafficking between these two tissue compartments occurs at all stages of HIV/SIV infection, not simply during active viral replication (450). Interestingly, our data did not support preferential viral infection as a predominant mechanism of CCR6+CD161- CD4+ T cell loss from the blood. CCR6+CD161- CD4+ T cells harbored similar levels of SIV to other CD4+ T cell subsets in the blood and LN, which differs from previous studies that demonstrated higher levels of integrated HIV DNA in CCR6+CD4+ T cells, when compared to CCR6(-) cells, in viremic ART-naïve humans (223). This discrepancy may arise from the differences in viral DNA quantification, since measuring total cell-associated SIV DNA, as we did, may dilute the enrichment of integrated SIV DNA in CCR6+CD4+ T cells. Additionally, due to limited cell subset numbers in the rectal mucosa (resulting from an overall CD4+ T cell depletion), we were unable to quantify levels of viral infection in CCR6+CD161- CD4+ T cells in the gut. However, the finding that CCR6+CD161- CD4+ T cells are maintained at an elevated frequency in the gut mucosa supports a mechanism where their loss from the blood during SIV infection is primarily a result of their migration to the gut mucosa as opposed to their distinct susceptibility to viral infection. In addition to their redistribution throughout the body, our data also support an increased susceptibility of CCR6+CD161- and CCR6+CD161+ CD4+ T cells to apoptosis (Figure 3.8), which is an additional mechanism contributing to their loss from the blood during HIV/SIV infection.

In our study, the elevated frequencies of CCR6+CD161- CD4+ T cells in the gut mucosa following SIV infection were diminished upon ART administration (Figure 3.9A). In agreement

with these results, a recent study found reduced production of CCL20 by enterocytes of the small intestine in ART-treated, HIV-infected individuals when compared to uninfected individuals, resulting in reduced CCR6+CD4+ T cell frequencies in the gut (450). Furthermore, we found that SIV-infected SMs maintain comparable or lower frequencies of CCR6+CD161- and CCR6+CD161+ CD4+ T cells, respectively, in the mucosa (Figure 3.9C), which strengthens our model in which redistribution of CCR6+CD4+ T cells to the mucosa is ultimately damaging to the host. The damage that accompanies CCR6+CD161- and CCR6+CD161+ (CCR6+) CD4+ T cell accumulation in the mucosa may result from a number of mechanisms. First, recruiting CCR6+CD4+ T cells to the mucosa may increase the inflammation present there, as these cells are able to produce IFN-γ and TNF-α in addition to IL-17, thus creating a pro-inflammatory microenvironment that is able to promote viral replication and weaken intestinal structural integrity (220, 462). Alternatively, the accumulation of CCR6+CD4+ T cells could be a consequence of the high levels of inflammation present during pathogenic HIV/SIV infection, rather than the cause of it, since inflammation and microbial translocation increase CCL20 production by epithelial cells (451, 452). In addition to epithelial cells, CCR6+CD4+ T cells are also able to produce CCL20 (223), which attracts more CCR6+CD4+ viral targets to the gut mucosa as well as CCR6-expressing plasmacytoid dendritic cells (pDCs). These pDCs can subsequently recruit CCR5+ targets through their MIP-1a and MIP-1b production and produce high levels of Type-I IFNs (456). In fact, blocking CCL20 production using the inhibitor glycerol monolaurate was able to prevent SIV acquisition in vaginally-challenged RMs (456). Thus, recruiting and accumulating CCR6+CD4+ T cells to the gut mucosa may be damaging to the host by accelerating recruitment of viral targets, which then expands the pool of virally infected cells, and, invariably, the viral reservoir. Indeed, CCR6+CXCR3+ CD4+ T cells were recently shown to be enriched in integrated HIV DNA in long-term ART-suppressed HIV-infected individuals, when compared to other memory CD4+ T cell subsets (234); thus, accumulating CCR6+CD4+ T

cells in the mucosa can damage the host due both to their own susceptibility to infection but also to their ability to persist and sustain the viral reservoir.

Apart from its functional role in coordinating the migration of CD4+ T cells, CCR6 also is used to phenotypically identify Th17 cells, most often in combination with CCR4 and CD161 (441, 442, 445, 446). In our study, the accumulation of CCR6+CD161- and CCR6+CD161+ CD4+ (CCR6+CD4+) T cells in the gut mucosa of SIV-infected RMs did not correspond to an increase in mucosal Th17 cells (data not shown), likely due to a combination of factors. First, it is possible that environmental factors promoting the polarization of Th17 cells are not present in this setting. The differentiation of Th17 cells requires a combination of cytokines, including IL-6, transforming growth factor-β (TGF-β), and IL-1β, and later IL-21 and IL-23 for their expansion and survival (463, 464). Although some studies have demonstrated an upregulation of a number of Th17 polarization cytokines during HIV infection, including TGF-β, IL-6, and IL-23 (465, 466), other studies have found reduced production of IL-21 in the RB of SIV-infected RMs, which could impact the maintenance of CCR6+CD4+ T cells as Th17 cells in the mucosa (226). Second, even if CCR6+CD4+ T cells accumulated in the presence of Th17-polarizing cytokines, this subset may express lower levels of cytokine receptors on their surface, thus leaving them unable to receive the Th17-polarizing signals. While the expression levels of these receptors remains to be tested specifically on CCR6+CD4+ T cells, both IL-6R and TGF-βR levels are decreased on bulk CD4+ T cells during HIV and SIV infection, respectively (467, 468). Finally, an upregulation of negative regulatory genes, such as PIAS3, SHP2, and SOCS3, may suppress the expression of IL-17 in these accumulated CCR6+CD4+ T cells during SIV infection, as these genes are upregulated in bulk CD4+ T cells from SIV-infected RMs (469). Understanding how these mechanisms coordinate specifically within mucosal CCR6+CD4+ T cells to suppress Th17 expression will be an important future question in elucidating their role in disease progression.

Along with identifying the redistribution of CCR6+CD161- and CCR6+CD161+ CD4+ T cells to the gut mucosa as a main mechanism for their loss from the blood, we found that CD161+CCR6- CD4+ T cells were depleted from all tissue compartments during SIV infection and harbored significantly higher levels of viral RNA. These results underline the heightened susceptibility of CD161+CCR6- CD4+ T cells to SIV infection, which is likely facilitated by their high levels of CCR5 expression (Figure 3.2A, Figure 3.S3A). While high levels of CXCR3 expression on CD161+CCR6- CD4+ T cells (Figure 3.S4B) favor a Th1 phenotype and agree with their increased IFN-γ production, their CXCR3 expression may also suggest their contribution to a subset of germinal center TFH cells that are preferentially infected in LN, given their high levels of cell-associated SIV RNA (Figure 3.7C) (458). Indeed, higher levels of SIV RNA with comparable levels of SIV DNA would suggest an increased transcriptional activity of CD161+CCR6- CD4+ T cells in the LN, as has been shown in TFH from HIV-infected persons (278). We also found a loss of CD161+CD8+ T cells in the blood of SIV-infected RMs (Figure 3.3C). Circulating CD161+CD8+ MAIT cells are similarly decreased from the blood of HIV-infected individuals, where their depletion has been proposed to result from increased activation-induced cell death following interaction with microbial products in mucosal sites (454).

The preservation of CD161+CCR6- CD4+ T cells in the blood of SIV-infected natural host SMs supports a model where the infection and loss of these cells promotes disease progression. Indeed, elite controllers (ECs) also maintain their levels of circulating CD161+CD4+ T cells (470). Preservation of CD161+CCR6- CD4+ T cells in these two models is still compatible with viral infection being a key contributor to the depletion of this cell subset during progressive HIV/SIV infection. The ability of SMs to preserve CD161+CCR6- CD4+ T cells despite high levels of plasma viremia may result from their markedly lower levels of CCR5 expression on CD4+ T cells as compared to RMs (161, 179), while preservation of CD161+ CD4+ T cells in ECs may result from their significantly lower levels of HIV viremia when compared to normal progressors (102).

In conclusion, CCR6 and CD161-expressing CD4+ T cells are preferentially lost from the blood during pathogenic SIV infection in RMs, but their loss disrupts immune homeostasis by distinct mechanisms. Increased levels of the SIV co-receptor CCR5 likely contribute to the enriched viral targeting of CD161+CCR6- CD4+ T cells and their eventual depletion from multiple tissue compartments during chronic infection. On the other hand, CCR6+CD161- and CCR6+CD161+ CD4+ T cells traffic from the blood to the gut mucosa during SIV infection, migrating towards increased levels of CCL20 produced there. Therefore, while the loss of CCR6+CD4+ T cells from circulation is indicative of disease progression, their accumulation in the gut mucosa further promotes damage to the host, as evidenced by their maintained levels in the mucosa of SIV-infected SMs. Moreover, ART is only able to partially reverse the redistribution and cell loss of these CD4+ T cell subsets incurred during active viral replication, both in the blood of humans (223) and in tissues here, thus underscoring the need for additional therapies that can restore CCR6 and CD161-expressing CD4+ T cell homeostasis throughout the body and halt disease progression.

Figures

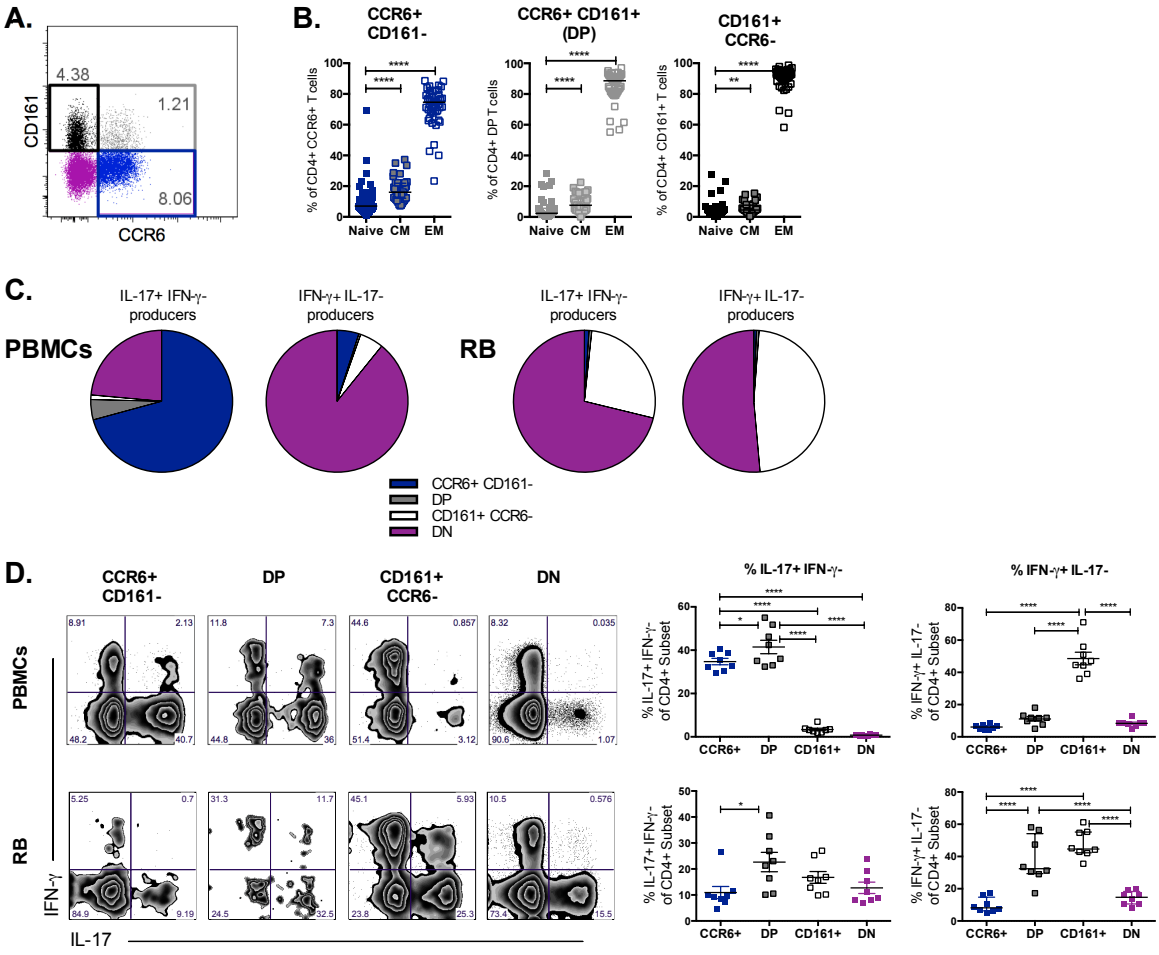

Figure 3.1. CCR6+ and CD161+ CD4+ T cells contribute to IL-17 production in the blood and tissues. (A) Representative flow plot for the measurement of CD4+ T cells by their CCR6 and CD161 expression is shown from the blood of an uninfected rhesus macaque (RM). (B) Memory phenotypes were evaluated within CCR6 and CD161-expressing subsets in 42 healthy RMs using the following definitions: naïve (CD28+CD95-), central memory (CM; CD95+CD62L+), and effector memory (EM; CD95+CD62L-). The frequencies of naïve and memory subsets are shown as a percentage of the CD4+ T cell subsets. Medians are indicated by the horizontal bars on each graph (±IQR). **, $p<0.01$; ****, $p<0.0001$, as determined by Mann-Whitney U tests. (C) PBMCs or lymphocytes isolated from RB of 8 healthy RMs were stimulated for 4 hours with PMA/Ionomycin, containing GolgiStop and Brefeldin A, in order to quantify

their production of IL-17 and IFN-γ. The average contribution by each CCR6 and CD161-expressing CD4+ T cell subset to IL-17 and IFN-γ production within the blood and RB are represented by the pie charts. (D) Representative flow plots are shown for the levels of IL-17 and IFN-γ production produced by each CCR6 and CD161-expressing subset following 4 hour PMA/Ionomycin stimulation (n=8). The frequencies of IL-17+IFN-γ- as well as IFN-γ+IL-17-producing cells within these subsets are shown for 8 uninfected RMs. Averaged data are presented as the mean±SEM, and ANOVAs using Tukey's adjustment for multiple comparisons were used to compare differences between subsets. *, $p<0.05$; ****, $p<0.0001$.

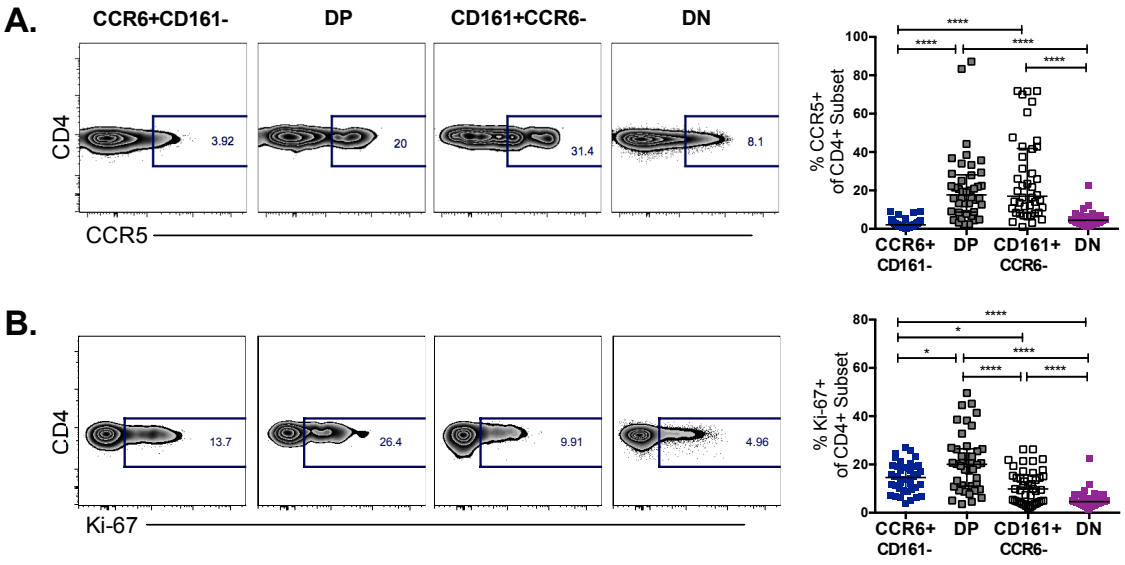

Figure 3.2. CCR6+ and CD161+ CD4+ T cells are susceptible to SIV infection. Representative flow plots of CCR5 (A) and Ki-67 (B) expression within CCR6 and CD161-expressing CD4+ T cells are shown from the blood of an individual uninfected RM. The frequencies of CCR5+ (A) and Ki-67+ cells (B) within these subsets are shown for 42 uninfected RMs. Medians are indicated by the horizontal bars on each graph (±IQR). *, $p<0.05$; **, $p<0.01$; ****, $p<0.0001$, as determined by Mann-Whitney U tests.

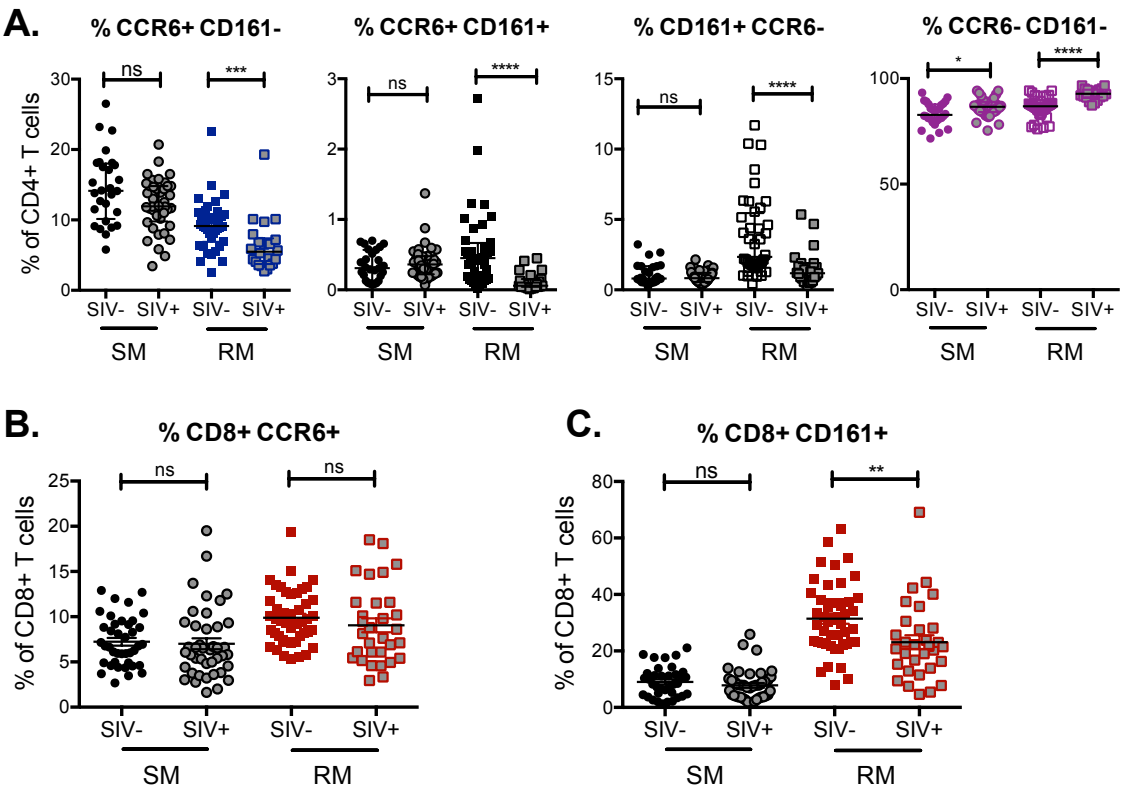

Figure 3.3. Only pathogenic SIV infection of RMs results in a significant depletion of peripheral CCR6+ and CD161+ CD4+ T cells. (A) Frequencies of CCR6 and CD161-expressing CD4+ T cell subsets were measured in whole blood and compared between uninfected and SIV-infected sooty mangabeys (SMs) and RMs. In the SIV-infected animals, the average lengths of infection were 8.91 ± 0.90 years for SMs, and 1.02 ± 0.12 years for RMs (see Methods). Medians are indicated by the horizontal bars on each graph (±IQR). *, $p<0.05$; ***, $p<0.001$; ****, $p<0.0001$, as determined by Mann-Whitney U tests. The frequencies of CD8+ T cells expressing CCR6 (B) and CD161 (C) were compared between uninfected and SIV-infected SMs and RMs. Sample means are indicated by the horizontal bars (±SEM), and Mann-Whitney U tests were used to compare frequencies. **, $p<0.01$.

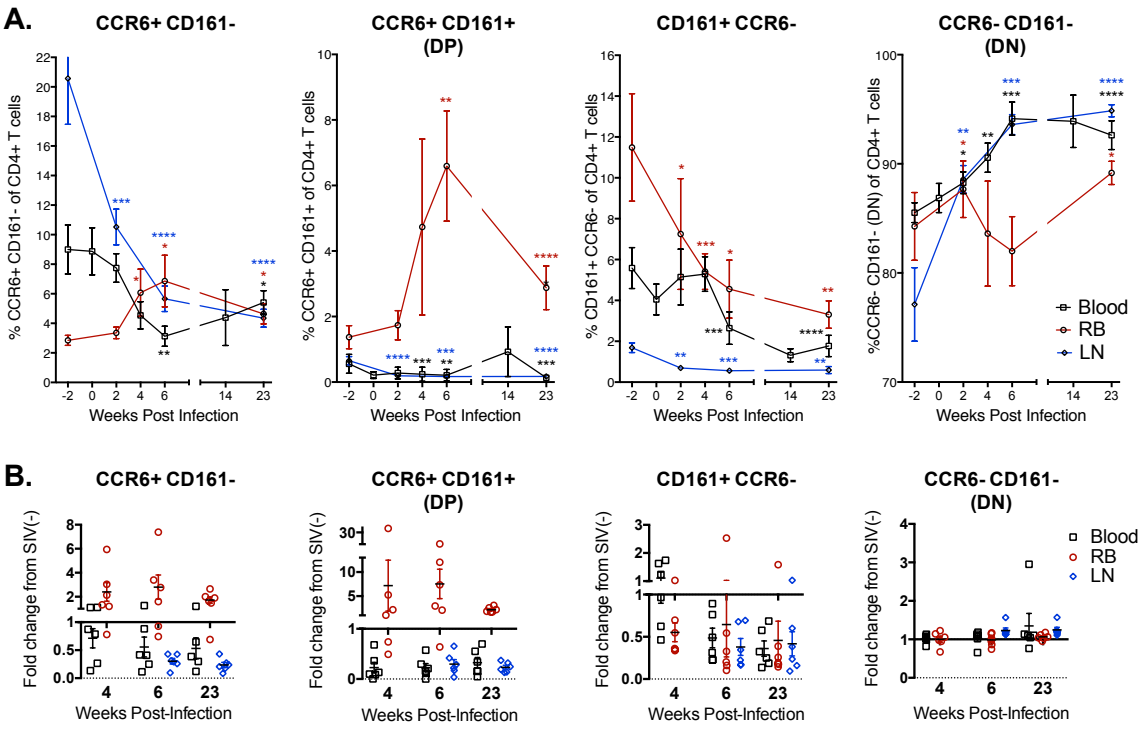

Figure 3.4. CCR6+CD4+ T cells accumulate in the rectal mucosa during SIV infection, but CD161+CCR6- CD4+ T cells are depleted in all tissues. (A) The frequencies of CCR6 and CD161-expressing T cells, expressed as a percentage of live CD4+ T cells, were measured longitudinally in the blood, rectal mucosa (RB), and lymph nodes (LN) during the course of SIV infection for 6 RMs. Data are represented as mean ± SEM. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$, as determined by repeated-measures analyses. (B) Longitudinal assessment of the fold change of CCR6 and CD161-expressing CD4+ T cell subsets relative to their percentages of CD4+ T cells prior to infection (Week -2) within the blood, RB, and LN. Sample means are indicated by the horizontal bars (±SEM).

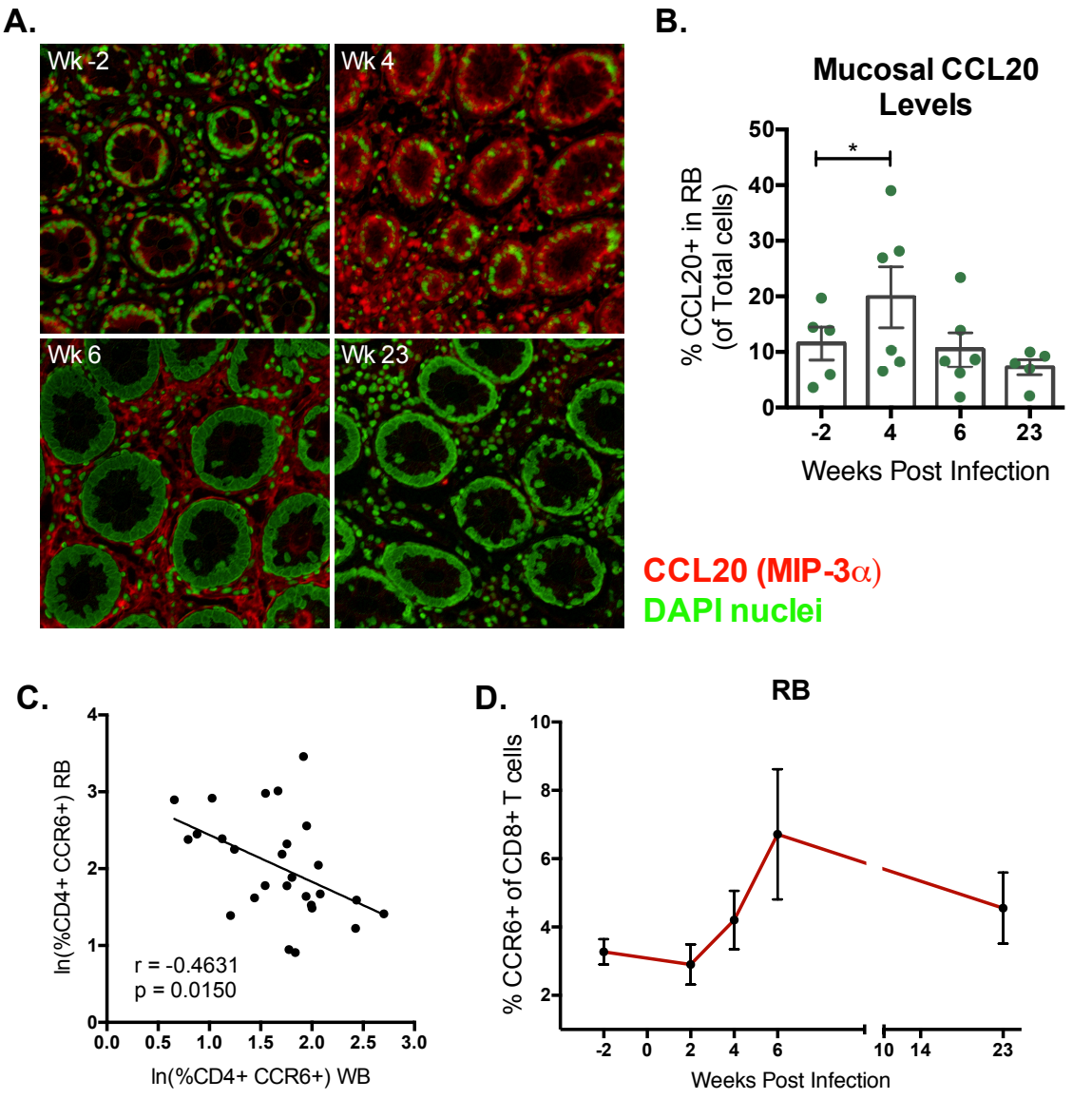

Figure 3.5. Increases in mucosal CCL20 production correspond to an accumulation of CCR6+ T cells during SIV infection. (A) Fixed rectal mucosal tissues underwent in situ hybridization for CCL20 (MIP-3α, red) and counterstaining for nuclei (DAPI, green) longitudinally during SIV infection. Representative images (40x) of each time point are shown from an individual RM (RLz8)- upper row, Weeks -2 and 4; bottom row, Weeks 6 and 23. (B) Quantitative image analysis demonstrating the fraction of total cells in the rectal mucosa that express CCL20 at 4 separate time points before and during SIV infection. Sample means are indicated by the horizontal bars (±SEM), and Mann-Whitney U tests were used to compare

percentages. *, $p<0.05$. (C) CCR6+CD4+ T cell frequencies from WB and RB were ln transformed (log base e) prior to analysis by a mixed-effects model. The estimated rate of linear decline for CCR6+CD4+ T cells in the RB was statistically different from zero ($p=0.0150$), indicating a linear relationship between ln(%CCR6+CD4+) in RB and ln(%CCR6+CD4+) in WB. (D) The frequencies of CCR6+ T cells, expressed as a percentage of live CD8+ T cells, were measured longitudinally in the rectal mucosa (RB) during the course of SIV infection for 6 RMs. Data are represented as mean ± SEM.

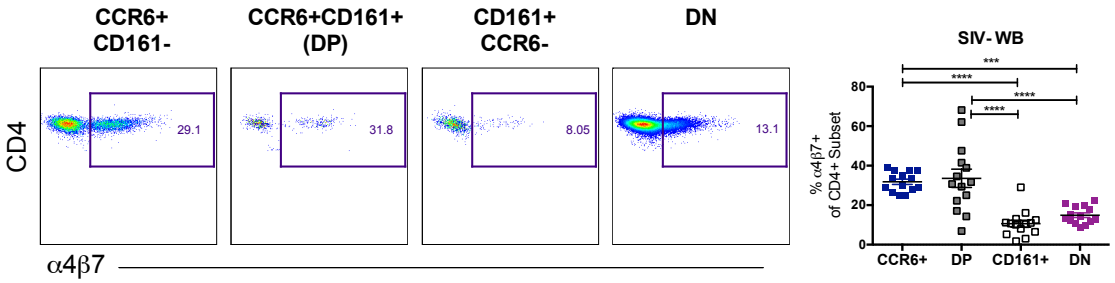

Figure 3.6. CCR6+ CD4+ T cells express heightened levels of α4β7. Representative flow plots of α4β7 expression within CCR6 and CD161-expressing CD4+ T cells are shown from the blood of an individual uninfected RM. The frequencies of α4β7+ cells within these subsets are shown for 14 uninfected RMs. Averaged data are presented as the mean ± SEM, and ANOVAs using Tukey's adjustment for multiple comparisons were used to compare differences between subsets. ***, $p<0.001$; ****, $p<0.0001$.

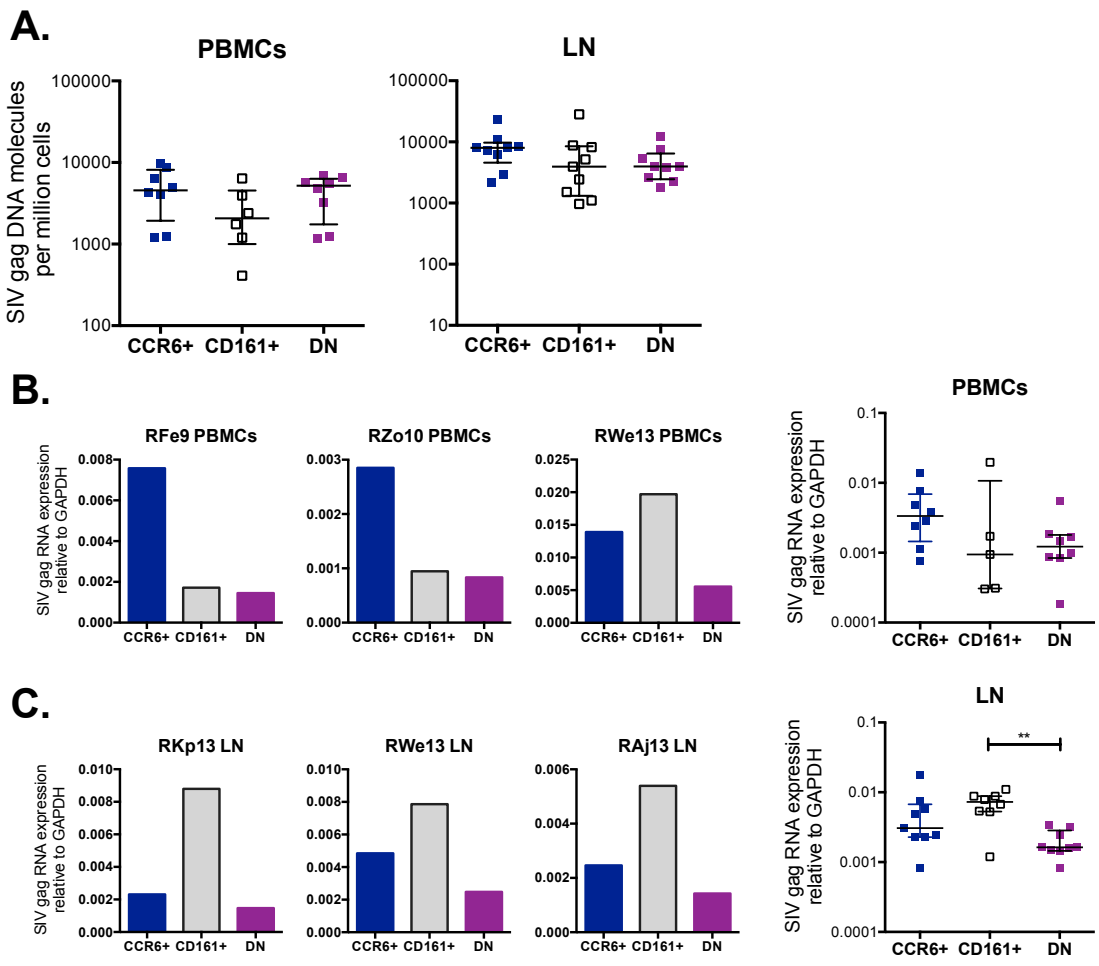

Figure 3.7. CD161+CCR6- CD4+ T cells harbor the highest levels of SIV RNA in lymphoid tissues. (A) Levels of cell-associated SIV*gag* DNA were quantified from CCR6 and CD161-sorted subsets in the blood and LN of 10 SIV-infected RMs. Representative SIV RNA levels in the PBMCs (B) and LN (C) for 3 RMs during chronic SIV infection. Cumulative data for SIV RNA levels in the CCR6 and CD161 memory CD4+ T cell subsets is shown in the scatter plots. DNA and RNA measurements were excluded for samples in which <15,000 cells were sorted, due to assay sensitivity limitations; two additional data points were excluded due to technical error. Medians are indicated by the horizontal bars on each graph (±IQR). **, $p<0.01$, as determined by Mann-Whitney U tests.

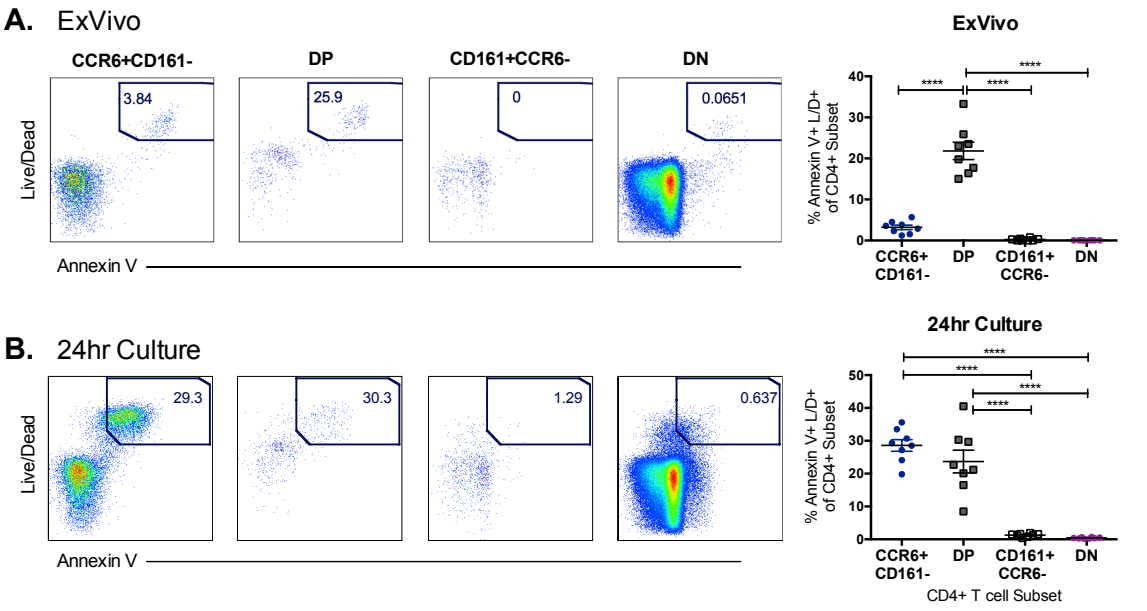

Figure 3.8. CCR6+CD161- and CCR6+CD161+ CD4+ T cells are more susceptible to cell death than CD4+ T cells not expressing CCR6. (A) PBMCs from 8 healthy RMs were stained ex vivo with Annexin V and Live/Dead; a representative flow plot within each of the CCR6 and CD161-expressing CD4+ T cell subsets is shown. The frequencies of AnnexinV+Live/Dead+ (Dead) cells within these subsets are shown for the 8 uninfected RMs. (B) Isolated PBMCs were cultured for 24 hours in the presence of complete RPMI media alone, prior to staining for cell death; representative flow plots within each of the CCR6 and CD161-expressing CD4+ T cell subsets are shown. The frequencies of AnnexinV+Live/Dead+ cells within these subsets are shown for the 8 uninfected RMs. Averaged data are presented as the mean ± SEM, and ANOVAs using Tukey's adjustment for multiple comparisons were used to compare differences between subsets. ****, $p<0.0001$.

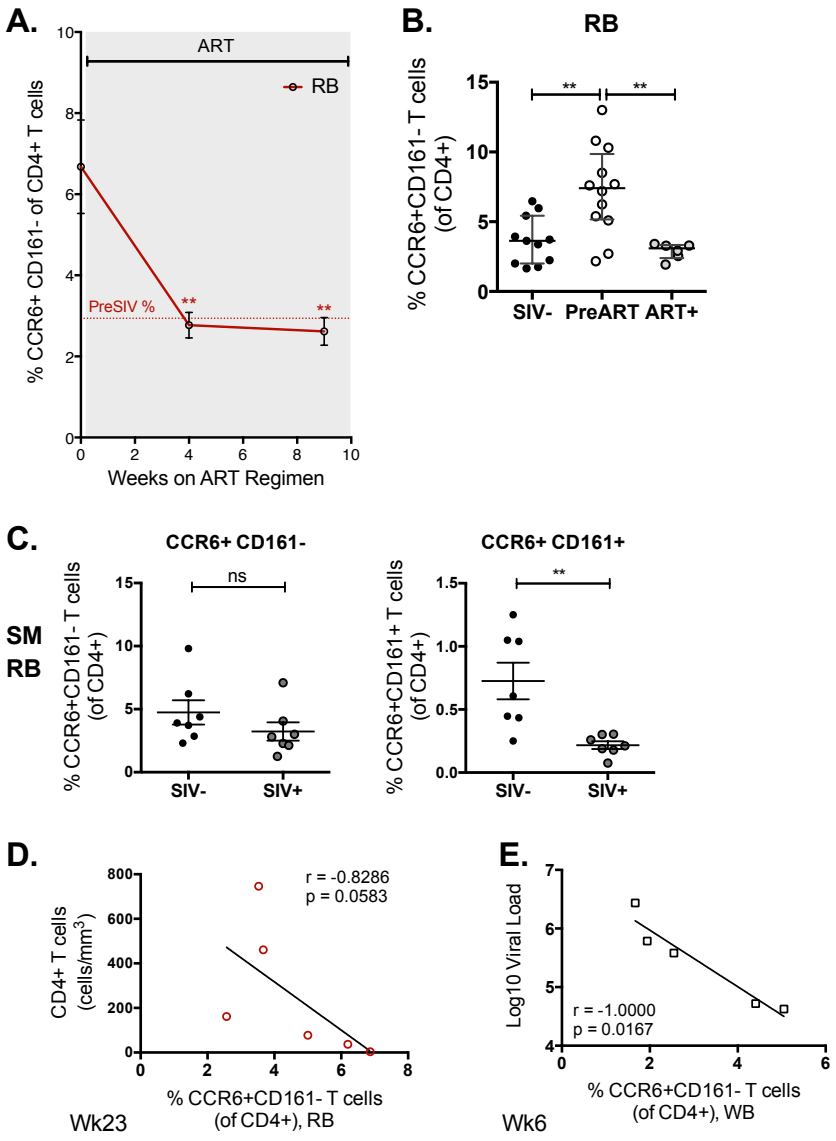

Figure 3.9. Increased CCR6+CD161- CD4+ T cell levels in the mucosa are associated with SIV disease progression. (A) The frequencies of CCR6+CD161- T cells, expressed as a percentage of live CD4+ T cells, were measured longitudinally in RB during ART administration in 6 SIV-infected RMs. Data are represented as mean ± SEM. **, $p<0.01$, as determined by repeated-measures analyses. The shaded area represents ART; the dotted red line indicates the baseline levels of CCR6+CD161- CD4+ T cells in the RB of SIV-uninfected historical RM controls. (B) Cross-sectional analysis of CCR6+CD161+ CD4+ T cells in RB of uninfected (n=12), untreated (PreART; n=14), and ART-suppressed (n=8) SIV-infected RMs. Medians are

indicated by the horizontal bars on each graph (±IQR). *, $p<0.05$; **, $p<0.01$, as determined by Mann-Whitney U tests. (C) Frequencies of CCR6+CD161- and CCR6+CD161+ CD4+ T cell subsets were measured in RB and compared between uninfected and SIV-infected sooty mangabeys (SMs). Sample means are indicated by the horizontal bars (±SEM). **, $p<0.01$, as determined by unpaired *t* tests. (D) The correlation between the percentage of mucosal CCR6+CD161- CD4+ T cells and absolute CD4+ T cell counts are shown for SIV-infected RMs (n=6) at week 23 post-infection. (E) The correlation between the percentage of CCR6+CD161- CD4+ T cells in the blood and viral load, expressed following log transformation, are shown for SIV-infected RMs (n=6) at week 6 post-infection. Statistical analyses were performed using Spearman rank correlation tests.

CD4+ CCR6+CD161- rate of change *(per year of SIV infection)*	
SIV+ RMs	**SIV+ SMs**
-2.97%	+0.12%

Table 3.S1. CCR6+CD161- CD4+ T cells are preserved in naturally SIV-infected SMs compared to SIV-infected RMs. The fraction of CCR6+CD161- CD4+ T cells in the blood was related to the duration of SIV infection at the time of sampling for RMs (n=18) and SMs (n=40) in order to estimate a linear rate of change of this cell subset per year of SIV infection. Slopes were determined by linear regression.

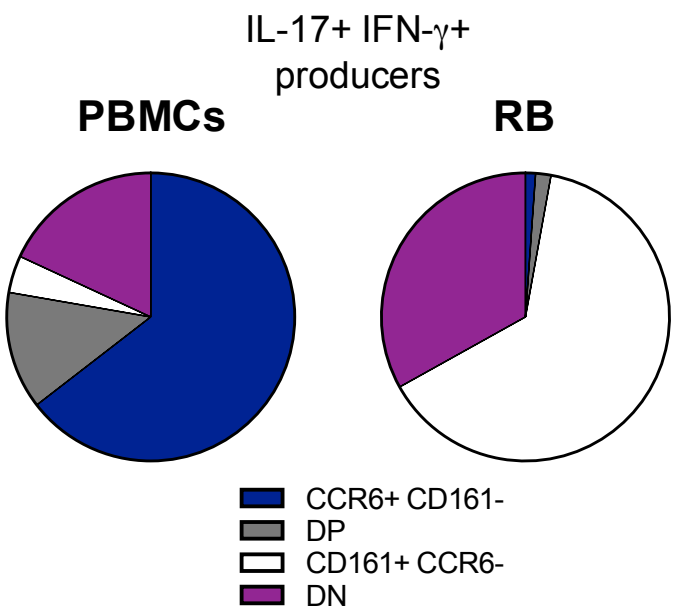

Figure 3.S1. The contributions by CD4+ T cell subsets to dual IL-17 and IFN-γ production vary by anatomic location. PBMCs or lymphocytes isolated from RB (RBMCs) of 8 SIV-uninfected RMs were stimulated for 4 hours with PMA/Ionomycin, containing GolgiStop and Brefeldin A, in order to quantify their co-production of IL-17 and IFN-γ. The average contribution by each CCR6 and CD161-expressing CD4+ T cell subset to co-IL-17 and IFN-γ production within the blood and RB are represented by the pie charts.

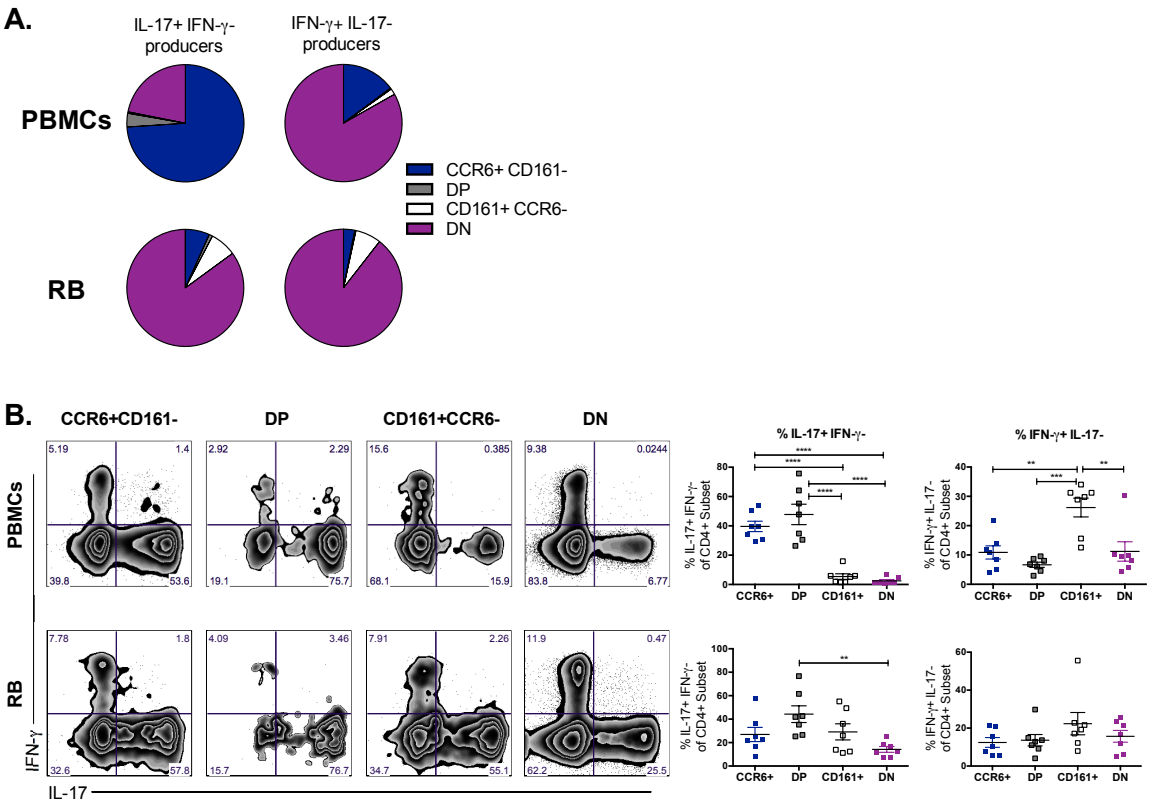

Figure 3.S2. CCR6+ and CD161+ CD4+ T cells in SMs are functionally comparable to their counterparts in RMs. (A) PBMCs or RBMCs of 7 SIV-uninfected SMs were stimulated for 4 hours with PMA/Ionomycin, containing GolgiStop and Brefeldin A, in order to quantify their production of IL-17 and IFN-γ. The average contribution by each CCR6 and CD161-expressing CD4+ T cell subset to IL-17+(IFN-γ-) and IFN-γ+(IL-17-) production within the blood and RB are represented by the pie charts. (B) Representative flow plots are shown for the levels of IL-17 and IFN-γ production produced by each CCR6 and CD161-expressing subset following 4 hour PMA/Ionomycin stimulation (n=7). The frequencies of IL-17+IFN-γ- as well as IFN-γ+IL-17-producing cells within these subsets are shown for 7 SIV-uninfected SMs. Averaged data are presented as the mean ± SEM, and ANOVAs using Tukey's adjustment for multiple comparisons were used to compare differences between subsets. **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

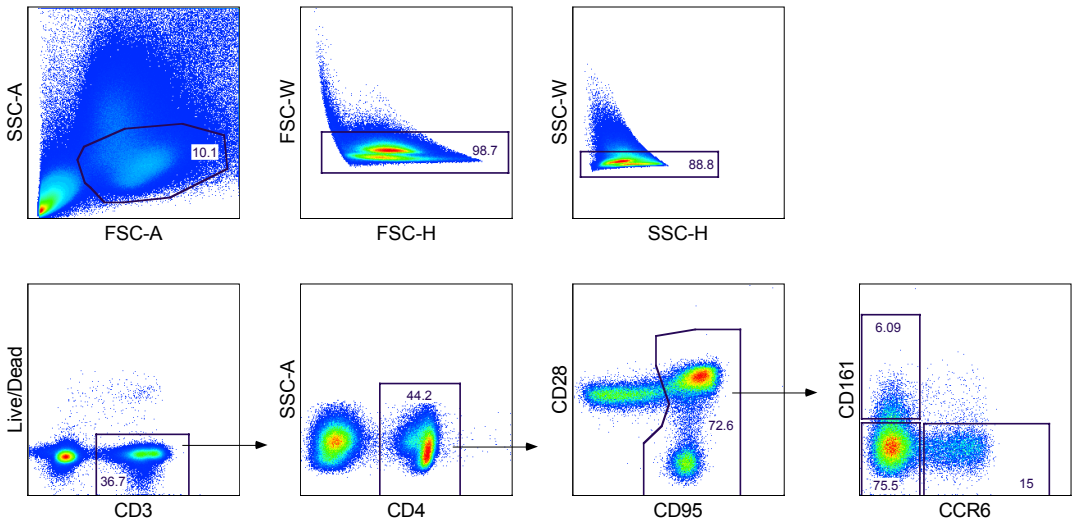

Figure 3.S3. Gating strategy for sorted CCR6+ and CD161+ memory CD4+ T cell subsets. Lymphocytes were isolated from the blood and LN of chronically SIV-infected RMs at necropsy, and memory CD4+ T cell subsets (CD95+) were sorted on the basis of CCR6 and CD161 expression using a FACS AriaII (BD Biosciences). PBMC staining from a representative SIV-infected RM is shown.

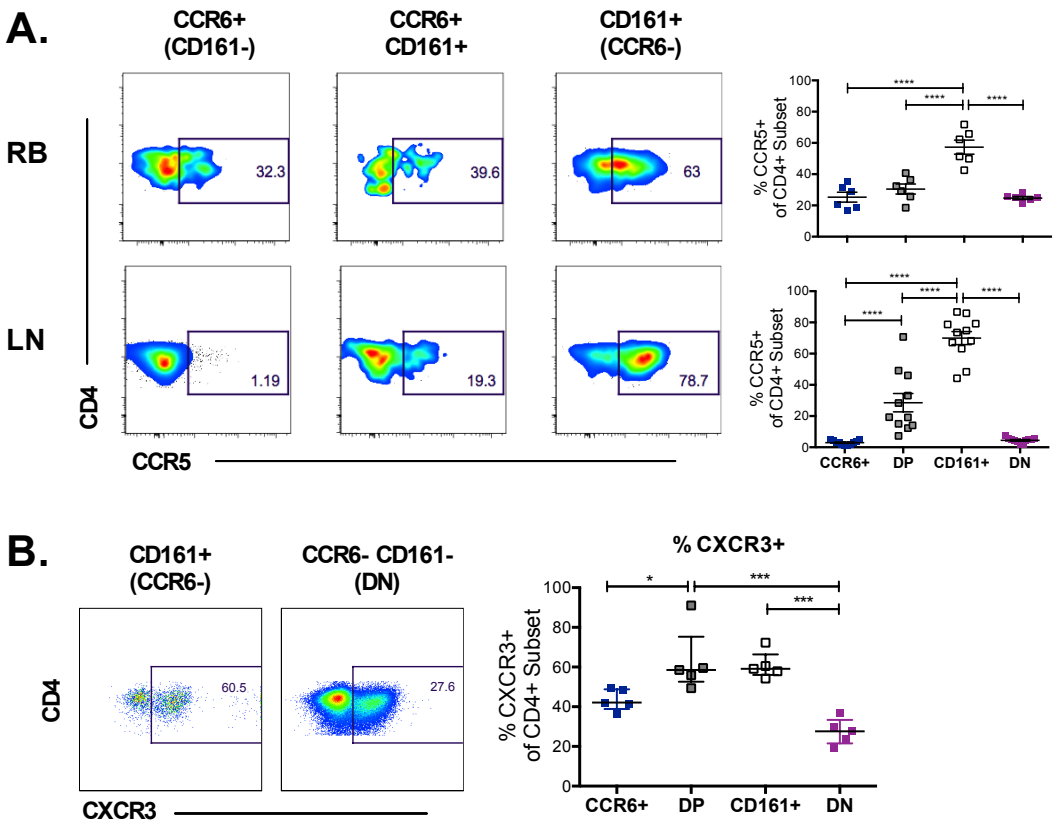

Figure 3.S4. CD4+ T cells expressing CD161, with or without CCR6, co-express high levels of the SIV co-receptor CCR5 in tissues. (A) Representative flow plots of CCR5 expression on CCR6+CD161-, CCR6+CD161+, and CD161+CCR6- CD4+ T cells in RB and LN of a healthy RM are shown. The frequencies of CCR5+ cells within the CCR6 and CD161-expressing subsets are shown for 6 (RB) and 12 (LN) uninfected RMs. Sample means are indicated by the horizontal bars (±SEM). (B) Representative flow plots of CXCR3 expression within CD161+CCR6- and CCR6-CD161- (DN) CD4+ T cells are shown from the LN of an individual ART-treated, SIV-infected RM. The frequencies of CXCR3+ cells within the CCR6 and CD161-expressing subsets are shown for 5 ART-treated, SIV-infected RMs. Medians are indicated by the horizontal bars on each graph (±IQR). *, $p<0.05$; ***, $p<0.001$; ****, $p<0.0001$, as determined by ANOVA using Tukey's adjustment for multiple comparisons.

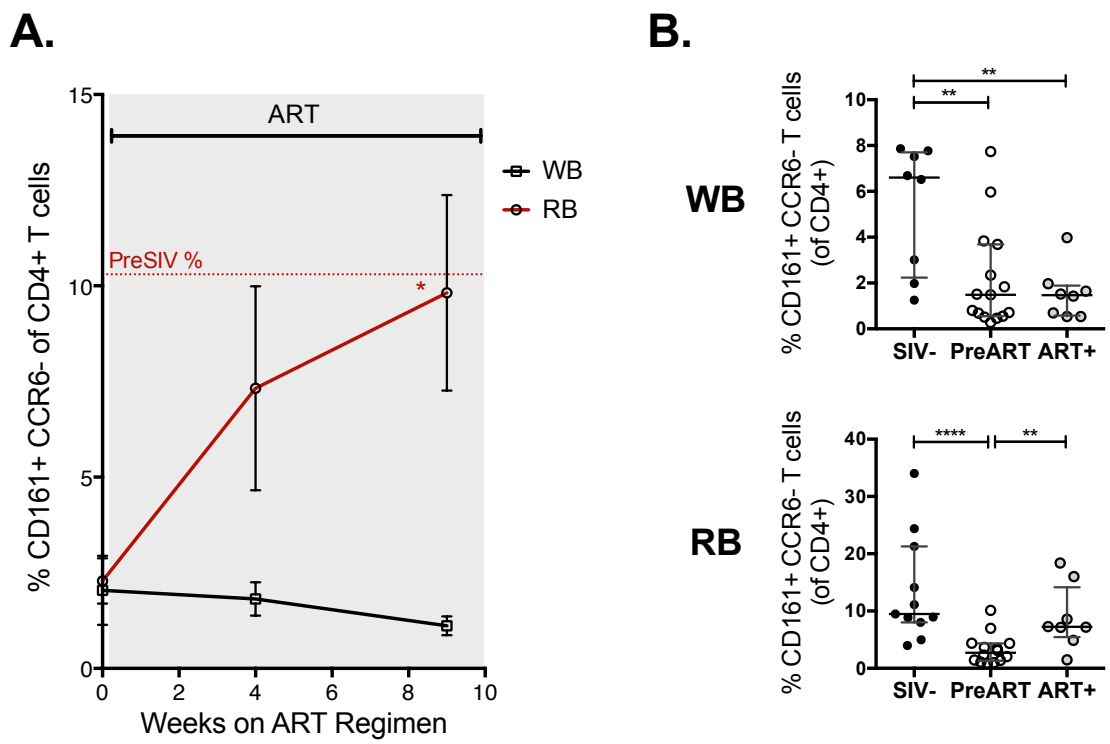

Figure 3.S5. ART restores CD161+CCR6- CD4+ T cell levels in the mucosa, but is ineffective in restoring frequencies to pre-infection levels in the blood. (A) The frequencies of CD161+CCR6- T cells, expressed as a percentage of live CD4+ T cells, were measured longitudinally in the blood (WB, black squares) and RB (red circles) during ART administration in 6 SIV-infected RMs. Data are represented as mean ± SEM. The shaded area represents ART; the dotted red line indicates the baseline levels of CD161+CCR6- CD4+ T cells in the RB of SIV-uninfected historical RM controls. *, $p<0.05$, as determined by repeated-measures analyses. (B) Cross-sectional analysis of CD161+CCR6- CD4+ T cells in WB and RB of uninfected (n=12), untreated (n=14), and ART-suppressed (n=8) SIV-infected RMs. Medians are indicated by the horizontal bars on each graph (±IQR). **, $p<0.01$; ****, $p<0.0001$, as determined by Mann-Whitney U tests.

Chapter Four

CTLA-4 and PD-1-expressing CD4+ T-cells are key contributors to viral persistence in ART-suppressed SIV-infected rhesus macaques*

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Abstract

Antiretroviral therapy (ART) suppresses HIV plasma viremia, but is unable to eliminate the latent viral reservoir from HIV-infected individuals. Defining the cellular and anatomic localization of persistently infected cells is critical for designing HIV cure strategies. Using SIV-infected rhesus macaques, we show that CD4+ T-cells expressing CTLA-4 and/or PD-1 contribute nearly 90% of SIV-DNA content after ART-mediated viral suppression in multiple tissues, with the highest contribution found in the lymph nodes (LN). Furthermore, our findings indicate that during ART, CTLA-4+PD-1- CD4+ T-cells, which phenotypically overlap with Tregs, are infected at significantly higher frequencies than other CD4+ T-cell subsets in multiple tissues, contain replication-competent virus, and persist outside the LN B-cell follicle. Remarkably, the contribution of CTLA-4+PD-1- cells to SIV DNA in the LN increases with ART and both their frequencies and viral seeding predict SIV persistence on-ART. Therefore, efforts targeting both CTLA-4 and PD-1 hold the potential to affect the large majority of latently infected cells.

Introduction

The ability of antiretroviral therapy (ART) to effectively suppress HIV-1 replication has been paramount in reducing HIV morbidity and mortality (300, 301). Despite this success, HIV-infected individuals must remain on ART for their lifetime due to the persistence of latently infected cells containing transcriptionally silent, integrated provirus, which allows them to evade immune detection (357, 358, 471-473). A small fraction of these latently infected cells contain proviruses that are replication competent, constituting the latent viral reservoir that is responsible for the rebound of viremia upon treatment interruption; this reservoir represents the main barrier to eradicating HIV from individuals (323, 474). Even the initiation of ART during acute infection is unable to prevent the establishment and elimination of the viral reservoir, due to the rapid seeding of cellular reservoirs during the earliest stages of infection (319, 366, 473). Therefore, strategies that target and eliminate latently infected cells are critically needed to achieve a functional cure for HIV.

Efforts to reduce the size of the HIV reservoir using "latency reversing agents" (LRAs) have not produced meaningful reductions in the viral reservoir nor have they resulted in antiretroviral-free HIV remission (475). One limitation of these LRAs may be their non-specific targeting of the latent reservoir (476), since the frequency of latently infected cells is approximately 1 per one million CD4+ T-cells (358, 359). Determining cellular subsets that preferentially harbor proviral DNA, then, may facilitate the specific targeting of latent reservoirs. Resting memory CD4+ T-cells are a well-characterized cellular reservoir, with numerous data suggesting the enrichment of proviral DNA within central, transitional, and, more recently, stem cell memory cells (199, 202, 477). Yet, these subsets of memory CD4+ T-cells are highly heterogeneous in their expression of surface markers, thus necessitating the identification of additional markers that more strictly define latently infected cells.

Co-inhibitory receptors (Co-IRs) were initially studied on antiviral CD8+ T-cells in chronic HIV infection, where their increased expression was associated with increased levels of CD8+ T-cell functional impairment, HIV viral load and declining CD4+ T-cell count (126, 143, 144, 478). Further analysis revealed that virus-specific CD4+ T-cells similarly upregulate multiple Co-IRs, including programmed death-1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and T-cell Ig domain and mucin domain 3 (TIM-3), in the setting of HIV and SIV infection (124, 125, 143, 479). Upregulation of these receptors occurs soon after antigenic stimulation as part of a negative feedback loop that prevents further activation and proliferation of T-cells and leads to T-cell quiescence. Thus, the expression of Co-IRs on memory CD4+ T-cells could contribute to the persistence of the latent viral reservoir by hindering the activation of virally infected T-cells. In agreement with this, PD-1 was shown to identify memory CD4+ T-cells enriched in HIV proviral DNA from long-term ART-suppressed individuals (202, 278). Moreover, PD-1 and CTLA-4 also distinguish functional CD4+ T-cell subsets, with PD-1 characterizing follicular helper T-cells (TFH), and CTLA-4 constitutively expressed on regulatory T-cells (Tregs) (267, 480-482). Given the ability of these receptors to maintain cells in a quiescent state and the additive effects of their co-expression (479), we hypothesized that memory CD4+ T-cells expressing Co-IRs would harbor high levels of proviral DNA.

Here, using ART-treated, SIV-infected rhesus macaques (RMs), we were able to identify Co-IRs that mark persistently infected memory CD4+ T-cells in multiple tissue compartments. Our results indicate, for the first time, that CTLA-4 and PD-1-expressing T-cells are the major contributors to the SIV DNA pool within memory CD4+ T-cells after ART-mediated viral suppression in the blood, lymph nodes (LN), spleen, and gut, and that these cells overlap phenotypically and functionally with TFH cells. Our findings also highlight the previously unappreciated viral enrichment of CTLA-4+PD-1- memory CD4+ T-cells following ART-mediated suppression, a subset comprised predominantly of Tregs that persist outside the B-cell

follicle. Additionally, the frequency and viral seeding of these CTLA-4+PD-1- memory CD4+ T cells predicts SIV persistence on ART, a feature specific to this Treg-like subset. As such, efforts that target both CTLA-4 and PD-1 hold the potential to affect a majority of latently infected cells.

Materials and Methods

Animals, SIV-infection and antiretroviral therapy

Ten female Indian rhesus macaques (RMs; *Macaca mulatta)*, all housed at the Yerkes National Primate Research Center, Atlanta, GA, were included in this study. All RMs were infected intravenously with 1000 TCID50 of SIVmac251 (Figure 4.1A). Approximately 7 weeks post-infection, all animals initiated an antiretroviral therapy (ART) regimen consisting of tenofovir (PMPA; 20-25 mg/kg/d, s.c.), emtricitabine (FTC; 30-50 mg/kg/d, s.c.), raltegravir (100-150 mg/bid, oral), darunavir (400-700 mg/bid, oral), and ritonavir (50 mg/bid, oral), which was maintained daily for the duration of the study (Table 4.1). To enhance control of viral load, this regimen was intensified with the addition of maraviroc (100-150 mg/bid, oral) 21-24 weeks after ART initiation in 7 RMs. Animals remained on ART until plasma viremia was undetectable (limit of detection: 60 copies viral RNA/mL) for at least 3 months (5.0±1.4 months from first undetectable plasma viral load). Following persistent viral suppression, animals underwent elective necropsy, with the exception of one RM who exhibited rapid disease progression and had to be sacrificed following ten days of ART (RKa13; Table 4.1). Longitudinal analysis accounts for all RMs, while cell-associated DNA and RNA quantitation includes only the 9 animals which reached study completion.

Study Approval

All animal experimentations were conducted following guidelines established by the Animal Welfare Act and the NIH for housing and care of laboratory animals and performed in accordance with Institutional regulations after review and approval by the Institutional Animal Care and Usage Committees (IACUC; Permit number: 2002089) at the Yerkes National Primate Research Center (YNPRC). Anesthesia was administered prior to performing any procedure, and proper steps were taken to minimize the suffering of the animals in this study.

Sample collection and processing

Blood, lymph node (LN) and rectal biopsies (Gut) were performed longitudinally and at necropsy. Blood samples were used for a complete blood count and routine chemical analysis, and plasma separated by centrifugation within 1 hr of phlebotomy. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density gradient centrifugation. To obtain rectal biopsy punches, an anoscope was placed a short distance into the rectum for the collection of up to 20 pinch biopsies with biopsy forceps. Gut-derived lymphocytes were digested with 1 mg/mL collagenase for 2 hours at 37°C, and then passed through a 70-um cell strainer to remove residual tissue fragments. Intestinal samples, which included segments of the rectum, colon, ileum, and jejunum, were collected from necropsied animals into RPMI 1640 medium supplemented with 10%FBS, 100 IU/mL penicillin, and 100 ug/mL streptomycin. Intestinal samples were cut into small pieces, and digested with 1 mg/mL collagenase by shaking for 2 hr at 37°C. To enrich for lymphocytes, filtered samples were layered over a discontinuous Percoll density gradient (GE Life Sciences), centrifuged and washed in PBS prior to staining and/or sorting. For LN biopsies, the skin over the axillary or inguinal region was clipped and surgically prepped. An incision was then made in the skin over the LN, which was exposed by blunt dissection and excised over clamps. LNs were then homogenized and passed through a 70-um cell strainer to isolate lymphocytes. All samples were processed, fixed (1% paraformaldehyde), and analyzed within 24 hours of collection.

Flow cytometric analysis

Fourteen-parameter flow cytometric analysis was performed on peripheral blood, LN, and gut-derived cells according to standard procedures using a panel of monoclonal antibodies that we and others have shown to be cross-reactive with RMs (227). The following antibodies were used at predetermined optimal concentrations: anti-CD3-APC-Cy7 (clone SP34-2), anti-Ki-67-Alexa700 (clone B56), anti-CD95-PE-Cy5 (clone DX2), anti-CCR7-PE-Cy7 (clone 3D12), anti-

CTLA-4-BV421 (clone BNI3), all from BD Biosciences; anti-CD4-BV605 (clone OKT4), anti-PD-1-PE-Cy7 (clone EH12.2H7), anti-CD95-BV605 (clone DX2), anti-CD25-BV711 (clone BC96), and anti-FoxP3-APC (clone 150D) from Biolegend; anti-CD8-Qdot705 (clone 3B5) and Aqua Live/Dead amine dye-AmCyan from Invitrogen; anti-CD28-ECD (clone CD28.2) from Beckman Coulter; anti-CD127-PECy5 (clone eBioRDR5) and anti-CXCR5-PerCp-Cy5.5 (clone MU5UBEE) from eBioscience; and anti-TIM-3-PE (clone344823) from R&D. To detect the expression of CTLA-4 on the cell surface with improved sensitivity, mononuclear cells were stimulated for 3 hours at 37°C with phorbol myristate acetate (PMA; 80 ng/mL) and ionomycin (500 ng/mL), and CTLA-4 antibody was added to the stimulation media at the start of stimulation. To detect the expression of FoxP3 intracellularly, mononuclear cells were fixed and permeabilized with FoxP3 Fix/Perm solution (Tonbo), and subsequently stained intracellularly for FoxP3. Flow cytometric acquisition was performed on at least 100,000 CD3+ T cells on an LSRII cytometer driven by the FACS DiVa software, or at least 10,000 CD3+ T cells for gut-derived cells. The data acquired were analyzed using FlowJo software (version 9.8.5; TreeStar).

Intracellular cytokine staining

The production of IL-21 by Co-IR-expressing subsets was determined by examining the frequency of memory CD4+ T-cells that produced IL-21 following a 3 hour *in vitro* stimulation with PMA and ionomycin (see above). PBMCs and LN-derived cells, isolated as described above, were resuspended to $3x10^6$ cells/mL in complete RPMI 1640 media containing PMA, ionomycin, Brefeldin A, and BD Golgistop. Cells were then incubated at 37°C for 3 hours. Stimulation was stopped by washing cells with PBS. Following a 30 minute surface stain, cells were fixed and permeabilized prior to staining intracellularly with anti-IL-21-PE (clone 3A3-N2.1; BD Biosciences) for 45 minutes at room temperature. Following staining, cells were washed and fixed in PBS containing 1% paraformaldehyde (PFA), and acquired on a BD LSRII cytometer.

FACS cell sorting

Mononuclear cells isolated from blood, LN, spleen, and pooled gut mucosal tissues (including right and left colon, ileum, jejunum, and rectum) were stimulated for 3 hours with PMA and Ionomycin, as described above, with anti-CTLA-4 antibody added with the stimulation media, and subsequently stained with anti-CD3, anti-CD4, anti-CD8, anti-CD28, anti-CD95, anti-PD-1, anti-CTLA-4 and anti-TIM-3. Memory CD4+ T-cells were then sorted based on their expression of PD-1, CTLA-4, and TIM-3 using a FACS AriaII (BD Biosciences). With the exception of gut mucosal tissues, memory CD4+ T-cells sorted at necropsy were first separated into PD-1 positive and negative populations. These subsets were then sorted into four subsets based on their expressions of CTLA-4 and TIM-3 (Figure 4.S2). Memory CD4+ T-cells sorted during the study, and from gut mucosal tissues at necropsy, were sorted by their PD-1 and CTLA-4 expression alone, due to a lower quantity of starting material. Sorted CD4+ T-cell subsets were on average >96% pure as determined by post-sorting FACS analysis.

Determination of viral load RNA

Quantitative real-time RT-PCR was performed to determine SIV plasma viral load as previously described (413).

Quantitation of cell-associated SIV DNA and RNA

Cellular DNA and RNA were extracted from at least 10,000 sorted memory CD4+ T-cells lysed in RLT Plus buffer (Qiagen) and isolated using the AllPrep DNA/RNA Mini Kit (Qiagen) per the manufacturer's instructions. cDNA was synthesized from extracted RNA, and quantification of SIVmac *gag* DNA and cDNA was performed on samples using the QX100™ Droplet Digital™ PCR system (Bio-rad). Total SIV DNA and RNA were quantified for these samples using SIVmac *gag* primers and probes, and normalized to the GAPDH gene (primer/probe set found below). Data was analyzed using the Quantasoft analysis software 1.3.2.0 (Bio-rad).

Primer and Probe sets used for analysis of SIV DNA/RNA.

SIV Forward: 5'-GCAGAGGAGGAAATTACCCAGTAC-3'

SIV Reverse: 5'- CAATTTTACCCAGGCATTTAATGTT-3'

SIV Probe: 5'-TGTCCACCTGCCATTAAGCCCGA-3'

GAPDH Forward: 5'-GCACCACCAACTGCTTAGCAC-3'

GAPDH Reverse: 5'-TCTTCTGGGTGGCAGTGATG-3'

GAPDH Probe: 5'-TCGTGGAAGGACTCATGACCACAGTCC-3'

SIV DNA in situ hybridization and immunofluorescent detection for confocal phenotypic analysis

Viral DNA detection (DNAscope) was performed and validated as described in detail below. Briefly, following HIER (Pretreat 2 step; ACD) and proteinase digestion (Pretreat 3 step, ACD), the slides underwent a short denaturation step by incubating slides in a hybridization chamber at 60°C with warmed (60°C) SIV sense probes for 10-15 min in a HybEZ oven (ACD), following which the hybridization chamber was immediately transferred to an oven set at 40°C and incubated overnight (between 18-21 hours). Amplification steps were performed according to the ACD protocol with the exception that all wash steps used a 0.5X wash buffer. Amplification reagents from the RNAscope 2.0 HD Red Detection Kits were used for Fast Red chromogenic detection or Brown Detection Kits for Tyramide Signal Amplification (TSA™) Plus Cy3.5 immunofluorescence detection. Following amplification step 6, but prior to development of vDNA, immunophenotyping was performed by incubating slides overnight at 4^{o}C with specific antibodies. We combined DNAscope detection (Fast Red chromogen or TSA™ Plus Cy3.5) with immunofluorescence targeting cell markers using rabbit polyclonal anti-PD-1 (1:200; Cat#HPA035981; SIGMA) and goat polyclonal anti-hCTLA-4 (1:200; Cat#AF-386-PB; R&D Systems). Slides were washed, incubated with secondary donkey anti-goat IgG-Alexa 488 and

anti-rabbit IgG-Alexa 647 (all from Molecular Probes/ThermoFisher Scientific) for 1 hr at room temperature, and washed 2 times for 5 min in TBS + tween (0.05% v/v). To decrease auto-fluorescence, the tissues were incubated with Sudan Black solution [0.1% in 80% ethanol (ENG Scientific, Inc.) in 1x TBS]; for 20-30 min at room temperature and then washed, counterstained with DAPI (RTU; ACD) for 10 min, washed, and then developed with either Fast Red chromogen or TSA Plus Cy3.5 to reveal vDNA (development time varied depending on the tissue type, but was typically 6-8 min), washed in TBS and cover slipped with #1.5 GOLD SEAL® cover glass (EMS) using Prolong® Gold reagent (Invitrogen).

To quantify the number of vDNA+ cells and proportion that were PD-1+/CTLA4-, PD-1+/CTLA4+, PD-1-/CTLA4+, and PD-1-/CTLA4-, high magnification confocal images were collected from regions of interests and manually counted (depending of the tissue size, 5 to 10 B cell follicles and 5 to 10 within the T cell zone) using an Olympus FV10i confocal microscope using a 60x phase contrast oil-immersion objective (NA 1.35) imaging using sequential mode to separately capture the fluorescence from the different fluorochromes at an image resolution of 1024x1024 pixels.

Assessment of STAT5 phosphorylation

The levels of phosphorylated STAT5 within CTLA-4 and PD-1-expressing subsets were measured by phospho-flow cytometry in ART-treated, SIV-infected RM LN samples, both *ex vivo* and following a three hour *in vitro* stimulation with PMA and ionomycin (see above). A minimum of 10^6 cells were stained with anti-CD3, anti-CD4, anti-CD8, anti-CD20 (clone 2H7; BD Biosciences), anti-PD-1, anti-CTLA-4 and a viability stain (Live/Dead Fixable Aqua, Invitrogen) for 20 minutes at 4°C. Samples were fixed for 10 minutes with 4% PFA at 37°C, permeabilized for 20 minutes with Perm Buffer III (BD Biosciences) on ice, and then stained intracellularly with anti-pSTAT5 (clone 47/Stat5(pY694); BD Biosciences) for 30 minutes at

room temperature. Following staining, cells were washed and fixed in 1% PFA, and acquired on a BD LSR Fortessa.

Determination of replication competent virus in memory CD4+ T cells during ART

Cryopreserved LN cells from ART-treated, SIV-infected RMs were sorted to high purity into memory CD4+ T-cell subsets based on their expression of CTLA-4 and PD-1. Due to limited cryopreserved LN cell numbers, 3 RM LN were sorted- 1 SIVmac251-infected RM followed in this study (RJf13, 108 days with undetectable viral load), and 2 SIVmac239-infected RMs from another study (RPu12, 106 days undetectable; RLm12, 165 days undetectable). Purified CTLA-4 and PD-1-expressing memory CD4+ T-cell subsets were stimulated for 6 hours with anti-CD3 and anti-CD28, and then co-cultured at a 1:1 ratio with the CEMx174 cell line (NIH AIDS Research and Reference Reagent Program), with starting cell numbers ranging from 1.5 to 2.5 x 10^5 memory CD4+ T-cells per well. Cocultures were maintained in complete RPMI 1640 media supplemented with 10%FBS, 100 IU/mL penicillin, 100 ug/mL streptomycin and IL-2 (100 U/mL). Cells were split, fed with fresh media containing IL-2, and harvested weekly (days 7, 14, 21, 28, and 35) for 35 days. The presence of replication competent virus was detected by positive SIV*gag* p27 expression within CEMx174 cells, using flow cytometry (227, 279), and by quantifying SIV*gag* RNA in the supernatant using qRT-PCR (see Methods above).

Statistical Analysis

Repeated-measures analyses for each memory CD4+ T-cell subset outcome were performed with a means model via the SAS MIXED Procedure (version 9.4; SAS Institute, Cary, NC), providing separate estimates of the means by time on study. The statistical model included one predictor (time on study) with up to 17 categorical levels, and was fit separately for each anatomic location. A compound-symmetric variance-covariance form in repeated measurements was assumed for each outcome and robust estimates of the standard errors of parameters were used to perform

statistical tests and construct 95% confidence intervals. The model-based means are unbiased with unbalanced and missing data, so long as the missing data was non-informative (missing at random, MAR). Three specific statistical tests were done within the framework of the mixed effects linear model for each anatomic location. All statistical tests were two-sided and unadjusted for multiple comparisons. A p-value ≤ 0.05 was considered statistically significant for each of the specific statistical comparisons. Results from these studies also focused on the magnitude of the differences for each outcome as reflected by confidence intervals, consistency of findings and biological significance. Similar repeated-measures analyses were performed for $\log_{10}$ SIV DNA over time by anatomic location. Comparisons of cell-associated DNA and contributions were calculated using t-tests and Mann-Whitney *U* tests, respectively, as determined by sample distribution (normal or nonnormal). DNA measurements were excluded for samples in which <10,000 cells were sorted and values fell outside the assay limit of detection. Longitudinal comparisons of cell-associated DNA were calculated using paired t-tests. Spearman rank correlation coefficients were used to estimate linear associations for SIV DNA data. Data showing continuous outcomes are represented as mean ± SEM. Analyses of SIV DNA and contributions were conducted using GraphPad Prism 6.0.

Results

Expression of CTLA-4 defines a new subset of virally enriched CD4+ T-cells during ART in multiple tissues

Ten RMs were infected intravenously (i.v.) with 1000 TCID50 SIV_{mac251} (day 0; Figure 4.1A) and, after 7 weeks of infection (45-52 days post-SIV infection), treated with an ART regimen consisting of PMPA, FTC, raltegravir and ritonavir-boosted darunavir (Table 4.1) for up to 14 months. One animal (RKa13) experienced rapid disease progression and was euthanized ten days into ART administration. Overall, the combined ART regimen was effective in suppressing plasma viremia (at least 99.94% reduction from pre-ART, Figure 4.S1A), with 7 of 9 animals demonstrating durable virus suppression by 290 days of uninterrupted therapy with a single viral blip in only one of these animals. The two RMs with the highest pre-treatment viral loads similarly had undetectable virus levels after 288 and 317 days of ART, though both experienced two viral blips of less than 500 copies/mL prior to subsequent viral load reductions. At the time of necropsy, 8 of 9 RMs had undetectable plasma viral loads for an average of 125±76 days, thus ensuring that measurements of cell-associated SIV DNA across different CD4+ T-cell subsets were representative of persistent infection in the absence of measurable ongoing viral replication (Figure 4.1B; limit of detection: <60 copies/mL). Consistent with HIV-infected humans (483-485), ART successfully elevated both CD4+ T-cell frequencies (Figure 4.1C) and absolute counts (data not shown) in the blood and tissues of the SIV-infected RMs, although to levels that still were significantly lower compared to pre-infection (Blood: $p=0.0009$; Gut: $p=0.012$). The efficacy of ART in suppressing viral replication was also evident in tissues, with a nearly 4-fold decrease in SIV DNA levels in LN when compared to pre-ART levels (Figure 4.S1B, S1C). Altogether, these results demonstrate the ability of our ART regimen to suppress virus replication in SIV-infected RMs and recapitulate immunologic effects seen in ART-treated, HIV-infected humans.

To define the contribution of memory subsets that express different co-inhibitory receptors (Co-IR) to the pool of latently infected cells as well as to identify their anatomic localization, we measured levels of cell-associated SIV*gag* DNA in CD95+ memory CD4+ T-cells sorted from the blood, LN, spleen, and gut after a minimum of 3 months (and up to 7 months) following their first undetectable viral load. Memory CD4+ T-cells were sorted based on their expression of Co-IRs (Figure 4.S2), as these cell surface molecules, namely PD-1, CTLA-4, and TIM-3, are upregulated on CD4+ T-cells following HIV-1 infection (124, 125, 143, 486). Although intracellular CTLA-4 expression is routinely used to measure nascent production of CTLA-4 molecules (487), we performed enhanced surface staining (see Methods) to visualize surface CTLA-4 by stimulating isolated lymphocytes for 3 hours with PMA/Ionomycin, prior to sorting based on its expression. This short stimulation captured rapidly expressed surface CTLA-4 and has previously been shown to reflect intracellular CTLA-4 levels (124). We found a consistent enrichment of SIV DNA in CTLA-4+PD-1- memory CD4+ T-cells in the ART-treated SIV-infected RMs at animal necropsy, with the average frequency of infection among the different tissues ranging from 760 to 923 copies of SIV per million CTLA-4+PD-1- cells (Figure 4.1D, E). In fact, CTLA-4+PD-1- memory CD4+ T-cells harbored significantly higher levels of SIV DNA when compared to both PD-1+CTLA-4+ and PD-1-CTLA-4- cells in the blood ($p=0.0367$), LN ($p=0.0203$), and spleen ($p=0.0386$), and compared to PD-1+CTLA-4+ cells in the gut ($p=0.0198$) following an extended period of undetectable plasma viremia (Figure 4.1E; average of 125±76 days of undetectable plasma viral load). Of note, SIV DNA levels did not differ significantly between TIM-3+ and TIM-3- populations within each CTLA-4 and PD-1-expressing subset (data not shown), confirming a previous finding (486). Of note, only the frequencies of infected CTLA-4+PD-1- memory CD4+ T-cells in the PBMCs at necropsy, and not of other Co-IR subsets, were 1.9-fold higher than those found at an earlier (45 days prior; MidART) time point on ART ($p=0.176$; Figure 4.S3A,B). These results demonstrate that CTLA-4, in the absence of PD-1

expression, identifies a unique subset of memory CD4+ T-cells highly enriched in vDNA, and that these cells persist during ART-mediated viral suppression in several lymphoid tissues.

CTLA-4 and PD-1-expressing CD4+ T-cells are major contributors to viral persistence in blood and multiple tissue sites

We next investigated the kinetics of CTLA-4 and PD-1-expressing cells following SIV infection and ART-mediated viral suppression. Levels of PD-1+CTLA-4+ memory CD4+ T-cells increased in the early chronic phase of SIV infection, which may reflect high levels of viral antigen driving the expression of multiple Co-IRs; as a result, PD-1 and CTLA-4-single expressing subsets remained stable or decreased in frequency (Figure 4.S4A, B). ART initially increased levels of PD-1+CTLA-4+ memory CD4+ T-cells, but prolonged administration decreased their frequencies to pre-SIV infection levels in the blood ($p=0.459$) and gut ($p=0.725$), but not in the LN. Instead, PD-1+CTLA-4+ memory CD4+ T-cells significantly increased in the LN from their pre-ART frequencies ($p<0.0001$; Figure 4.S4B), suggesting that the effects of ART on CD4+ T-cell subset reconstitution and/or its ability to penetrate tissues can differ by anatomic location.

We then determined the relative contribution of Co-IR-expressing cells to the SIV DNA pool during ART. We found that cells expressing either PD-1 and/or CTLA-4 (Co-IR+) made up the vast majority of the SIV DNA pool within memory CD4+ T-cells in all tissues sampled at necropsy (Figure 4.2A). The summed contributions of these Co-IRs+ subsets were, on average, 81.7% of the memory CD4+ SIV DNA pool, with the highest contribution found in the LN (88.5%). When comparing the contributions of the different Co-IR-expressing subsets to the total SIV DNA pool, PD-1+CTLA-4+ cells were the highest contributors to the SIV DNA pool within the memory CD4+ T-cell population across multiple tissue sites, including PBMCs (31.3%), LN (49.8%), and gut (45.5%), and compared to PD-1- subsets in the spleen (Figure 4.2B). Despite their low frequency within the memory CD4+ T-cell compartment, CTLA-4+PD-1- cells were the

second highest contributors to the total SIV DNA pool within memory CD4+ T-cells in PBMCs, LN, and gut, as a result of their high frequency of infection, although their contribution was limited in the spleen (Figure 4.2B). Together with Figure 4.1, these results revealed that the expression of multiple Co-IRs, particularly CTLA-4 and PD-1, distinguishes memory CD4+ T-cells that significantly contribute to SIV viral persistence in ART-treated RMs.

CTLA-4 and PD-1 subsets share characteristics with regulatory and follicular helper T-cells

CTLA-4 is one of the markers that define regulatory T-cells (Tregs); thus, we next investigated the overlap between the above defined CTLA-4+PD-1- T-cells and CTLA-4+ Tregs in ex vivo conditions (482, 488) (Figure 4.3A). We found that, in our cohort of ART-treated SIV-infected RMs, CTLA-4+PD-1- memory CD4+ T-cells were significantly enriched in CD25+CD127low cells that expressed FoxP3, the master transcription factor of Tregs, when compared to both bulk memory CD4+ T-cells and the other CTLA-4 and PD-1-expressing subsets (Figure 4.3B; $p<0.0001$) (237, 489). In fact, Tregs were the dominant functional subset, as they comprised 66.2±1.8% and 59.0±2.2% of CTLA-4+PD-1- memory CD4+ T-cells in the LN and PBMCs, respectively. To confirm the functionality of the FoxP3 locus within these subsets, we monitored the expression of the chromatin modifier SATB1, an epigenetic modifier whose expression inhibits FoxP3 transcriptional activity (490). We found that CTLA-4-expressing CD4+ T-cells included significantly higher frequencies of FoxP3+SATB1- cells as compared to CTLA-4- CD4+ T-cells (68.4±3.7% vs. 34.9±4.8% and 32.7±3.5%; $P<0.0001$), consistent with the FoxP3 locus being transcriptionally active (Figure 4.S5). Therefore, these results demonstrate that CTLA-4+PD-1- memory CD4+ T-cells include functional Tregs and represent a novel subset that significantly contributes to SIV persistence during ART.

Recent reports demonstrated that T_{FH} cells harbor heightened levels of HIV-1 DNA in the LN of ART-suppressed individuals (278), and that productive persistent SIV infection in elite controller

RMs, but not typical progressors, is markedly restricted to T_{FH} (279). In line with these reports, we identified T_{FH} cells phenotypically as CXCR5+PD-1hi memory CD4+ T-cells and functionally by their production of IL-21 (Figure 4.3C-E) (267, 273, 481). PD-1+CTLA-4+ memory CD4+ T-cells included significantly higher levels of T_{FH} as defined by higher levels of Bcl6 expression, the master transcriptional regulator of T_{FH}, than PD-1+CTLA-4- cells in the LN (Figure 4.3C,D; $p<0.0001$). Consistent with this result, PD-1+CTLA-4+ memory CD4+ T-cells also produced significantly higher levels of IL-21 when compared to the other subsets, and to levels comparable to those produced by CXCR5+PD-1hi T_{FH} in the LN (Figure 4.3E). Interestingly, CTLA-4+PD-1- memory CD4+ T-cells included significantly lower levels of CXCR5+ cells when compared to the other Co-IR+ subsets (including CTLA-4-PD-1-), suggesting that this Treg-enriched subset is less susceptible to trafficking into germinal centers (Figure 4.3F; $p<0.0001$), a finding that is compatible with current reports (491, 492). Altogether, these results highlight the fact that two functional Th subsets, i.e, Tregs and T_{FH} cells, that play a major role in the regulation of the adaptive immune system are also targeted by SIV to establish viral persistence.

Virally enriched CTLA-4+PD-1- T-cells localize outside the lymph node follicle and significantly contribute to viral persistence during ART-mediated viral suppression

CTLA-4+PD-1- memory CD4+ T-cells express low levels of CXCR5 (Figure 4.3F) suggesting that these cells could be localized outside of the BCF. We used a novel in situ hybridization assay that combines the detection of viral DNA (vDNA) in fixed tissue sections (termed DNAscope) with phenotypic analysis using immunofluorescence and confocal microscopy (493) to determine the cellular and anatomic localization of SIV DNA in the LN of RMs in situ, pre-ART and during ART (Figure 4.4A). Prior to ART initiation, vDNA in the T-cell zone (TCZ) was found predominantly in CTLA-4+PD-1- cells (Figure 4.4B), although the majority of vDNA+ cells in both the TCZ and the B-cell follicle (BCF) lacked Co-IR expression at this time point.

Remarkably, following ART initiation and viral suppression, CTLA-4+ subsets exclusively harbored vDNA in the TCZ, thus highlighting, for the first time, the contribution of this unique subset outside the BCF to viral persistence (Figure 4.4A, 4B). The proportion of vDNA+ cells expressing CTLA-4 in the TCZ was already increased 2.4-fold early after initiation of ART (average of 1.2 months on ART; Figure 4.4C), when compared to pre-ART; this frequency further increased an average of 3.0-fold with longer exposure to ART (average of 9.2 months on ART). In contrast, the contribution of CTLA-4- vDNA+ cells was reduced with exposure to ART 1.6-fold (early ART; Figure 4.4C) and 1.5-fold (Mid ART) in the TCZ. Consistent with published reports, the majority of vDNA+ cells in the BCF at Mid ART were PD-1+CTLA-4+ (the subset which predominantly includes T_{FH} cells), which represents a 4.2-fold increase when compared to their contribution preART to vDNA+ cells (Figure 4.4C) (278). Finally, the localization of CTLA-4+PD-1- vDNA+ cells outside the BCF was also confirmed in the spleen (data not shown). Altogether our results demonstrate, for the first time, the significant and increasing contribution of a T-cell subset residing outside the B cell follicle to viral persistence in multiple lymphoid tissues.

CTLA-4 and PD-1-expressing CD4+ T-cells harbor replication competent SIV

To confirm the contribution of these CTLA-4 and PD-1-expressing memory CD4+ T-cells to the latent SIV reservoir, we measured if these subsets contained replication competent virus using a modified viral outgrowth assay previously described for SIV-infected RMs (227). Memory CD4+ T-cells were sorted into CTLA-4 and PD-1-expressing subsets from the LN of 3 ART-treated, SIV-infected RMs after a prolonged period of viral suppression, and subsequently cocultured with the CEMx174 cell line to measure productive viral infection. Frequencies of CEMx174 cells expressing p27 and levels of SIV*gag* RNA released in the supernatant were measured weekly for 35 days of coculture. By day 35 of coculture, we found replication competent virus within each memory CD4+ T cell subset expressing CTLA-4 (Figure 4.5A, S6A). Replication competent

virus, as identified by the presence of p27+ cells, was detected in PD-1+CTLA-4+ memory CD4+ T-cells from all 3 RMs, and from 2 RMs in CTLA-4+PD-1- cells. These results were confirmed by the exponential increase of SIV RNA in the coculture supernatants (Figure 4.5B, S6B). Altogether, these results demonstrate that CTLA-4 and PD-1-expressing cells harbor replication competent SIV and, given their viral enrichment across multiple anatomic sites, constitute the majority of the latent SIV reservoir in ART-treated, SIV-infected RMs.

Seeding of virus in CTLA-4+PD-1- memory CD4+ T-cells predicts viral persistence

To investigate the relationship between the levels of CTLA-4+PD-1- and PD-1+CTLA-4+ memory CD4+ T cells and viral persistence, we longitudinally monitored the frequencies of these cells in the LN during SIV infection. Because CTLA-4+PD-1- memory CD4+ T-cells show a partial phenotypic overlap with Tregs, we hypothesized that this cell subset is infected by SIV during acute infection as its frequencies are elevated to dampen the high levels of immune activation resulting from viral replication. Consistent with our hypothesis, we found a positive correlation (r=0.8694, p=0.0556; Figure 4.6A) between the frequencies (by flow cytometry) of CTLA-4+PD-1- memory CD4+ T-cells in the LN at peak SIV infection (day 14 p.i.) and the fraction (by DNA scope) of SIV-DNA+ CTLA-4+PD-1- cells in the LN TCZ pre-ART initiation (day 52 p.i.); this association was specific for CTLA-4+PD-1- memory CD4+ T-cells as frequencies of PD-1+CTLA-4+ T_{FH}-like cells did not show this correlation (Figure 4.6B). We next tested the hypothesis that the frequencies of CTLA-4+PD-1- memory CD4+ T-cells at acute infection could predict SIV persistence at later time points of ART exposure. We found that the frequencies of CTLA-4+PD-1- memory CD4+ T-cells at peak SIV infection correlated with the levels of SIV DNA (by ddPCR) within memory CD4+ (r=0.7413, p=0.0353; Figure 4.6C) and memory PD-1+CTLA-4+ CD4+ T-cells (r=0.7688, p=0.0258; Figure 4.6D) in the LN at necropsy, thus up to 14 months after ART initiation. Importantly, these same correlations were not found with PD-1+CTLA-4+ memory CD4+ T-cells (Figure 4.6E and data not shown).

Further confirming the relationship between viral seeding in CTLA-4+PD-1- cells and viral persistence, we also found a positive association between the fraction of SIV-DNA+ CTLA-4+PD-1- cells in the TCZ pre-ART initiation and the time to viral load suppression following ART initiation (r=0.7855, p=0.0641; Figure 4.6F). Additionally, there was a significant positive correlation between CTLA-4+PD-1- SIV-DNA+ levels in the TCZ pre-ART and levels of SIV-DNA in PD-1+CTLA-4+ memory CD4+ T cells in the LN at necropsy (r=0.8586, p=0.0286; Figure 4.6G). In contrast, the seeding of virus in PD-1+CTLA-4+ cells in the BCF prior to ART initiation did not predict the SIV DNA levels in this LN subset at necropsy (Figure 4.6H). Hence, we were able to identify a positive correlation between vDNA+ CTLA-4+PD-1- cells in the TCZ prior to ART and persistence of virus in both TCZ and BCF during ART. Altogether, these data are consistent with CTLA-4+PD-1- Treg-like memory CD4+ T-cells uniquely contributing to the establishment and maintenance of the viral reservoir through their early seeding and long-term persistence.

CTLA-4+PD-1- CD4+ T-cells have upregulated levels of cell survival molecules

Memory CD4+ T-cells harbor latent HIV/SIV infection due to their long half-lives and potential for homeostatic proliferation (202). We found that CTLA-4+PD-1- CD4+ T-cells expressed significantly higher levels of the anti-apoptotic molecule Bcl2 ex vivo, both by MFI and frequency of Bcl-2+ cells, when compared to the other subsets (Figure 4.7A; $p<0.0001$). Consistent with the increased levels of Bcl2, CTLA-4+PD-1- CD4+ T-cells also expressed significantly higher levels of the phosphorylated form of STAT5 (pSTAT5) as compared to CTLA-4- cells (Figure 4.7B; $p<0.0001$). Activation of pSTAT5 is triggered by the cytokines IL-2, IL-7, and IL-15, all known to contribute to HIV persistence, and results in increased survival of CD4+ TCM cells (494). Altogether, these results indicate that the increased persistence of virally infected CTLA-4+PD-1- CD4+ T-cells during ART is associated with their elevated expression

of functional markers of cell survival and homeostatic proliferation. Consistent with a mechanistic link between increased capacity for survival and viral persistence, we found that the frequencies of CTLA-4+PD-1- CD4+ T-cells expressing pSTAT5 early after ART initiation (38-41 days post-ART initiation) were significantly correlated with the fraction of CTLA-4+PD-1- vDNA+ cells in the TCZ at a later ART timepoint (MidART) ($r=0.9132$, $p=0.0110$; Figure 4.7C). However, this relationship was not seen in PD-1+CTLA-4+ CD4+ T-cells in the LN, suggesting that distinct mechanisms control the persistence of these two subsets (Figure 4.7D). These data support a mechanism where CTLA-4+PD-1- memory CD4+ T-cells are seeded early during SIV infection (Figure 4.6A), and, due to their increased capacity for cell survival (Figures 4.7A, 7B), persist during ART to increasingly contribute to the latent viral reservoir.

Discussion

Reducing the persistent HIV/SIV reservoir remains an essential milestone for developing a functional cure for HIV-1 and has been significantly hindered by the lack of knowledge surrounding which phenotypic markers identify persistently infected cells as well as the anatomic location of these cells. The best characterized cellular reservoir, to date, are resting memory CD4+ T-cells (202, 358, 473). Latency is established in these cells following either direct infection of resting CD4+ T-cells or upon infection of activated CD4+ T-cells that then revert to a resting state (361, 364). Because Co-IRs are upregulated following activation to maintain cells in a resting state (495), we hypothesized that memory CD4+ T-cells expressing Co-IRs would harbor higher levels of persistent virus during ART than cells lacking their expression. We chose to test this hypothesis in the RM model of SIV infection for a number of reasons. First, because we could control the timing, duration, and adherence to the ART regimen, we minimized the variability in the size and composition of the viral reservoir (320, 496-498). Second, because ART regimens now suppress plasma viremia in SIV-infected RMs to levels similar to those seen in ART-treated, HIV-infected humans (227, 394, 396), we were able to achieve effective viral suppression (less than 60 copies/mL) in all of our animals, thus allowing us to investigate the viral reservoir in the absence of detectable viral replication. Additionally, because of our access to the RMs longitudinally, we could examine the immunophenotype of latently infected cells not only at multiple time points during viral suppression, but also from multiple tissue compartments, including at elective necropsy, thus allowing for an improved understanding of reservoir dynamics and location that is nearly impossible in the human clinical setting.

Our findings highlight the previously underappreciated contribution of CTLA-4+PD-1- memory CD4+ T-cells to the persistent viral reservoir. Indeed, the frequency of CTLA-4+PD-1- memory CD4+ T-cells harboring SIV DNA was higher than in other memory CD4+ T-cell populations both in PBMCs and in all sampled tissues, including the LN, spleen, and gut, following prolonged

(up to 14 months) ART treatment. Tregs constitute a major proportion of these cells, and, importantly, they persist outside of the BCF, as *in situ* analysis of SIV-DNA+ cells in the LN demonstrated the persistence of virus exclusively in CTLA-4+ cells in the TCZ following ART-mediated viral suppression (Figure 4.4B). These findings, together with the detection of replication competent virus within CTLA-4+PD-1- memory CD4+ T-cells, support the presence of latent HIV genomes in a previously unrecognized subset of cells characterized by expression of CTLA-4, within an anatomic location distinct from T_{FH} cells, and constituting an important immune modulatory function on T cell homeostasis and function.

This unique enrichment of CTLA-4+PD-1- CD4+ cells in SIV-DNA may result from a number of different mechanisms. First, it may indicate their preferential infectivity, as demonstrated by recent work in ART-naïve HIV-infected subjects showing that the frequency of memory CD4+ T cells carrying integrated HIV-DNA is up to 18-fold higher in CTLA-4+ as compared to CTLA-4- cells (499). Previous studies have also demonstrated high levels of CTLA-4 on HIV-specific CD4+ T-cells (124, 500), which are preferential targets for HIV (210). The enrichment of CTLA-4+PD-1- memory CD4+ T-cells may also arise from an increased propensity for cell survival (501). In agreement with this hypothesis, we found higher levels of Bcl2 and pSTAT5, and an increasing frequency of infection within CTLA-4+PD-1- memory CD4+ T-cells over time (Figure 4.S3B), all of which support an increased persistence of this virally enriched subset. Interestingly, Bach2, which has been previously shown to promote the differentiation, activation, and survival of FoxP3+ Tregs, was found to be a frequent integration site for HIV, which is consistent with our model of increased infectivity and persistence of Tregs (502, 503). Additionally, we found that CTLA-4+PD-1- memory CD4+ T-cells were predominantly CCR7+, and hence were included in the pool of long-lived central memory CD4+ T cells (data not shown). Finally, the result that the majority of vDNA in both the TCZ and the BCF was found in cells that lacked PD-1 and CTLA-4 expression before ART initiation, while in cells that express

CTLA-4 (alone in TCZ or together with PD-1 in the BCF) after prolonged ART is consistent with a model in which infected cells downregulate CTLA-4 during active viral transcription via Nef (499, 504) but re-express it when they become latent. In agreement with this potential mechanism, we found that PD-1+CTLA-4- memory CD4+ T-cells contained significantly higher levels of SIV RNA when compared to PD-1+CTLA-4+ CD4+ T-cells (data not shown). Thus, PD-1 expression in the absence of CTLA-4 seems to identify a less latent CD4+ T-cell subset that is more enriched in ongoing viral transcription, consistent with recent human data (278).

Overall, we found that PD-1+CTLA-4+ CD4+ T-cells – due to their high frequency within memory CD4+ T-cells - were the greatest contributors to the SIV DNA pool across multiple tissue compartments, despite their lower frequencies of SIV infection than CTLA-4+PD-1- CD4+ T-cells (Figure 4.2B). Of note, replication competent virus was detected in PD-1+CTLA-4+ memory CD4+ T-cells in all 3 RM LN tested, while the PD-1+CTLA-4- subset only produced virus in a single animal (Figure 4.S6A). This animal, RPu12, had the shortest period of ART-mediated viral load suppression of the 3 RMs prior to sampling, which suggests that PD-1 expression alone identifies a less latent CD4+ T-cell subset that is more enriched in ongoing viral transcription (Figure 4.S6A, S6B). These observations strongly suggest that PD-1 expression alone only captures a fraction of the persistent reservoir, but that a stronger link is found between the expression of multiple Co-IRs - here CTLA-4 and PD-1- on CD4+ T-cells and viral persistence.

An important issue raised by our findings is the consequence, in terms of both immunologic function and T cell differentiation, of these highly infected CTLA-4 expressing CD4+ T-cells. Previous studies have demonstrated that T_{FH} are highly enriched in HIV-DNA in both viremic and aviremic individuals (271, 272, 278). Consistent with these studies, we found that PD-1+CTLA-4+ cells, the subset that contributes most to T_{FH} cells, were the dominant contributors to

the viral DNA pool in the LN. However, we also found persistent virus outside of the follicle in the TCZ in CTLA-4+PD-1- cells, which phenotypically overlap with Tregs (Figure 4.3B). These findings build on two prior studies that showed heightened levels of HIV-1 DNA in Tregs from the blood of ART-treated HIV-infected individuals (256, 505); herein we reveal, for the first time, their high viral DNA content in multiple tissues, their localization in lymphoid tissues outside the BCF, and their growing contribution to viral persistence during ART. Treg migration and expansion in tissue sites of HIV replication, including LNs, has been suggested to inhibit antiviral immune responses during HIV infection (158, 506, 507). Thus, we speculate that CTLA-4+PD-1- memory CD4+ T-cells are seeded with virus during acute SIV infection while functioning as Tregs to dampen T-cell activation. In support of this mechanism, we found that, prior to ART, the levels of CD4+ T-cell proliferation, as measured by Ki-67+ frequencies, negatively correlated with the frequency of CTLA-4+PD-1- memory CD4+ T-cells in the LN (data not shown), suggesting that this CTLA-4 subset expands to reduce immune activation in the absence of ART. While expression of CTLA-4 during acute infection may indicate activated T-cells, independent of their Treg or non Treg function, the fact that these cells remain PD-1- (a molecule upregulated in activated T-cells) and inversely correlate with the extent of T-cell proliferation strongly supports their nature as Tregs. Remarkably, we found that the frequency of CTLA-4+PD-1- memory CD4+ T-cells within the LN during peak SIV infection (day 14) predicted the levels of SIV DNA both prior to ART initiation, within CTLA-4+PD-1- Tregs in the TCZ (Figure 4.6A), and at necropsy, within memory CD4+ and memory PD-1+CTLA-4+ CD4+ T-cells in the LN (Figure 4.6C,D). We then find that CTLA-4+PD-1- CD4+ T-cells express higher levels of functional markers of cell survival (Figure 4.7A, 7B), supporting the persistence of these acutely infected cells and their specific ability to predict the size of the viral reservoir. In summary, our results highlight CTLA-4+PD-1- CD4+ Tregs as a novel bonafide SIV reservoir. How this virally infected subset preferentially survives in a latent state will be an important question to be

addressed in future studies aimed at understanding the maintenance of the viral reservoir under long-term ART.

Of note, our viral outgrowth assay also detected replication competent virus within CTLA-4-PD-1- cells (DN; data not shown). The detection of reactivated virus in DN cells was expected, given their phenotype as memory CD4+ T-cells, as well as the possibility that these cells may express Co-IR that were not included in our study, such as TIGIT and/or LAG-3 (486). Despite containing replication-competent virus, our data demonstrate that the contribution of DN cells to viral persistence rapidly decreases with prolonged periods of ART (Figure 4.4B, S3A), suggesting that their contribution could be relatively modest after many years of ART in HIV-infected humans. The mechanism responsible for this rapid decay in the contribution of DN CD4+ T cells to viral persistence remains to be determined. These cells express the lowest levels of pSTAT5 among all the memory subsets analyzed (Figure 4.7B), leading us to hypothesize that they have a shorter lifespan than Co-IR+ CD4+ T cells. Further studies that better characterize the fate and functional role of DN cells as well as their mechanism of persistence will help elucidate their contribution to the viral reservoir.

Reducing the latent viral reservoir will require targeting persistently infected cells to induce their reactivation and promote their elimination through cytolytic or cytotoxic pathways. In our study, we found that memory CD4+ T-cells expressing CTLA-4 and/or PD-1 constituted the majority of infected CD4+ T-cells during ART across all tissue compartments, contained replication competent virus, and that their contribution significantly increased following the initiation and maintenance of ART. However, while PD-1+CTLA-4+ CD4+ T-cells, located primarily within the BCF, were consistently the major contributors to the SIV DNA pool, our data also supports the significant enrichment of a population of CTLA-4+PD-1- memory CD4+ Tregs outside the BCF (Figure 4.1E), a unique subset that would be preserved by blocking PD-1 alone. Therefore,

we anticipate that targeting both CTLA-4 and PD-1 in a dual co-inhibitory blockade under a suppressive ART regimen may represent a novel strategy for impacting a majority of persistently infected CD4+ T-cells across all tissue compartments, while also reinvigorating dysfunctional antiviral CD8+ T-cells expressing heightened levels of Co-IRs. Co-inhibitory blockades of CTLA-4 and PD-1, individually, have produced significant antitumor effects in clinical cancer trials, and their combination has resulted in powerful synergistic effects greater than either therapy alone, albeit with more frequent immune-related adverse events (508). The use of PD-1 and CTLA-4 blockades has also been explored as monotherapies in viremic SIV-infected RMs, where their use has improved antiviral T-cell functions and viral reactivation, respectively (509-511). Importantly, a recent case report demonstrated the ability of ipilimumab (anti-CTLA-4 blockade) to increase cell-associated HIV-1 RNA in an ART-suppressed, HIV-infected melanoma patient (512). This case study, by revealing the impact of targeting extracellular CTLA-4, supports our use of enhanced surface staining to visualize rapidly expressed CTLA-4 on CD4+ T-cells as well as the capacity of immunotherapeutic blockades to bind this transiently expressed molecule. These studies also support the ability of co-inhibitory blockades to target latently infected cells, facilitate viral reactivation, and strengthen the antiviral CD8+ T-cell response necessary to eliminate recently activated cells. Further studies are needed in non-human primates to determine if combining CTLA-4 and PD-1 blockades in the context of ART suppression is effective and well-tolerated in reducing the viral reservoir.

In summary, CTLA-4 and PD-1-expressing memory CD4+ T-cells are major contributors to SIV viral persistence and constitute the majority of the viral reservoir in ART-treated, SIV-infected RMs. Remarkably, our study identifies CTLA-4+PD-1- memory CD4+ T-cells as a novel subset enriched in replication competent SIV DNA that persist outside the B-cell follicle despite ART-mediated viral suppression. By demonstrating the functional overlap between CTLA-4+PD-1- memory CD4+ T-cells and Tregs, as well as the enrichment of TFHs within PD-1+CTLA-4+

memory CD4+ T-cells, our results highlight that SIV is able to establish and maintain viral persistence through the specific targeting of two CD4+ T-cell subsets critical for the regulation of the adaptive immune system.

Figures

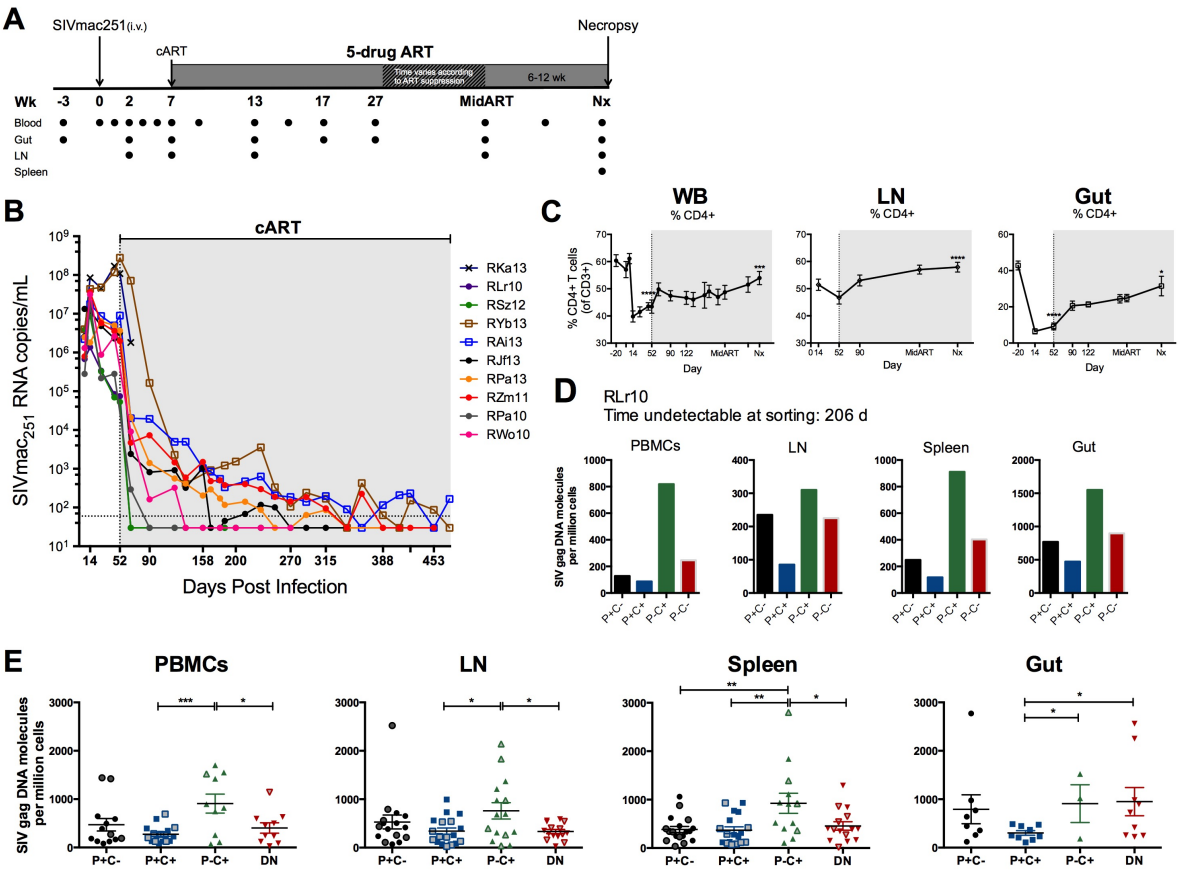


Figure 4.1. CTLA-4+PD-1- memory CD4+ T-cells harbor higher levels of SIV DNA following ART-mediated, viral load suppression. (A) Schematic of the study design. Ten RMs were infected i.v. with 1000 TCID50 SIVmac251 (day/week 0), and at approximately 7 weeks post-infection, initiated a five-drug combination ART regimen (cART: PMPA, FTC, raltegravir, and ritonavir-boosted darunavir). All animals were maintained on the daily cART regimen until plasma viremia was undetectable (limit of detection: 60 copies viral RNA/mL) for at least 3 months. Peripheral blood (WB), rectal biopsy (Gut), and lymph node (LN) biopsies were collected longitudinally at the indicated time points and multiple organs were processed and harvested at the time of elective necropsy. Sorting of memory CD4+ T-cells by Co-IR expression was performed at two time points during ART-mediated viral suppression- first, at midART

(approximately 1 month following undetectable viral load measurement); and second, at necropsy. (**B**) Plasma viral loads are shown for the 10 individual RMs, quantified using the standard qRT-PCR assay (limit of detection of 60 SIV RNA copies/mL of plasma, LOD, represented by the horizontal dotted line). Undetectable plasma viral load measurements are plotted as one-half of the LOD (30 copies/mL). (**C**) CD4+ T-cell levels, expressed as the frequency of live CD3+ T-cells, were longitudinally measured in WB, LN, and gut biopsies of the 10 RMs. The gray shaded area represents time on cART (combination ART); Nx represents the measured values from animal necropsy. Averaged data are presented as the mean ± SEM. Repeated-measures analyses were performed using a means model (SAS Mixed Procedure, version 9.4) to determine statistical significance, with indicated tests of significance representing comparison to pre-SIV infection (WB, Gut) or pre-ART initiation (LN). (**D**) Representative SIV DNA levels in the PBMCs, LN, spleen, and gut tissues for an individual RM (RLr10) after 206 days of viral load suppression. (**E**) Levels of cell-associated SIV*gag* DNA were quantified from CTLA-4 and PD-1-sorted subsets of 9 ART-treated, SIV-infected RMs at least three months following the first undetectable viral load measurement (163 ± 42 d). Sorted TIM-3+ subsets are represented by open symbols in the PBMCs, LN, and spleen; closed symbols represent TIM-3- subsets. Data from subsets with less than 10,000 sorted cells were excluded when undetectable. Sample averages are indicated by the horizontal bar on each graph (± SEM), and t-tests were used to compare DNA levels between subsets (P, PD-1; C, CTLA-4). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

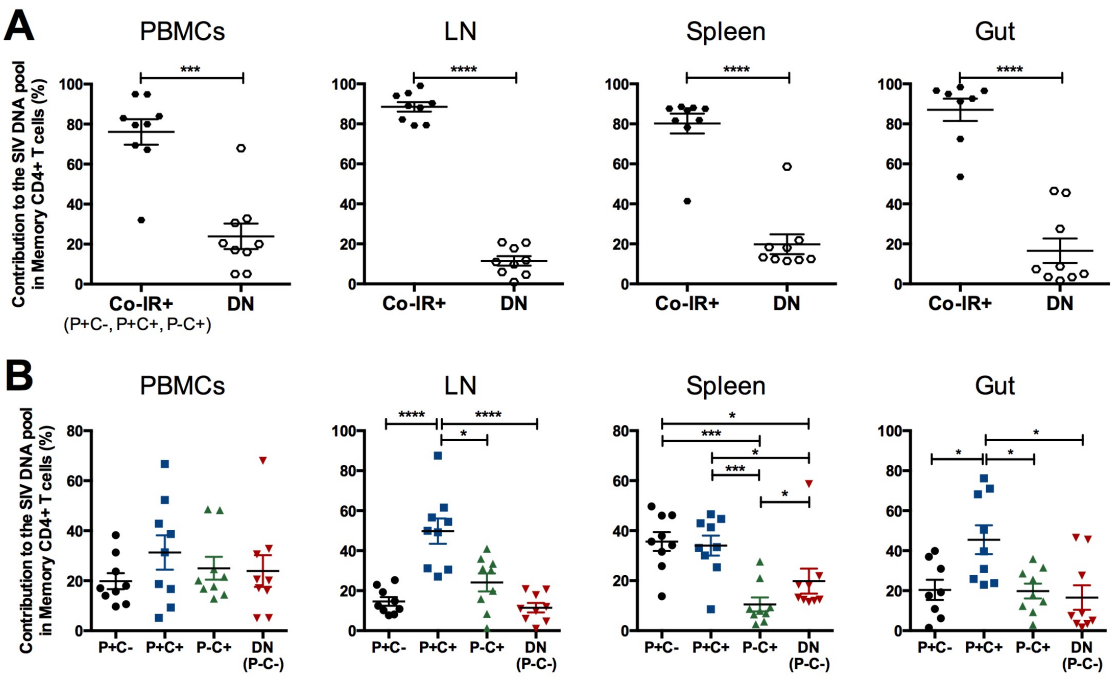


Figure 4.2. PD-1+ CTLA-4+ CD4+ T-cells are major contributors to viral persistence across anatomic locations. (**A**) The summed contributions of CTLA-4 and PD-1-expressing subsets (Co-IR+: P+C-, P+C+, and P-C+) to the total SIV DNA pool were compared to the contribution of PD-1-CTLA-4- (DN) memory CD4+ T-cells at the time of necropsy for 9 ART-treated, SIV-infected RMs. (**B**) Contributions of individual CTLA-4 and PD-1-expressing subsets to the total memory SIV DNA pool at the time of necropsy were determined using the level of SIV DNA per cell and the frequency of each Co-IR-expressing subset within memory CD4+ T-cells. Sample averages are indicated by the horizontal bar on each graph (± SEM), and Mann-Whitney *U* tests were used to compare DNA levels between subsets (P, PD-1; C, CTLA-4). *, $p<0.05$; ***, $p<0.001$; ****, $p<0.0001$.

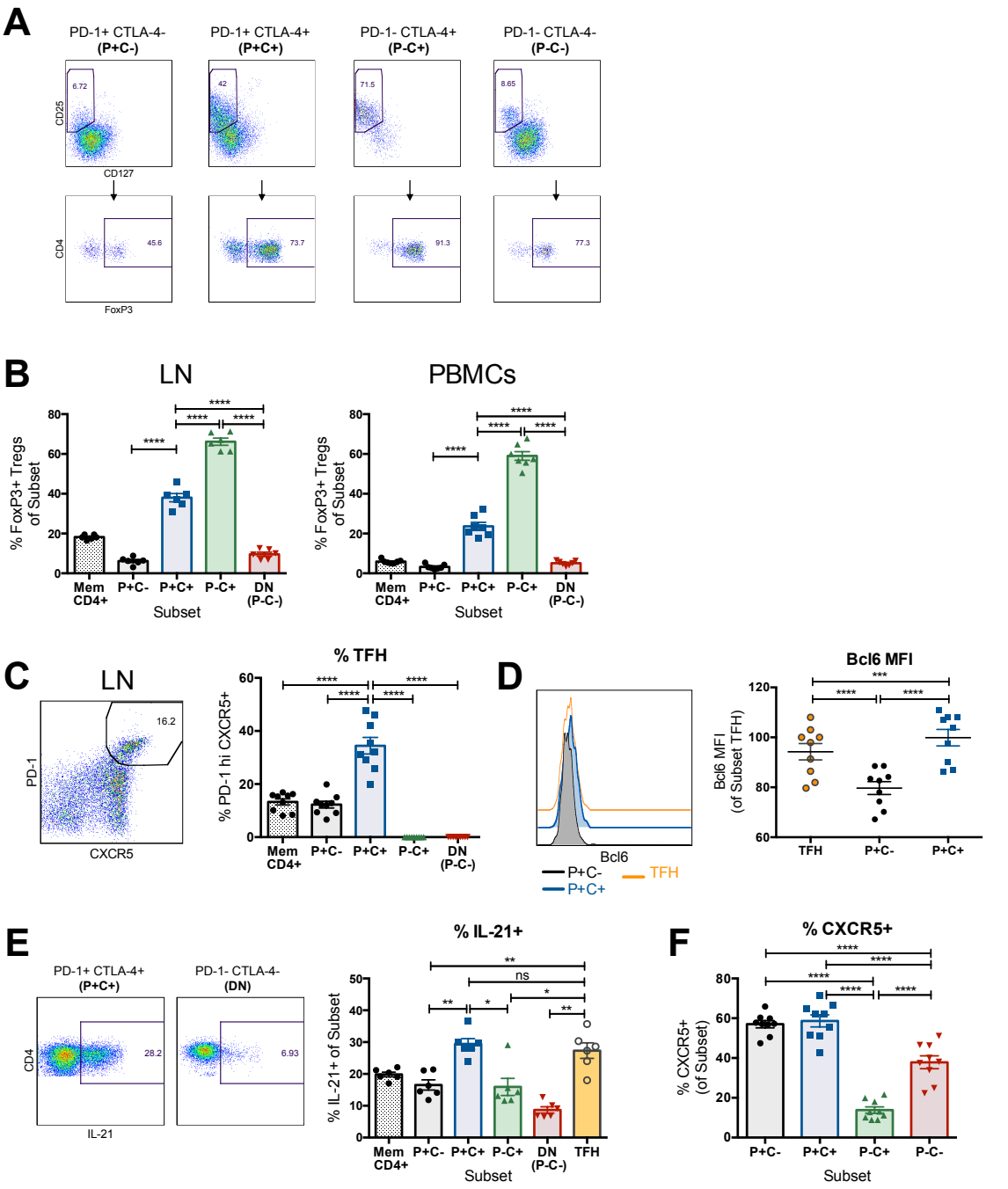


Figure 4.3. CTLA-4 and PD-1+ memory CD4+ T-cells overlap with regulatory T and follicular helper T-cells. (**A**) Representative flow plots of regulatory T cells (Tregs), defined as CD25+CD127low and FoxP3+, within CTLA-4 and PD-1-expressing memory CD4+ T-cell subsets are shown from the LN of an individual ART-treated, SIV-infected RM at necropsy. The results shown are from unstimulated lymphocytes, where CTLA-4 expression was measured

intracellularly; similar results were obtained following PMA/Ionomycin stimulation. (**B**) The frequencies of FoxP3+ Tregs within memory CD4+ T-cell subsets are shown from the LN and PBMCs of 6 ART-treated, SIV-infected RMs. (**C**) Representative flow plot demonstrating the gating for follicular helper cells (TFH) within the LN of an ART-treated, SIV-infected RM, defined as PD-1hi CXCR5+ cells. Frequencies of TFH cells were quantified in the LN of 9 ART-treated, SIV-infected RMs in the absence of stimulation at day 90 post-SIV infection (38-41 days post-ART initiation). (**D**) The representative histograms and aggregate mean fluorescence intensity (MFI) of Bcl6 within TFH cells from PD-1-expressing memory CD4+ T-cell subsets ex vivo are shown from the LN of 9-ART-treated SIV-infected RMs at day 90 post-SIV infection. (**E**) Representative flow plots of IL-21 production by PD-1+CTLA-4+ and PD-1-CTLA-4- (DN) memory CD4+ T-cells. Levels of IL-21 production by each CTLA-4 and PD-1-expressing subset were measured from the LN of 6 ART-treated, SIV-infected RMs following a 3 hour PMA/Ionomycin stimulation containing GolgiStop and Brefeldin A. (**F**) Frequencies of CXCR5+ cells were quantified between each CTLA-4 and PD-1-expressing subset in the LN of 9 ART-treated, SIV-infected RMs at day 90 post-SIV infection (38-41 days post-ART initiation). The results shown are from unstimulated lymphocytes. Averaged data are presented as the mean ± SEM, and ANOVAs using Tukey's adjustment for multiple comparisons were used to compare differences between subsets. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

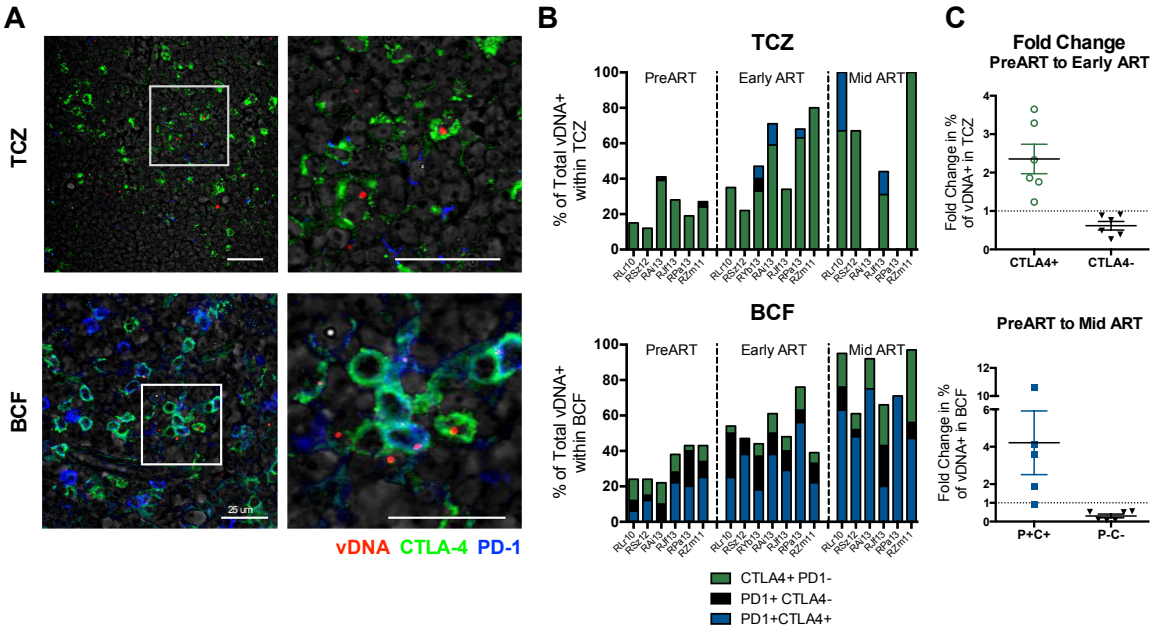


Figure 4.4. SIV DNA is found outside the B-cell follicle in CTLA-4+PD-1- cells. (A) Immunofluorescence staining for CTLA-4 (green) and PD-1 (blue) combined with DNAscope hybridization for SIV vDNA (red) in the LN T-cell zone (TCZ) and B cell follicle (BCF) of a representative RM (RJf13). Representative images are shown for each anatomic location within the LN following ART (RJf13: D315 p.i.; MidART) in the absence of detectable plasma viremia. Scale bars = 25 um. The white box highlights the expanded view on the right. (B) Quantitative image analysis for the TCZ and BCF of the LN demonstrating the fraction of SIV vDNA+ cells that express CTLA-4 and/or PD-1 in SIV-infected RMs (n=7) at 3 separate time points: PreART, Early ART (90 days post-SIV infection, 38-41 days post-ART initiation), and MidART (average time since last undetectable plasma viral load being approximately 80 ± 40 d). (C) Fold change in the fraction of vDNA+ cells in the TCZ that express CTLA-4, or lack its expression, was calculated using the DNAscope quantification of 6 RMs between the Early ART and PreART time points. Fold change is also shown for the fraction of vDNA+ cells in the BCF that express PD-1 and CTLA-4 (P+C+) and those that lack their expression (P-C-) using the DNAscope quantification of 6 RMs between the MidART and PreART time points. Data are presented as mean ± SEM, with a dashed line corresponding to FC=1 (no change).

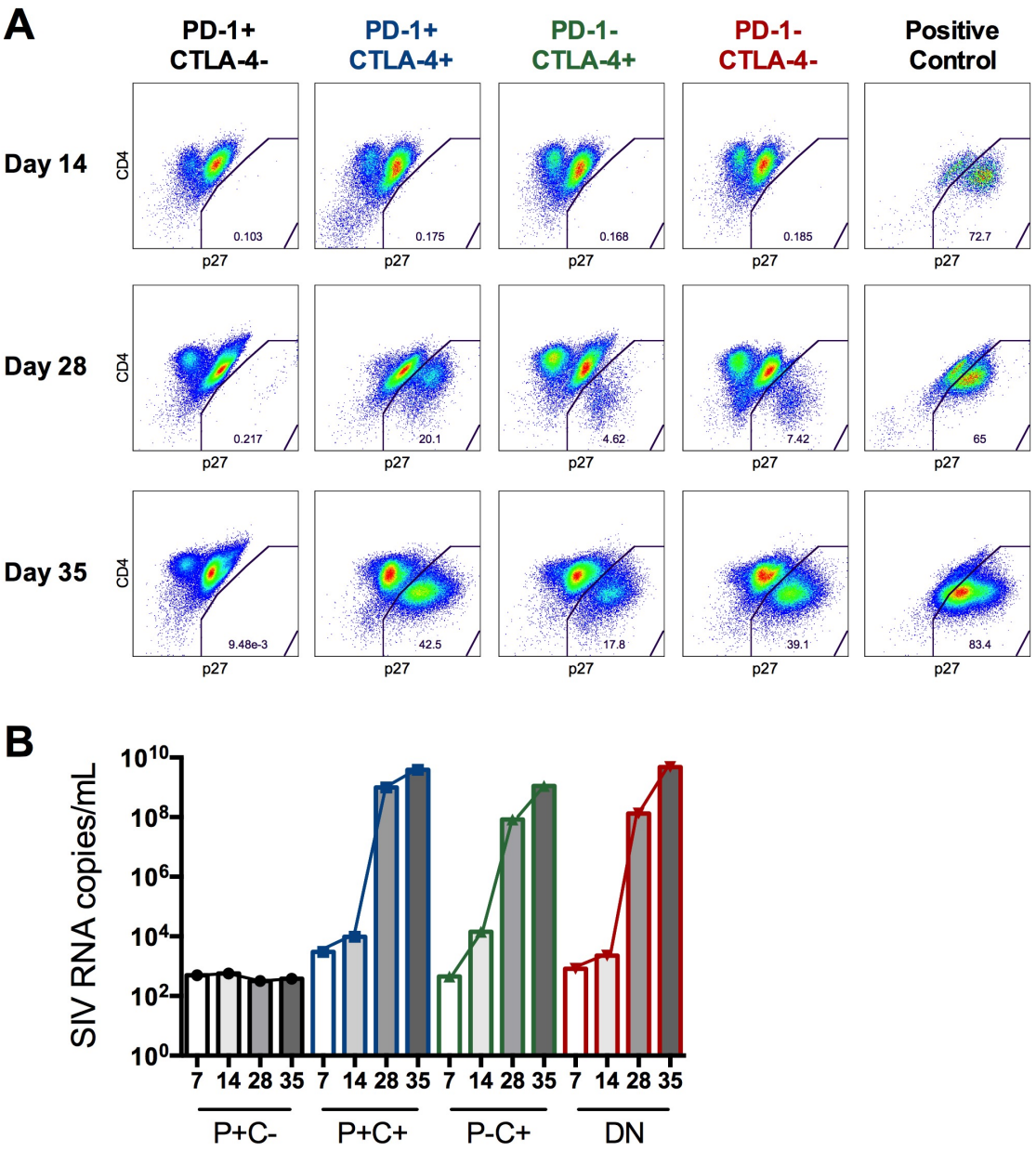


Figure 4.5. CTLA-4 and PD-1-expressing cells harbor replication competent SIV. (A) Memory CD4+ T-cells were sorted from ART-treated, SIV-infected RM LNs based on CTLA-4 and PD-1 expression (see Figure 4.S2) and co-cultured with CEMx174 cells for 35 days (n=3). Representative flow plots of intracellular SIV*gag* p27 in CEMx174 cells are shown for days 14, 28, and 35 of co-culture in the four CTLA-4 and PD-1 subsets from an individual ART-treated, SIV-infected RM (RJf13). The right column shows SIV*gag* p27 staining for a positive control

(CEMx174 cells infected directly with SIVmac). (B) SIV*gag* RNA was quantified longitudinally from the supernatant of the individual co-cultures. Due to cell sample limitations, only PD-1+CTLA-4+ memory CD4+ T cells had replicate wells; the SIV RNA data for this subset represents the average of the three replicate wells.

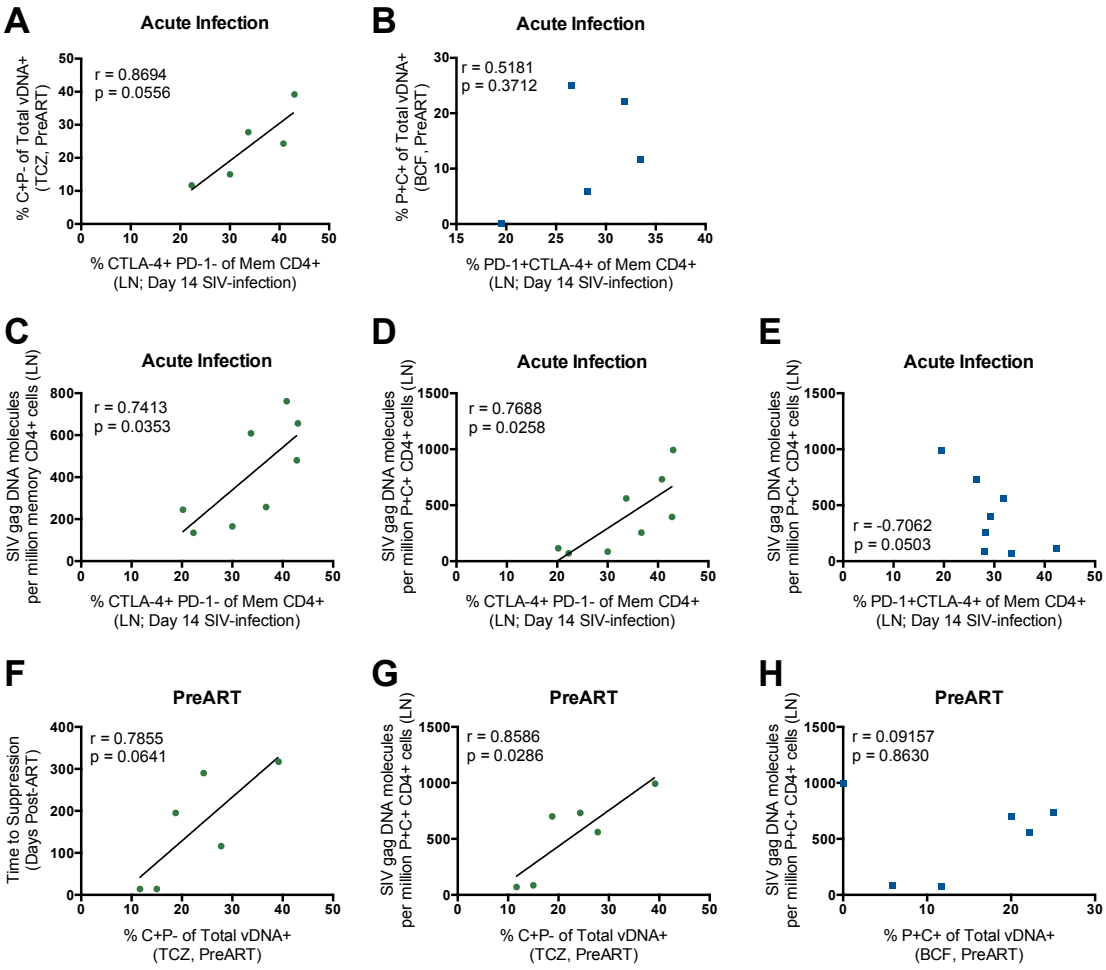


Figure 4.6. Frequencies of CTLA-4+PD-1- memory CD4+ T-cells and their viral seeding predicts viral persistence during ART. Correlations are shown between the frequencies of CTLA-4+PD-1- (A) or PD-1+CTLA-4+ (B) memory CD4+ T-cells in the LN at the peak of SIV infection (day 14) and the fraction of viral DNA (vDNA) found within C+P- in the TCZ (A) and P+C+ in the BCF (B) prior to ART initiation by DNAscope. Only 5 SIV-infected RMs had measurements to relate in this analysis; all are shown. The frequencies of CTLA-4+PD-1- memory CD4+ T-cells in the LN at the peak of SIV infection (day 14) are significantly associated with the frequencies of bulk memory (C; p=0.0353) and memory PD-1+CTLA-4+ (D; p=0.0258) CD4+ T-cells harboring SIV*gag* DNA in the LN at necropsy (average time of ART-mediated viral suppression: 125 ± 76 days; n=8). (E) The correlation is also shown between the frequency

of PD-1+CTLA-4+ memory CD4+ T-cells in the LN at day 14 and the frequencies of memory PD-1+CTLA-4+ CD4+ T-cells harboring SIV DNA in the LN at necropsy. The fraction of SIV DNA+ cells expressing CTLA-4+PD-1- in the TCZ preART initiation was positively correlated with time to viral load suppression (F; p=0.0641) and SIV DNA levels in PD-1+CTLA-4+ memory CD4+ T cells in the LN (G; p=0.0286) at necropsy. (H) The correlation is also shown between the fraction of SIV DNA+ cells expressing PD-1+CTLA-4+ in the BCF preART initiation and the frequencies of memory PD-1+CTLA-4+ CD4+ T-cells harboring SIV DNA in the LN at necropsy. All statistical analyses were performed using Pearson product-moment correlation tests.

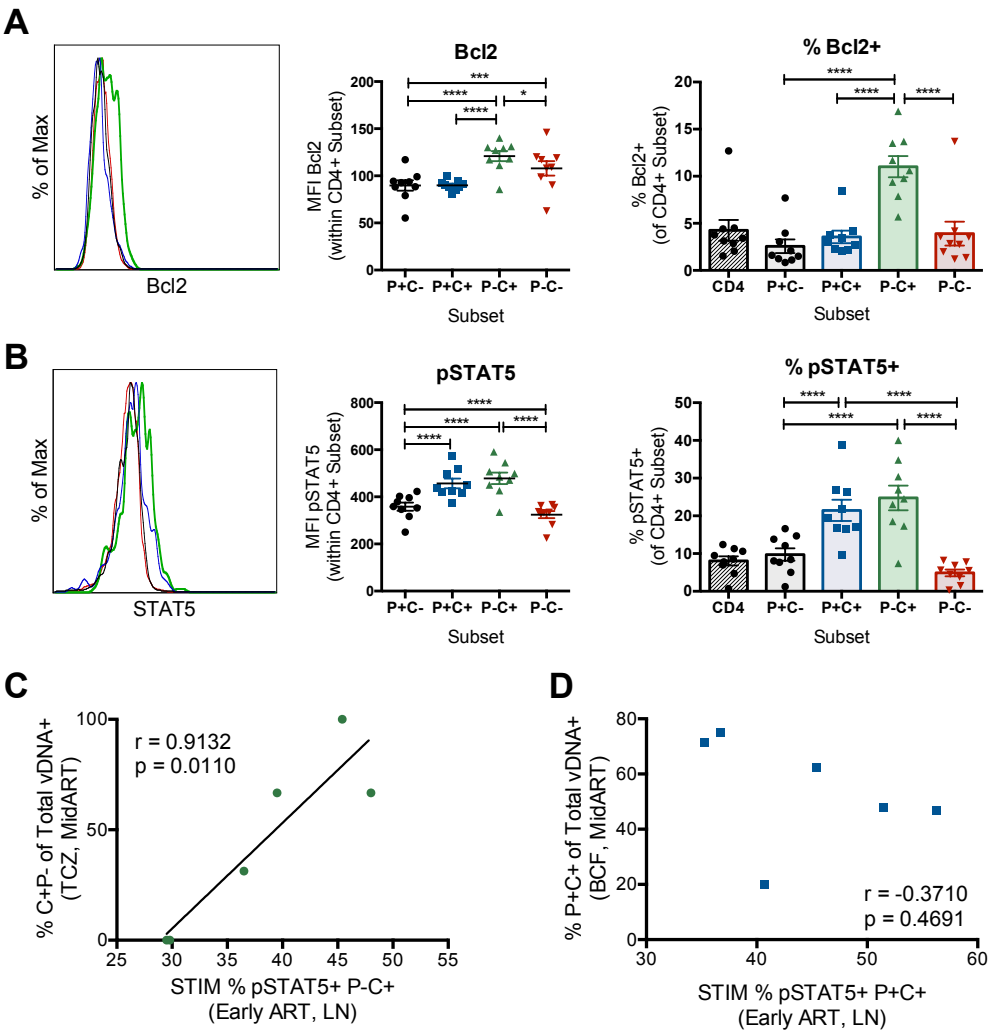


Figure 4.7. CTLA-4+PD-1- CD4+ T-cells demonstrate increased potential for survival and homeostatic proliferation. The representative mean fluorescence intensity (MFI) of Bcl2 (**A**) and pSTAT5 (**B**) expression is shown for CTLA-4 and PD-1-expressing memory CD4+ T-cells from the LN of an individual ART-treated, SIV-infected RM. Aggregate MFI data are then shown for each molecule for 9 ART-treated, SIV-infected RMs. Frequencies of Bcl2+ (**A**) and pSTAT5+ (**B**) cells were quantified in the LN of 9 ART-treated, SIV-infected RMs at day 90 post-SIV infection (38-41 days post-ART initiation). Of note, Bcl2 and pSTAT5 expression were determined ex vivo, with CTLA-4 expression measured intracellularly. Averaged data are presented as the mean ± SEM, and ANOVAs using Tukey's adjustment for multiple comparisons

were used to compare differences between subsets. *, $p<0.05$; ***, $p<0.001$; ****, $p<0.0001$. Correlations are shown between the frequencies of CTLA-4+PD-1- (**C**) or PD-1+CTLA-4+ (**D**) memory CD4+ T-cells in the LN during early ART (38-41 days post-ART initiation) expressing pSTAT5 and the fraction of viral DNA (vDNA) found within C+P- in the TCZ (**C**) and P+C+ in the BCF (**D**) at MidART by DNAscope (MidART average time since last undetectable plasma viral load being approximately 80 ± 40 d). Statistical analyses were performed using Pearson product-moment correlation tests.

Macaque	Age (years)	VL at ART initiation[1] (copies RNA/mL)	ART regimen[2]	Time to first suppression[3] (days)	Mid-ART time point		Necropsy time point	
					Time since first supp. (days)	VL (copies RNA/mL)	Time since first supp. (days)	VL (copies RNA/mL)
RKa13	4.39	1.09×10^8	PMPA, FTC, RAL, DRV/r	n/a*	n/a*	n/a*	n/a*	n/a*
RLr10	9.20	7.46×10^4	PMPA, FTC, RAL, DRV/r, MRV	14	134	<60	206	<60
RSz12	4.43	5.30×10^4	PMPA, FTC, RAL, DRV/r, MRV	14	134	<60	204	<60
RYb13	4.35	2.80×10^8	PMPA, FTC, RAL, DRV/r, MRV	288	83	151	133	<60
RAi13	4.33	8.99×10^6	PMPA, FTC, RAL, DRV/r, MRV	317	64	233	108	166
RJf13	4.35	3.20×10^6	PMPA, FTC, RAL, DRV/r, MRV	116	147	<60	211	<60
RPa13	4.44	3.65×10^6	PMPA, FTC, RAL, DRV/r, MRV	195	68	85.6	136	<60
RZm11	7.34	1.98×10^6	PMPA, FTC, RAL, DRV/r, MRV	290	62	<60	114	<60
RPa10	10.44	2.81×10^5	PMPA, FTC, RAL, DRV/r	41	111	<60	201	<60
RWo10	9.50	2.61×10^6	PMPA, FTC, RAL, DRV/r	83	69	<60	153	<60

Table 4.1. Characteristics of the 10 RMs infected with SIVmac251.

[1]Viral load was measured by quantitative RT-PCR with a limit of detection of 60 copies/mL of plasma.

[2]Antiretroviral therapy (ART) abbreviations: PMPA, tenofovir; FTC, emtricitabine; RAL, raltegravir; DRV/r, darunavir boosted by ritonavir; MRV, maraviroc

[3]Time to first suppression represents the number of days RMs were on ART before their first measurement of undetectable plasma viremia. This time point was then used to establish MidART and necropsy time points, which reflect at least one and three months after this time, respectively.

A

Macaque	Days on ART *(years)*	% VL Reduction *(Nx/PreART VL)*
RKa13	n/a*	n/a*
RLr10	220	100.0%
RSz12	218	99.96%
RYb13	421	99.94%
RAi13	425	100.0%
RJf13	327	100.0%
RPa13	331	100.0%
RZm11	404	100.0%
RPa10	242	99.99%
RWo10	236	100.0%

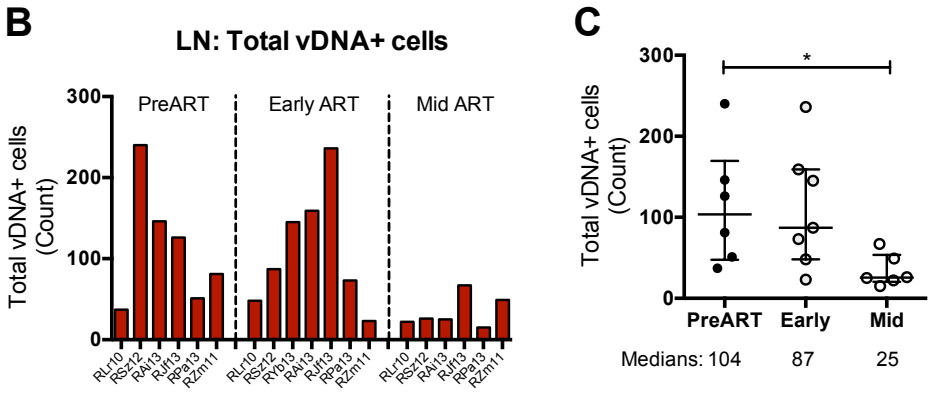


Figure 4.S1. ART significantly reduces plasma viremia and SIV DNA levels in lymphoid tissues of SIV-infected RMs. (A) The above table illustrates the duration of uninterrupted ART administration and the reduction in plasma viremia as a result of this ART regimen. The percent reduction was calculated between plasma viral load measurements at animal necropsy and the final time point prior to ART initiation (PreART), where undetectable plasma viral load measurements are represented as one-half of the LOD (30 copies/mL). (B) Quantitative image analysis following DNAscope hybridization for the LN represents the number of vDNA+ cells present in SIV-infected RMs (n=7) at 3 separate time points: PreART, Early ART (90 days post-

SIV infection, 38-41 days post-ART initiation), and MidART (average time since last undetectable plasma viral load being approximately 80 ± 40 d). (C) The aggregate data of vDNA+ cells in the LN are shown for 6 SIV-infected RMs (7 at Early ART). Medians are indicated by the horizontal bars on the graph (±IQR) and numerically below. *, $p<0.05$.

Supplemental Figure 2

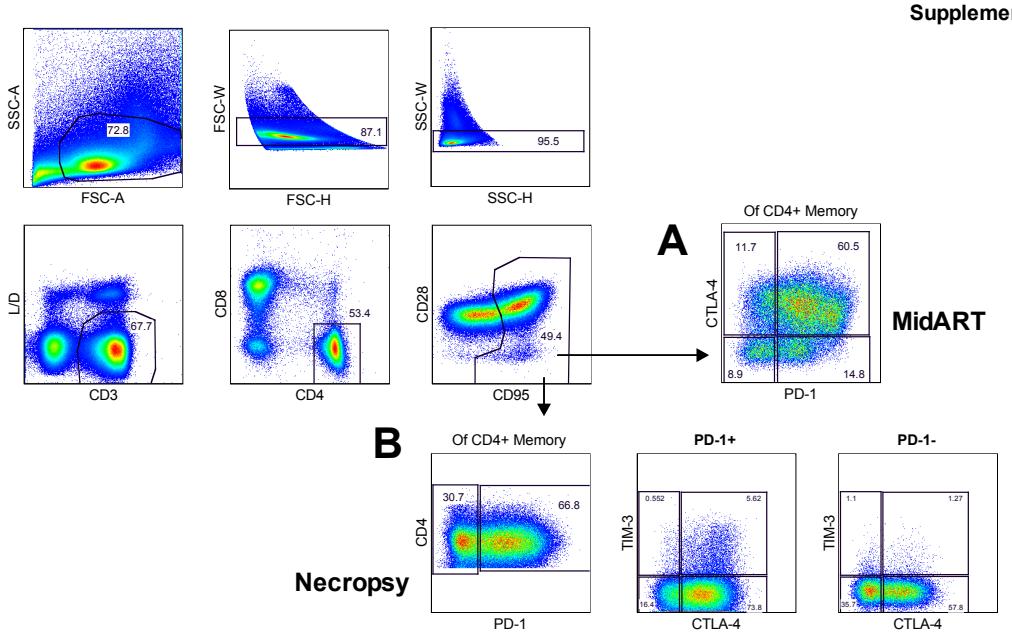


Figure 4.S2. Gating strategy for sorted CTLA-4 and PD-1 memory CD4+ T-cell subsets. Memory CD4+ T-cell subsets were sorted on the basis of Co-IR expression using a FACS AriaII (BD Biosciences). Isolated lymphocytes were first stimulated for 3 hours with PMA and Ionomycin (See Methods), during which anti-CTLA-4 antibody was added with the stimulation media. Memory CD4+ T-cells (CD95+) were then sorted from the PBMCs and LN at midART (A) according to their expression of CTLA-4 and PD-1. At necropsy (B), memory CD4+ T-cells were first sorted from the PBMCs, LN, and spleen based on PD-1 expression, and then each PD-1 subset was further sorted according to TIM-3 and CTLA-4 expression. Pooled gut tissue samples at necropsy were sorted solely by their CTLA-4 and PD-1 expression, given their low starting material. LN staining from a representative ART-treated, SIV-infected RM is shown.

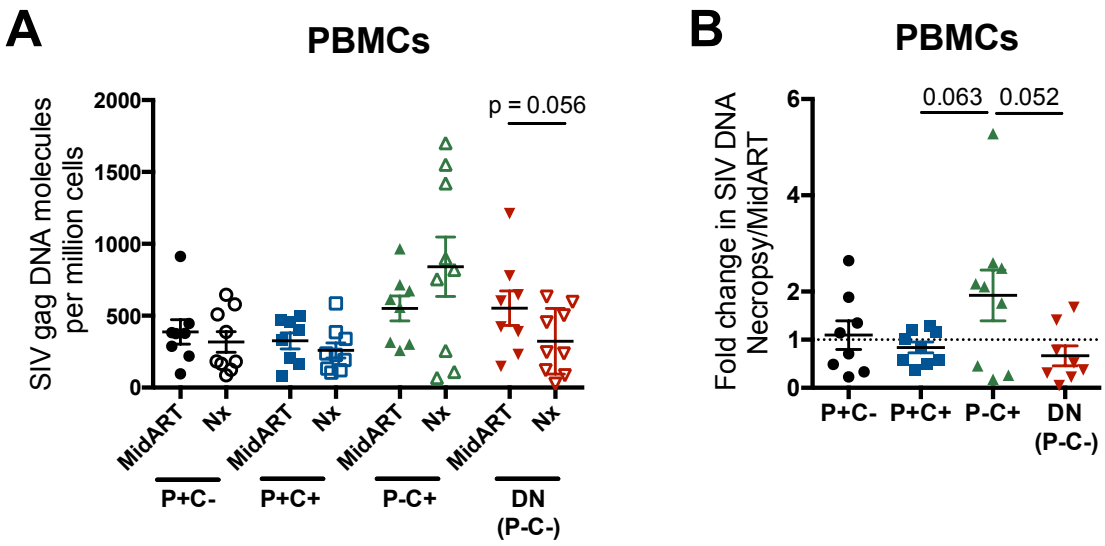


Figure 4.S3. Cell-associated SIV DNA decreases in memory CD4+ T-cells lacking co-inhibitory receptor expression over time. (A) Levels of cell-associated SIV*gag* DNA were measured in CTLA-4 and PD-1-sorted subsets at two time points during ART-mediated viral load suppression (MidART and necropsy) from the PBMCs of 9 RMs. Sample averages are indicated by the horizontal bar on each graph (± SEM), and paired t-tests were used to compare DNA levels within subsets between time points (P, PD-1; C, CTLA-4). (B) Fold change of SIV DNA is quantified within CTLA-4 and PD-1-expressing subsets between necropsy and MidART. The dotted line at 1 represents no change between time points. Sample averages are indicated by the horizontal bar on each graph (± SEM), and t-tests were used to compare fold change values between subsets.

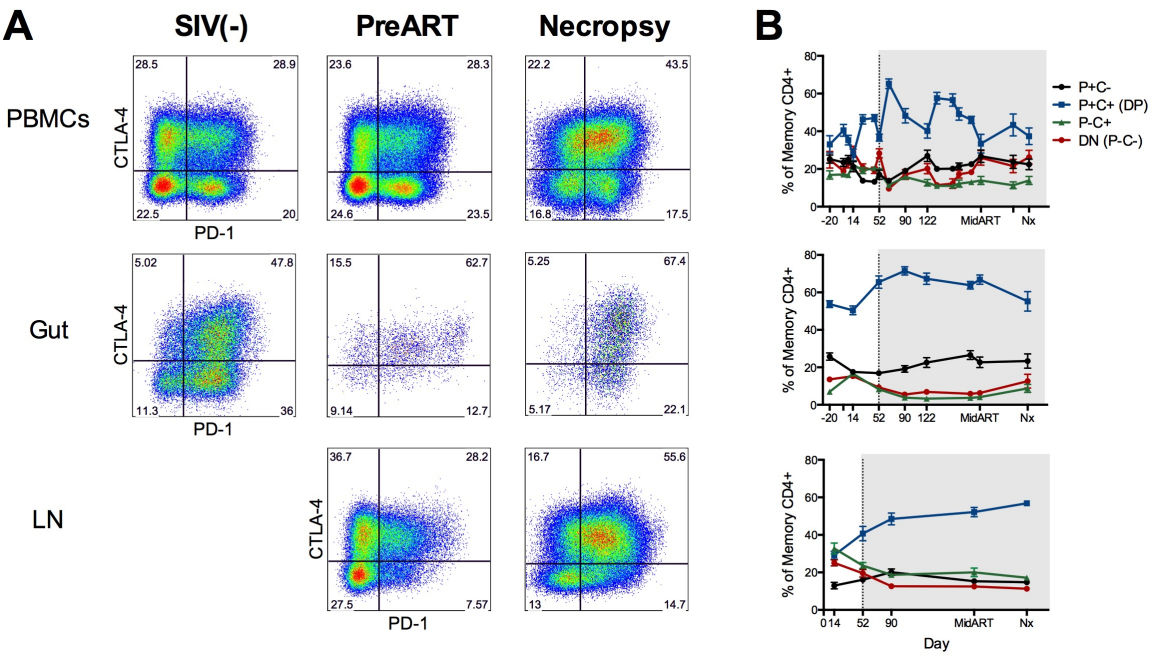


Figure 4.S4. PD-1+ CTLA-4+ CD4+ T-cells comprise the majority of memory CD4+ T-cells. (A) Representative flow plots of CTLA-4 and PD-1 expression on memory CD4+ T-cells (CD4+ CD95+) in PBMCs, Gut, and LN of an individual RM at multiple time points during the study (PreSIV/SIV(-), PreART, and at necropsy). CTLA-4 expression was visualized extracellularly using enhanced surface staining, as described in the Methods section. (B) Frequencies of CTLA-4 and PD-1-expressing memory CD4+ T-cells, expressed as percentages of memory CD4+ T-cells, were measured longitudinally during SIV infection and ART suppression (gray box) for 9 RMs. Averaged data are presented as the mean ± SEM. The MidART time point represents values measured at the time of sorting for each animal, with the average time since last undetectable plasma viral load being approximately 80 ± 40 d; similarly, necropsy (Nx) refers to the animal's final access point, with average time of ART-mediated viral suppression being 125 ± 76 days.

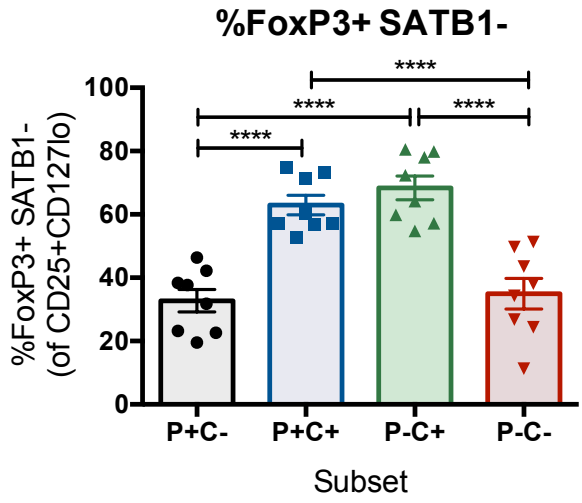


Figure 4.S5. CTLA-4-expressing memory CD4+ T-cells contain higher frequencies of functional FoxP3. Within CD25+CD127low memory CD4+ T cells, frequencies of FoxP3+ SATB1- cells were quantified between each CTLA-4 and PD-1-expressing subset in the LN of 9 ART-treated, SIV-infected RMs at day 90 post-SIV infection (38-41 days post-ART initiation). The results shown are from unstimulated lymphocytes, where CTLA-4 expression was measured intracellularly. Averaged data are presented as the mean ± SEM, and ANOVAs using Tukey's adjustment for multiple comparisons were used to compare differences between subsets. ****, $p<0.0001$.

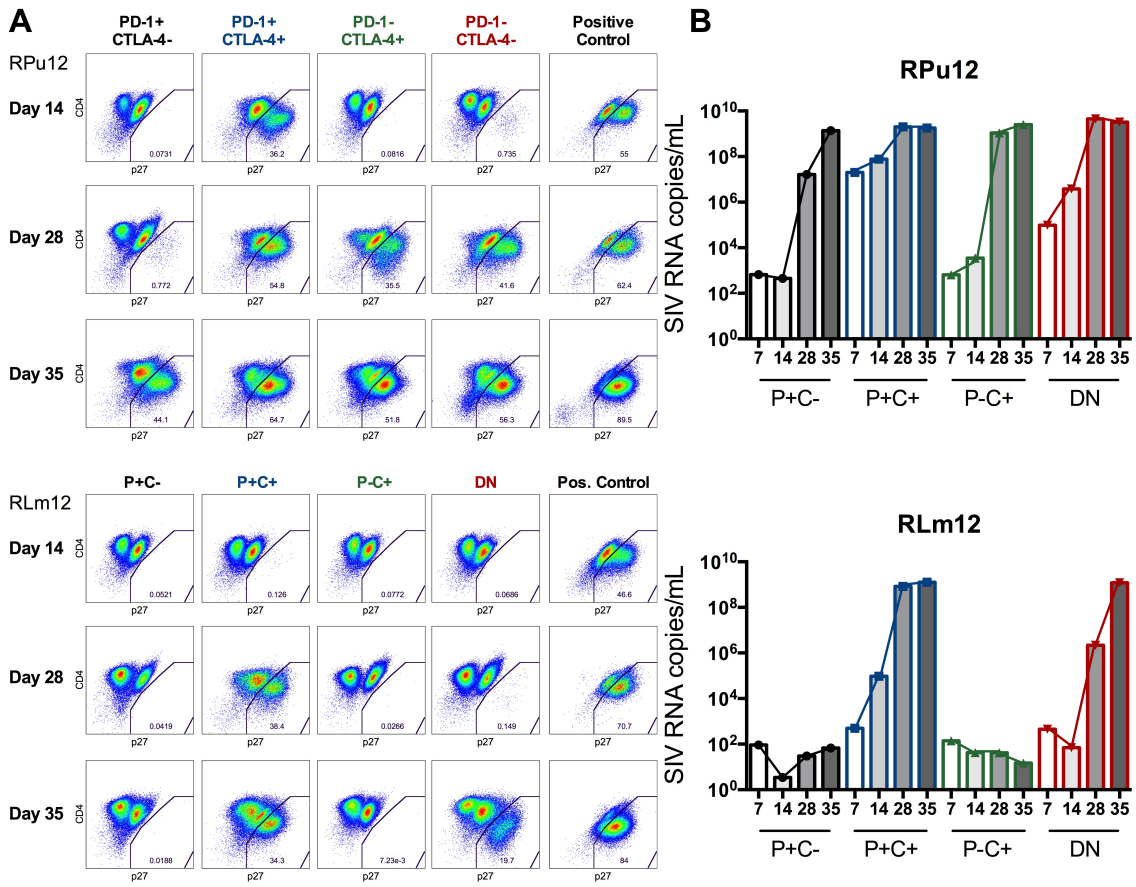


Figure 4.S6. CTLA-4 and PD-1-expressing cells harbor replication competent SIV. (A) Representative flow plots of intracellular SIV*gag* p27 in CEMx174 cells are shown for days 14, 28, and 35 of co-culture in the four CTLA-4 and PD-1 subsets from 2 ART-treated, SIV-infected RM (RPu12, RLm12). Note, these RMs were SIVmac239-infected and were ART-suppressed for 106 and 165 days, respectively. The right column shows SIV*gag* p27 staining for a positive control (CEMx174 cells infected directly with SIVmac). (B) SIV*gag* RNA was quantified longitudinally from the supernatant of the individual co-cultures. Due to cell sample limitations, only PD-1+CTLA-4+ memory CD4+ T cells had replicate wells; the SIV RNA data for this subset represents the average of two replicate wells.

Chapter Five

Discussion

Since the start of the HIV/AIDS epidemic, a fundamental goal has been the development of a preventative vaccine to stop the spread of disease. Thirty years of research efforts and failed trials, though, have demonstrated the need for a deeper understanding of HIV pathogenesis and persistence to facilitate the design of improved vaccine candidates and therapeutics. While the loss of circulating CD4+ T cells was an early documented hallmark of HIV/SIV infection, the preferential loss of CD4+ T cell subsets has been a more recent discovery that has been shown to impact disease progression and severity. These CD4+ T cell subsets have been classified by their memory differentiation state, their functions, and their locations. Therefore, depending on their immunologic roles, the preferential loss of these CD4+ T cell subsets can differentially affect features of the immune response to promote disease progression. Identifying particular CD4+ T cells that are preferentially infected will inform the design of preventative vaccines, for one, but also can elucidate how alterations in CD4+ T cell homeostasis contribute to disease progression.

Apart from their targeting and depletion, the infection of distinct CD4+ T cell subsets also shapes the size and composition of the latent viral reservoir that persists during ART. While memory CD4+ T cells are the predominant contributors to viral persistence, this subset of CD4+ T cells is highly heterogeneous in terms of their functionality and location. Thus, a major question which remains in the field is determining the specific phenotypic and functional profile of latently infected cells during ART. Identification of this profile is critical for the design of more targeted functional cure strategies for HIV-infected individuals. Given the essential role of CD4+ T cells to both viral pathogenesis and persistence, the research described in this dissertation sought to determine which CD4+ T cell subsets have a critical impact on HIV disease progression and persistence.

Contribution of CD4+ T cell subsets to HIV/SIV disease progression

Comparative analysis of SIV infection between natural host sooty mangabeys (SMs) and non-natural hosts rhesus macaques (RMs) has revealed specific patterns of SIV-infected cells *in vivo,* underscoring a model where the subset of CD4+ T cell infected is more critical to disease progression than the number of infected cells (161, 200, 403). For example, in SMs where SIV infection does not progress to AIDS, few CD4+ TCM and TSCM cells are infected by SIV, while, in contrast, they are highly infected in SIV-infected RMs, demonstrating that their preferential infection contributes to disease progression (161, 200). Therefore, protection of these CD4+ T cell subsets may be a key factor used by SMs to limit pathogenesis. Nevertheless, both of these memory CD4+ T cell subsets, which are less differentiated than their corresponding TTM and TEM subsets, possess the ability to repopulate other memory cell subsets (188, 190, 192). As a result, the homeostasis of CD4+ TCM and TSCM may be altered in response to the infection and depletion of more differentiated CD4+ TTM and TEM cells. In describing the relative protection of CD4+ TSCM from viral infection in SIV-infected SMs, Cartwright et al. found that CD4+ TSCM frequencies are maintained over the course of SIV infection in both RMs and SMs, and that their proliferation is critical for repopulating decreasing levels of CD4+ TCM (200). Yet, it was initially unclear if the protection of CD4+ TCM cells in SMs translated into their increased stability during SIV infection.

By examining the levels of CD4+ TCM cells between SIV-infected SMs and RMs, we found that the frequencies of these cells are differently regulated between pathogenic and non-pathogenic SIV infection (Figure 2.1). In fact, our data showed that CD4+ TCM levels are highly stable in SIV-infected SMs, where their decay rate was approximately 20 times slower than in SIV-infected RMs and more related to the animal's aging. These results suggest that, like CD4+ TSCM cells, the preservation of the CD4+ TCM compartment is critical for evading disease

progression. We also found that the fraction of CD4+ TCM cells proliferating increases only in RMs, but not in SMs, following SIV infection (Figure 2.3), an increase which is associated with measures of disease progression including the loss of circulating CD4+ T cells and increased viral load. In SIV-infected SMs, however, the levels of CD4+ TCM proliferation positively associate with levels of CD4+ T cells. These data demonstrate that overall CD4+ cell homeostasis is maintained through the preservation and proliferation of CD4+ TCM in SMs during SIV infection; however, proliferation of CD4+ TCM cells in SIV-infected RMs is unable to maintain CD4+ T cell homeostasis, and may in fact be deleterious by increasing viral targets. Altogether, this research is in agreement with previous work that demonstrated that the depletion of CD4+ TCM cells is the key factor dictating the tempo of progression to AIDS in SIV-infected RMs (160, 201, 409, 410). Moreover, this work adds to this model by illustrating that CD4+ TCM cells, through their increased proliferation, are unable to maintain CD4+ T cell homeostasis during SIV infection of RMs, but instead, promote further viral infection and disease progression. These data have been confirmed in a cohort of long-term non-progressors (LTNPs), supporting the clinical relevance of our findings and emphasizing the overall need to maintain TCM and TSCM CD4+ T cells in order to preserve CD4+ T cell homeostasis (513).

In addition to the tropism of HIV/SIV for distinct memory CD4+ T cell subsets, HIV also differentially targets functional CD4+ T cell subsets to contribute to viral pathogenesis and persistence. Th17 cells, a helper CD4+ T cell subset localized primarily in mucosal tissues, are preferentially lost from HIV/SIV-infected humans/RMs, but preserved in SIV-infected SMs (135-137, 180, 220). Given their functional role in antimicrobial defense and intestinal barrier integrity, the preferential loss of these Th17 cells is associated with numerous consequences which hasten disease progression, including permeability of the mucosal barrier, increases in microbial translocation, and systemic immune activation (136, 137, 180, 430, 431). Th17 cells can also be phenotypically characterized by their surface expression of CCR6 and CD161, two

molecules which coordinate the migration of Th17 cells and their precursors to the gut mucosa. Previous reports had demonstrated the loss of CCR6+ and CD161+ CD4+ T cell subsets from the blood of HIV-infected individuals. By examining the kinetics of these CD4+ T cell subsets longitudinally in different anatomic locations of RMs, we found that CCR6+CD161- CD4+ T cells are predominantly lost from circulation due to their accumulation in the gut mucosa during untreated SIV infection. This accumulation was detected as early as 4 weeks post infection in CCR6+CD161- CD4+ T cells (Figure 3.4A) and was maintained throughout chronic SIV infection.

However, previous reports have demonstrated the irreversible loss of mucosal Th17 cells during HIV infection (135). Therefore, our results would indicate that CCR6+CD161- and CCR6+CD161+ CD4+ T cells migrating to the mucosal tract during untreated HIV/SIV infection are not functioning as Th17 cells at this site. Indeed, when we examined the cytokine production of CCR6 and CD161-expressing CD4+ T cells in RB, we found that only 10.9±2.3% of CCR6+CD161- CD4+ T cells produced IL-17 in healthy RMs (Figure 3.1D). Instead, of CCR6 and CD161-expressing CD4+ T cells, CD161+CCR6- CD4+ T cells were greater contributors to IL-17 production in rectal tissues, although CCR6-CD161- contributed the most in the gut (Figure 3.1C). Additionally, we were unable to find any correlations between the frequencies of Th17 cells and CCR6+ CD4+ T cells during SIV infection of RMs (data not shown). These data suggest that the accumulation of CCR6+CD161- CD4+ T cells, in particular, is not beneficial in restoring mucosal Th17 cells in SIV-infected RMs. As elaborated in Chapter 3, the inability of CCR6+CD161- and CCR6+CD161+ CD4+ (CCR6+CD4+) T cells to act as Th17 cells in the mucosa during SIV infection may result from a combination of factors, including a lack of polarizing cytokines present in the mucosal environment, decreased levels of cytokine receptors on the surface of CCR6+ CD4+ T cells to prevent their further polarization, as well as an upregulation of negative regulatory genes to prevent IL-17 production. One alternative hypothesis

not discussed in Chapter 3, though, was the possibility that CCR6+CD4+ T cells accumulating in the rectal mucosa may be acting as regulatory T cells (Tregs), as opposed to Th17 cells. Mercer et al. identified a subset of IL-17-producing Tregs that could differentiate from naïve CCR6+ CD4+ T cells in the presence of IL-2, IL-1B, TGF-β, and IL-23 (514). Similar to the CCR6+CD161- CD4+ T cells described in our study, IL-17 Tregs accumulate in the gut of individuals with high levels of inflammation, where they possess pathogenic potential (515, 516). Additionally, IL-17 Tregs are preferentially reduced from the blood of HIV-infected patients, which parallels the kinetics of CCR6+CD4+ T cells and may similarly reflect their redistribution. Thus, future studies will be critical in determining if CCR6+CD161- CD4+ T cells have suppressive capacities, particularly in the gut mucosa, of SIV-infected RMs (514, 517).

We found that increased levels of CCL20, the exclusive ligand for CCR6, by cells in the rectal mucosa coordinated the migration and accumulation of CCR6+CD4+ T cells in SIV-infected RMs (Figure 3.5B). Heightened levels of CCL20 were expected during untreated SIV infection, due to the presence of microbial translocation and localized inflammation in the mucosa. However, following the initiation and maintenance of ART, the accumulation of CCR6+CD161- CD4+ T cells in the mucosa was lost, suggesting their migration out of the gut in response to diminished levels of CCL20 there (450). These results suggest, then, that mucosal accumulation may be a mechanism by which HIV/SIV facilitates viral spreading. In this model, HIV/SIV targets Th17 cells in mucosal tissues early during acute infection, causing preferential cell loss, increased levels of inflammation, and the early breakdown of intestinal barrier integrity. Resulting increases in CCL20 production recruit CCR6+CD4+ T cells to mucosal tissues, where, due to their activation status (Figure 3.2), they are susceptible to SIV infection. Infected CCR6+ CD4+ T cells that are not lost due to viral cytopathic effects are then able to migrate out of the mucosa following ART-mediated viral suppression, which promotes the spread of virus throughout the host. This model would be consistent with a recent study that found reduced levels

of CCL20 in the mucosa of ART-treated, HIV-infected individuals, and a subsequent lack of CCR6+ CD4+ T cells localized there (450). Moreover, preliminary data from 2 SIV-infected RMs demonstrated increased frequencies of CCR6+CD4+ T cells in the LN following prolonged ART suppression (data not shown). Interestingly, CCR6+CD4+ T cells also express significantly higher levels of α4β7 compared to CD4+ T cells lacking CCR6 expression (Figure 3.6), an integrin which facilitates migration of expressing cells to mucosal tissues (518, 519). Previous reports have found that CD4+ T cells expressing α4β7 are preferential targets of HIV and SIV (286-290). Thus, high levels of α4β7 expression on CCR6+CD4+ T cells likely contributes to both the accumulation of CCR6+CD4+ T cells in the mucosa of SIV-infected RMs, but also their susceptibility to SIV infection. Importantly, α4β7 blockade was recently found to yield virologic control to SIV-infected RMs that interrupted their ART regimen (520). We speculate that a contributing factor to this virologic control was the ability of α4β7 blockade to specifically block the migration of CCR6+CD4+ T cells to the mucosa, particularly after the cessation of ART and the restoration of active viral replication in the gut of these SIV-infected RMs. Examining the kinetics of CCR6+ CD4+ T cells in different anatomic sites during α4β7 blockade treatment would better elucidate the impact of this subset in effectively controlling viremia.

In addition to promoting viral spread throughout the host, the accumulation of CCR6+CD161- and CCR6+CD161+ CD4+ T cells in the gut mucosa may also be contributing to viral persistence in CCR6-expressing CD4+ T cells. In fact, Khoury et al. found that CXCR3+CCR6+ CD4+ T cells, within the central memory compartment, harbored the highest levels of integrated HIV DNA in HIV-infected patients on ART for a minimum of 3 years (234). These results suggest that either CCR6+CD4+ T cells are infected at higher frequencies during productive infection or that virally infected CCR6+CD4+ T cells have an increased capacity to persist during ART. Although we were unable to find a preferential infection of CCR6+CD161- CD4+ T cells in the blood or LN of SIV-infected RMs (Figure 3.7A), these findings do not exclude the possibility that

CCR6+CD4+ T cells are preferentially infected in the mucosal tissues, an analysis that we were unable to complete due to the low frequency of CD4+ T cells found there in late chronic SIV infection. Indeed, several groups have found that CCR6+ Th17 cells are highly infected during productive HIV infection (221, 223, 432, 521). Thus, the contribution of CCR6+CD4+ T cells to viral persistence likely results, at least in part, from their high infectivity during active viral replication. Th17 cells have also been shown to be self-renewing and undergo homeostatic proliferation (231). Therefore, the persistence of virus in CCR6+CD4+ T cells, particularly in the blood where they produce IL-17, may also result from these cells expanding through this proliferation mechanism. Finally, suboptimal penetration of ART in mucosal tissues may facilitate the ongoing infection of CCR6+CD4+ T cells in the gut of ART-treated HIV-infected patients (388). Further studies that are able to quantify the levels of viral DNA and RNA in mucosal CCR6+CD4+ T cells, both during active and ART-suppressed infection, will be critical for dissecting the major mechanism of viral persistence within CCR6+CD4+ T cells.

We found that the accumulation of CCR6+CD161- and CCR6+CD161+ CD4+ T cells in mucosal tissues was a distinguishing feature of pathogenic SIV infection, as levels of these cell subsets were maintained or decreased in RB of SIV-infected SMs (Figure 3.9C). These data demonstrate how CCR6+CD4+ T cell accumulation in the gut mucosa can be damaging to the host and contributes to disease progression. Therefore, blocking CCR6+CD4+ T cell accumulation to the gut represents a novel therapeutic strategy for HIV-infected individuals to potentially reduce inflammation, viral seeding and viral spreading. One strategy for blocking CCR6+CD4+ T cell accumulation would be blocking the expression of its ligand, CCL20. Previous studies that have tested glycerol monolaurate (GML), a molecule that inhibits CCL20 production by vaginal epithelial cells, demonstrated its ability to protect RMs from intravaginal SIV challenge if administered directly before challenge (456, 522). As a result, further research is being conducted to formulate a sustained release GML that could suppress chemokine levels for prolonged

periods, and ultimately reduce target cell recruitment to a major site of viral replication. An additional rationale for blocking CCR6+CD4+ T cell recruitment to mucosal tissues, we speculate, comes from the results of a recent α4β7 blockade study, in which reducing migration to the GALT sustained virologic control in SIV-infected RMs (520). Further studies are needed in RMs to determine if blocking CCL20, specifically, in the context of ART suppression is effective in reducing viral spread and persistence. Alternatively, CCR6 antagonists are currently being developed for the treatment of human autoimmune diseases, such as psoriasis and rheumatoid arthritis, in which pathogenic CCR6+ Th17 cells cause inflammation and tissue damage. If validated and potent in these disease systems, CCR6 antagonists could be tested in SIV-infected RMs to measure their potency in blocking CCR6+ CD4+ T cell accumulation in the gut mucosa and reducing subsequent damage to the host.

In examining the mechanisms behind CCR6+ and CD161+ CD4+ T cell loss during SIV infection of RMs, we also found that viral infection was a central contributor to the loss of CD161+CCR6- CD4+ T cells. Although additional mechanisms likely further the depletion of these cells during SIV infection, we found that CD161+CCR6- CD4+ T cells expressed significantly higher levels of CCR5 (Figure 3.2), harbored higher levels of SIV RNA (Figure 3.7C), and were depleted from all anatomic locations that we surveyed (WB, LN, and RB; Figure 3.4A), thus supporting a critical role of viral infection in depleting this cell subset. Moreover, the loss of CD161+CCR6- CD4+ T cells was unique to pathogenic SIV infection and were unseen in SIV-infected SMs (Figure 3.3A), demonstrating that the loss of this subset contributes to disease progression. The preferential depletion of CD161+CCR6- CD4+ T cells may contribute to the inability of ART to fully restore Th17 levels, since CD161 is expressed on Th17 precursor cells (445, 446). However, we did find that approximately 2 months of ART administration was able to increase CD161+CCR6- CD4+ T cell levels in the gut mucosa to near-normal (pre-SIV) levels (Figure 3.S5). Nevertheless, ART is unable to restore CD161+CCR6- CD4+ T cell levels in the blood

(Figure 3.S5). Therefore, it is unclear how CD161+CCR6- CD4+ T cells contribute to Th17 cell homeostasis- that is, does their preferential depletion contribute to the irreversible loss of Th17 cells during HIV/SIV infection? Additionally, given their high levels of IFN-γ production in the blood, further studies are needed to determine where CD161+CCR6- CD4+ T cells fall along the pro-inflammatory continuum.

Another potential impact of the preferential infectivity of CD161+CCR6- CD4+ T cells may be their contribution to TFH cells. In support of this model, we found CD161+CCR6- CD4+ T cells expressed significantly higher levels of CXCR3 than the other CCR6 and CD161-expressing CD4+ T cell subsets (Figure 3.S4B), a phenotypic feature of preferentially infected TFH cells (509). Consistent with this finding, we found CD161+CCR6- CD4+ T cells harbored significantly higher levels of RNA in the LN, but not in the blood (Figure 3.7B,C), despite comparable levels of DNA, supporting the increased transcriptional activity of this CD4+ T cell subset. In contrast, CD161+CCR6- CD4+ T cells express significantly high levels of the SIV co-receptor CCR5 in the LN (Figure 3.S4A), while TFH cells have been shown to express low levels of CCR5 (262, 523). Therefore, understanding the relationship between CD161 expression and TFH cells will be critical in dissecting the role of this cell subset's preferential infectivity in the LN. Overall, these findings underscore the importance of maintaining CD4+ T cell homeostasis to avoid disease progression and implicate both memory (Chapter 2, TCM) and functional (Chapter 3, Th17-like) CD4+ T cell subsets as viral targets that, when disrupted, advance disease progression in the infected host.

HIV Eradication

Despite the ability of combination ART to suppress viral replication to below clinical levels of detection, ART is unable to cure HIV infection due to the presence of a long-lived pool of latently infected cells that persists and invariably leads to viral rebound upon ART cessation (356). In

fact, studies that have examined the decay of persistently infected cells with ART have estimated that it would take over 60 years on ART for patients to eradicate the latent reservoir (356, 473, 524). Given these estimates, the task of eradicating HIV from infected persons was largely considered unachievable in the early 2000s. However, the report of the Berlin patient in 2009 restored hope in achieving HIV eradication (525). Timothy Ray Brown was an ART-suppressed, HIV-infected individual that was diagnosed with acute myelogenous leukemia (AML). In order to treat the AML, Brown received a bone marrow transplant from a donor who was homozygous for a 32 basepair depletion in the HIV-1 coreceptor CCR5 (CCR5 del32). As a result, Brown's immune cell compartment was reconstituted with HIV-resistant cells, so that upon interrupting ART, there has been no detectable viral rebound (525, 526). To this day, Brown, now known as the "Berlin patient," is the only patient that has been "cured" of HIV, and thus, serves as a benchmark for achieving HIV eradication and continuing research to find strategies for an HIV cure.

Given its success in the Berlin patient, hematopoietic stem cell transplantation (HSCT) emerged as a potential strategy for curing HIV infection. However, no studies, to date, have been able to repeat their results. In one report, two HIV-infected individuals (the "Boston patients") received allogeneic HSCT for refractory lymphoma, though from individuals with wild-type CCR5+ cells, and subsequently experienced viral rebound 12 and 32 weeks post-ART interruption (527, 528). A separate study performed autologous HSCT on ART-treated, SIV-infected RMs to assess its effects on reducing the size of the viral reservoir (529). Yet, while virus rebounded in only 2 of the 3 transplanted animals, SHIV DNA was detected in tissues from all 3 animals that had underwent myeloablation, demonstrating that the treatment was insufficient to reset the hematopoietic compartment and eliminate persistent virus in these animals. These studies highlight the challenges surrounding HSCT as an HIV eradication strategy, but also underscore

the potential mechanisms that were critical for the Berlin patient's cure, including a graft versus host effect as well as the use of a CCR5 del32 donor.

As highlighted throughout this dissertation, HIV is able to persist throughout ART because it integrates into the host genome of resting CD4+ T cells, which remain transcriptionally silent, thereby subverting detection by the immune system (356). This mechanism of persistence has been the rationale behind a second leading strategy for HIV eradication, known as "shock and kill." In this strategy, the administration of drugs that are able to reactivate the reservoir ("shock") will induce viral synthesis, production, and release, which would promote the killing of virally infected cells by the immune system ("kill"). Moreover, effector mechanisms have been proposed to complement these reactivating agents to boost the ability of the immune system to clear virally infected cells. If successful, this combination approach may be able to force HIV out of "hiding" and significantly reduce the pool of latently infected cells comprising the reservoir, thus establishing a functional cure.

The leading group of reactivating agents, latency reversing agents (LRAs), have shown modest effects in increasing HIV RNA production *in vivo* (530). However, few of these interventions have been able to demonstrate a meaningful reduction in HIV reservoir size nor resulted in HIV remission (475, 531, 532). LRAs may be limited in their reservoir reduction potential due to their non-specific targeting of latently infected cells, for one (476). Yet, an additional limitation of LRAs is their reliance on the host immune response to eliminate newly activated, infected cells, since several studies have demonstrated the waning antiviral CTL response following chronic HIV/SIV infection (106, 126, 144, 346, 347, 509). These findings support the incorporation of additional interventions that significantly enhance the antiviral immune response in order to complement the reactivating effects promoted by LRAs and effectively clear latently infected cells.

Therapeutic vaccines, monoclonal antibodies, and immune checkpoint blockades (ICBs) have all been explored as strategies to increase virus-specific immune responses. Although the majority of early generation therapeutic vaccines failed to show strong virologic benefit by expanding dysfunctional pre-existing T cell clones, next generation vaccines are currently being developed that expand CD8+ T cells specific for novel epitopes. For example, a recent study that used cytomegalovirus (CMV) vectors as a prophylactic vaccine found that over half of vaccinated RMs successfully cleared SIVmac251 upon challenge, due in part to the generation of unconventional class II-restricted CD8+ T cell responses (533). Nevertheless, the clinical development of these CMV vectors has been limited by their replicative nature. Broadly neutralizing monoclonal antibodies (bnAbs) to HIV have also been expanded recently, with the identification of a next generation of antibodies able to target novel epitopes on the HIV-1 envelope (534). As a result of their ability to neutralize free virus, these bnAbs could both reduce chronic immune activation to indirectly increase antiviral T cell responses, but also could be used in combination with LRAs to clear newly reactivated virus. However, the ability of these bnAbs to access anatomic sites of viral persistence, such as the CNS, remains to be seen.

A third strategy of augmenting the antiviral immune response has been the administration of ICBs, which possess the dual ability to increase HIV-specific CD8+ T cell function as well as potentially induce viral reactivation from latently infected CD4+ T cells. The success of PD-1 blockade in restoring T and B cell functionality in SIV-infected RMs, while having a moderate impact on viremia, has provided support to the idea of combining this intervention with ART in order to improve the function of exhausted antiviral T cells (509). Moreover, PD-1 has been shown to identify memory CD4+ T cells enriched in HIV DNA from long-term ART-suppressed individuals, particularly in the LN, suggesting that PD-1 blockade would also target virally enriched CD4+ T cells (202, 278). Yet, virus-specific CD4+ T-cells upregulate multiple Co-IRs,

including PD-1, CTLA-4, and TIM-3, in the setting of HIV and SIV infection (124, 125, 143, 479). Therefore, a remaining research question was if additional Co-IRs, apart from PD-1, denote latently infected cells that could be targeted by ICBs to promote a functional cure.

Contribution of CD4+ T cell subsets to HIV/SIV persistence during ART

Using ART-treated, SIV-infected RMs, we were able to identify CTLA-4+PD-1- memory CD4+ T cells as a previously unrecognized component of the SIV reservoir. We found that CTLA-4+PD-1- memory CD4+ T cells, a subset comprised predominantly of Tregs, were significantly enriched in SIV DNA in multiple tissue compartments, including the blood, LN, spleen, and gut, and contained robust levels of replication-competent virus. Additionally, the contribution of CTLA-4+PD-1- Treg cells to the viral DNA pool increased with prolonged ART-mediated viral suppression.

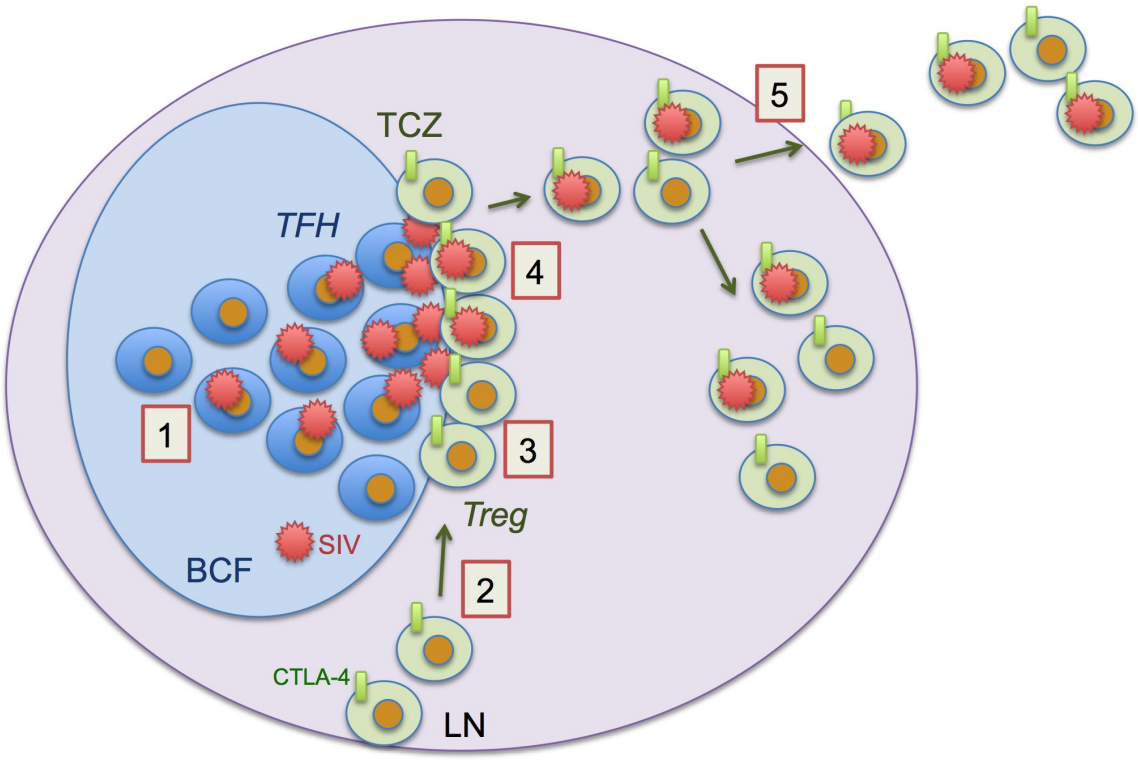
The preferential infection of these CTLA-4+PD-1- Tregs may be a result of both their function and their location. As discussed in *Functional CD4+ T cell subsets* (Chapter 1), the function of Tregs during HIV/SIV infection is unclear. While Tregs likely limit tissue damage resulting from CTLs, they may also impair the adaptive immune response by suppressing the elimination of virally infected cells. Even the kinetics of Tregs during HIV/SIV infection has been debated, with several studies noting decreased numbers in the blood but increases in the tissues, while others found depletion from tissues as well (158, 159, 239, 243, 248, 510, 535-538). We speculate that CTLA-4+PD-1- Tregs are highly susceptible targets for HIV/SIV infection while functioning to dampen activation during peak viral replication. In support of this model, we found that the levels of CTLA-4+PD-1- memory CD4+ T cells were inversely correlated with levels of proliferating CD4+ T cells, suggesting that this cell subset is associated with suppressing cell proliferation (data not shown). However, one limitation to this finding is that CTLA-4, in addition to its constitutive expression on Tregs, is transiently expressed by newly activated T cells; thus, CTLA-

4+PD-1- cells may not solely delineate Tregs in this setting. Nevertheless, due to their absence of PD-1, another marker for activated CD4+ T cells (539), and their enrichment in the Treg markers CD25+CD127lo FoxP3+ (Figure 4.3B), we believe that CTLA-4+PD-1- memory CD4+ T cells function predominantly as Tregs.

The viral enrichment of CTLA-4+PD-1- memory CD4+ T cells may also result from their location. We find that the frequencies of CTLA-4+PD-1- memory CD4+ T cells in the LN during peak viral infection (day 14) can predict the levels of virus seeded in these cells in the LN prior to ART initiation (Figure 4.6A) as well as their levels in memory CD4+ T cells after ART-mediated viral suppression at necropsy (Figure 4.6C). Interestingly, viral seeding in CTLA-4+PD-1- cells (PreART) is specifically associated with the levels of viral DNA found in PD-1+CTLA-4+ memory CD4+ T cells in the LN at necropsy (Figure 4.6G), a subset of cells which includes the highest fraction of TFH cells among the PD-1 and CTLA-4-expressing subsets (Figure 4.3C). These data support a model in which CTLA-4+PD-1- Tregs closely interact with productively infected TFH cells at the TCZ/BCF interface and that this cross talk promotes the preferential infection of CTLA-4+PD-1- CD4+ T cells. Several recent studies have described how Tregs control and regulate the numbers of TFH cells and GC formation through CTLA-4, supporting the direct interaction of these two subsets (540, 541). In fact, the depletion of Tregs, in these models, results in significant TFH/GC expansion and heightened B cell responses as a result. In our study, the level of virus found in PD-1+CTLA-4+ cells in the BCF prior to ART initiation could predict the frequencies of activated (CD39+) CTLA-4+PD-1- Tregs 38-41 days into ART administration (Figure 5.1A), highlighting that these Tregs are activated to suppress viral replication by the nearby TFH cells. Therefore, in our proposed model, CTLA-4+PD-1- Tregs in the LN interact with TFH (PD-1+CTLA-4+) cells at the TCZ/BCF interface in an effort to control their expansion and activation; yet, upon these interactions, TFH cells, with their high levels of viral RNA, are able to infect CTLA-4+PD-1- Tregs to increase their viral DNA levels and

facilitate their viral persistence (see Model 1 below). Indeed, the greater the expression of CXCR5 on CTLA-4+PD-1- memory CD4+ T cells at day 90 post-infection (38-41 days post-ART initiation), the higher their levels of viral DNA at necropsy (Figure 5.1B). Thus, in the few Tregs which express CXCR5 (Figure 4.3F), CXCR5 likely directs their migration to the TCZ/BCF border, where they are able to be directly infected by virus produced by TFH cells. Importantly, though, we found that virus persisted in CTLA-4+PD-1- CD4+ T cells outside the B-cell follicle (Figure 4.4A).

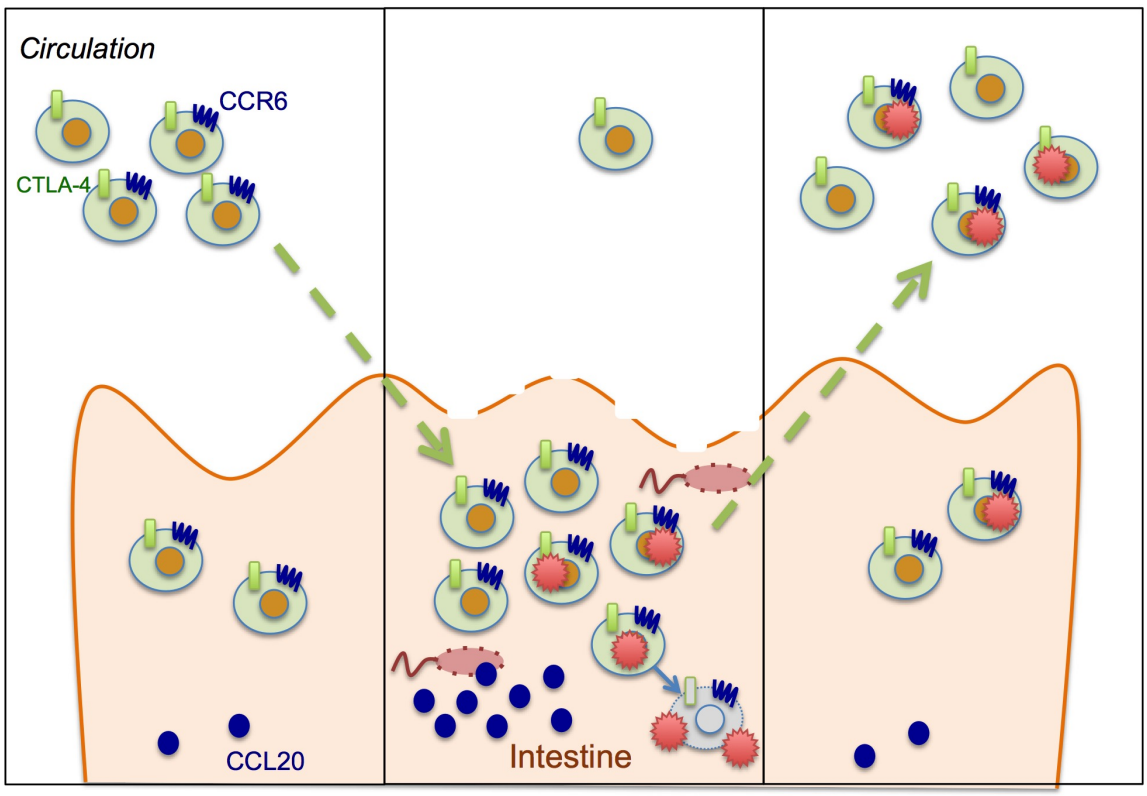
This relationship and infection mechanism is further supported by our data that the levels of SIV RNA within PD-1+CTLA-4+ TFH-like cells (in the LN) are directly associated with the SIV DNA levels within CTLA-4+PD-1- memory CD4+ T cells in the LN at necropsy (Figure 5.1C). These data are consistent with the recent finding by Banga et al. that PD-1+ TFH cells from long-term ART-treated, HIV-infected individuals constitute a more active viral reservoir in the LN (278). Therefore, despite ART-mediated viral suppression in the periphery, we speculate that viral infection may be ongoing between Tregs and TFH cells at the interface of the GC in the LN as a result of TFH active viral production.



Model 1. CTLA-4+PD-1- CD4+ T cell infection in the LN. 1) PD-1+CTLA-4+ TFH cells are localized in the B-cell follicle of the LN and actively produce virus. 2) CTLA-4+PD-1- Tregs migrate into the T-cell zone (TCZ) of the LN, through their CCR7 expression, where they move to the TCZ/BCF interface. 3) CTLA-4+PD-1- Tregs become activated (CD39+) as they suppress virus-producing TFH cells. 4) Activated CTLA-4+PD-1- Tregs become infected as a result of their interaction with productively infected TFH cells. 5) Infected Tregs are maintained within the LN or migrate out of LN, where they persist as a result of lower apoptosis levels.

In addition to the LN, CTLA-4+PD-1- memory CD4+ T cells may also be infected in the gut mucosa. We have found that CTLA-4+PD-1- memory CD4+ T cells express significantly higher levels of CCR6 than other PD-1 and CTLA-4-expressing subsets in the blood of healthy RMs (Figure 5.2A,B), which could direct their migration to the gut mucosa as discussed in Chapter 3. Consistent with the kinetics of CCR6+ CD4+ T cells that we demonstrated in Figure 3.4A, we found an accumulation of CTLA-4+PD-1- memory CD4+ T cells in the gut mucosa during acute

SIV infection (Figure 4.S4B). We hypothesize that this relocalization supports the increased infection of CTLA-4+PD-1- memory CD4+ T cells by accumulating in a major site of viral replication. Interestingly, we found that the levels of CTLA+PD-1- memory CD4+ T cells decrease in the gut mucosa upon ART initiation and maintenance (Figure 4.S4B), which agrees with the migration of CCR6+ CD4+ T cells out of the mucosa during ART (Figure 3.9A). Furthermore, the frequencies of CTLA-4+PD-1- memory CD4+ T cells expressing CCR6 in RB significantly decrease in the gut mucosa following ART initiation, while they increase in the blood (Figure 5.2A,B). These data, thus, support a model in which CTLA-4+PD-1- memory CD4+ T cells migrate to the gut mucosa during active viral infection, where they likely function as Tregs, become increasingly infected, and subsequently persist during ART by migrating out of the gut (see Model 2 below). This model of viral infection and persistence may also explain why the frequency of CTLA-4+PD-1- memory CD4+ T cells harboring SIV DNA increases in the blood following a prolonged period of ART, but not in tissues such as the LN (Figure 4.S3).



Model 2. CTLA-4+PD-1- CD4+ T cell infection in the gut mucosa. In a healthy state, the majority of CCR6+CTLA-4+PD-1- CD4+ T cells are found in circulation, with a small frequency localized in the gut mucosa. During acute SIV infection, CTLA-4+PD-1- memory CD4+ T cells accumulate in the gut mucosa, likely driven by their CCR6 expression, where they become activated and are susceptible to viral infection. While a fraction of CTLA-4+PD-1- CD4+ T cells die as a result of cytolytic effects of the virus, a fraction persist and, following ART initiation as CCL20 levels decrease, CTLA-4+PD-1- cells are able to reenter circulation where they persist and contribute to the viral reservoir.

Upon their preferential infection by HIV/SIV, CTLA-4+PD-1- memory CD4+ T cells increasingly contribute to the persistent viral reservoir, in part due to their heightened resistance

to apoptosis (Figure 4.7A, B). We found that CTLA-4+PD-1- CD4+ T cells expressed the highest levels of the antiapoptotic molecule Bcl2 (Figure 4.7A), in addition to the highest levels of pSTAT5 (Figure 4.7B), supporting their persistence despite high levels of viral infection. These data are consistent with previous reports that have found that Tregs are more resistant to apoptosis following LCMV infection, another chronic viral infection (542). Furthermore, Bach2 has been demonstrated to promote the differentiation, activation, and survival of FoxP3+ Tregs (502). Interestingly, recent data has found Bach2 to be a frequent integration site for HIV in long-term ART-suppressed patients, which agrees with our findings of increased infectivity and persistence of CTLA-4+PD-1- memory CD4+ T cells (503).

These findings and models underscore the need for future studies to further elucidate the mechanism of CTLA-4+PD-1-/Treg infectivity and viral persistence. First, additional studies will be essential for determining if CTLA-4+PD-1- memory CD4+ T cells function as Tregs in SIV-infected RMs. Preliminary data from our group has found decreased levels of IFN-γ production by CTLA-4+PD-1- memory CD4+ T cells upon PMA/Ionomycin stimulation compared to PD-1-expressing subsets (data not shown) in ART-treated SIV-infected RM PBMCs. Yet, further experiments that more comprehensively assess the cytokine production of these CTLA-4+PD-1- memory CD4+ T cells will help delineate the IL-2, IL-10, and TGF-β production levels of this subset, and subsequently, its Treg potential. Additionally, conducting a T-cell suppression assay using CTLA-4+PD-1- CD4+ T cells co-cultured with CD8+ T cells will be critical to assess this subset's ability to block the proliferation of T cells. A second question which arises from this set of data is understanding the plasticity between CTLA-4+PD-1- memory CD4+ T cells and the other Co-IR expressing subsets. While the low levels of CXCR5 (Figure 4.3F) and IFN-γ production (data not shown) by CTLA-4+PD-1- memory CD4+ T cells would suggest that this subset is distinct from the other PD-1 and CTLA-4-expressing subsets, it is possible that these memory CD4+ T cell subsets transiently up and downregulate their Co-IRs. Although this

plasticity will be difficult to test *in vivo*, examining the expression patterns of Co-IRs *in vitro* may provide some evidence of their regulation *in vivo*. Finally, we were unable to examine how these CTLA-4+PD-1- memory CD4+ T cells are maintained in a latent state. CTLA-4 is known to outcompete CD28 to bind CD80/CD86 and prevent T cell activation (543-545), which would support its ability to maintain CD4+ T cells in a resting state. Moreover, a high frequency of HIV-specific CD4+ T cells express CTLA-4; therefore, administration of CTLA-4 blockade, by preventing signaling through CTLA-4, may have the ability to increase T cell activation and viral production from these latently infected cells. Indeed, previous studies have found increased viral replication in SIV-infected RMs treated with CTLA-4 blockade (511). Therefore, determining if CTLA-4 blockade is able to promote viral reactivation and decrease the size of the viral reservoir in ART-treated, SIV-infected RMs will clarify the role of CTLA-4 in maintaining viral latency and promoting viral persistence.

In addition to the viral enrichment that we found within CTLA-4+PD-1- memory CD4+ T cells, our study also demonstrated that PD-1+CTLA-4+ memory CD4+ T cells are the major contributors to the viral reservoir throughout the body of ART-treated, SIV-infected RMs. Although PD-1+CTLA-4+ memory CD4+ T cells harbored lower levels of SIV DNA on a per cell basis when compared to CTLA-4+PD-1- cells, the high frequencies of PD-1+CTLA-4+ cells within the memory CD4+ T cell compartment account for their increased contribution to viral persistence (Figure 4.2B). Indeed, PD-1+CTLA-4+ cells made up the highest fraction of the memory CD4+ T cell compartment across all tissues accessed in the SIV-infected RMs at necropsy (Figure 4.S4B). An important implication of this finding comes from the phenotypic overlap of PD-1+CTLA-4+ memory CD4+ T cells and TFH cells (Figure 4.3C). Examination of their anatomic compartmentalization within the LN revealed that PD-1+CTLA-4+ cells are localized in the GC, which, in combination with their higher expression of Bcl6 (Figure 4.3D), is consistent with their TFH characterization (Figure 4.4A). Moreover, in agreement with our cell-

associated DNA measurements, we found that the GC in the LN BCF contained the highest levels of SIV with prolonged ART, harbored primarily in PD-1+CTLA-4+ cells (Figure 4.3B). These data confirm a recent report describing PD-1+ TFH cells as the major contributor to viral persistence in ART-treated, HIV-infected individuals (278). We have recently initiated a PD-1/CTLA-4 dual blockade trial to be administered in ART-suppressed, SIV-infected RMs in an effort to reduce the size of the viral reservoir. Given our findings revealing the enrichment and contribution within PD-1 and CTLA-4-expressing memory CD4+ T cells, combined with the known dysfunction of Co-IR-expressing antiviral CD8+ T cells, we hypothesize that this blockade strategy will result in a delay in viral rebound and potential virologic control of the host following ART cessation.

Summary

Ultimately, this dissertation contributes to our understanding of which CD4+ T cell subsets are critical for HIV pathogenesis and persistence. Chapter 2 revealed that the proliferation of CD4+ TCM is unable to restore CD4+ T cell homeostasis in SIV-infected RMs, but instead, augments disease progression. Chapter 3 demonstrated that CCR6+CD4+ T cells, through their loss from the blood and LN, accumulate in the gut mucosa to support disease progression. Additionally, the preferential loss of CD161+CCR6- CD4+ T cells also contributes to advancing the infection state and may influence the lack of Th17 restoration in HIV/SIV-infected hosts. Chapter 4 then focused on co-inhibitory expression and viral persistence to identify CTLA-4 and PD-1 as markers of virally enriched, persistent CD4+ T cells in ART-treated, SIV-infected RMs. This final chapter, in particular, highlighted the previously underappreciated role of CTLA-4+PD-1- Treg-like CD4+ T cells in viral persistence, where their high levels of infection and persistence result in increasing contributions to the viral reservoir over time. As a result, these findings will inform the design of novel HIV therapeutics aimed at restoring CD4+ T cell homeostasis and achieving a functional cure.

Figures

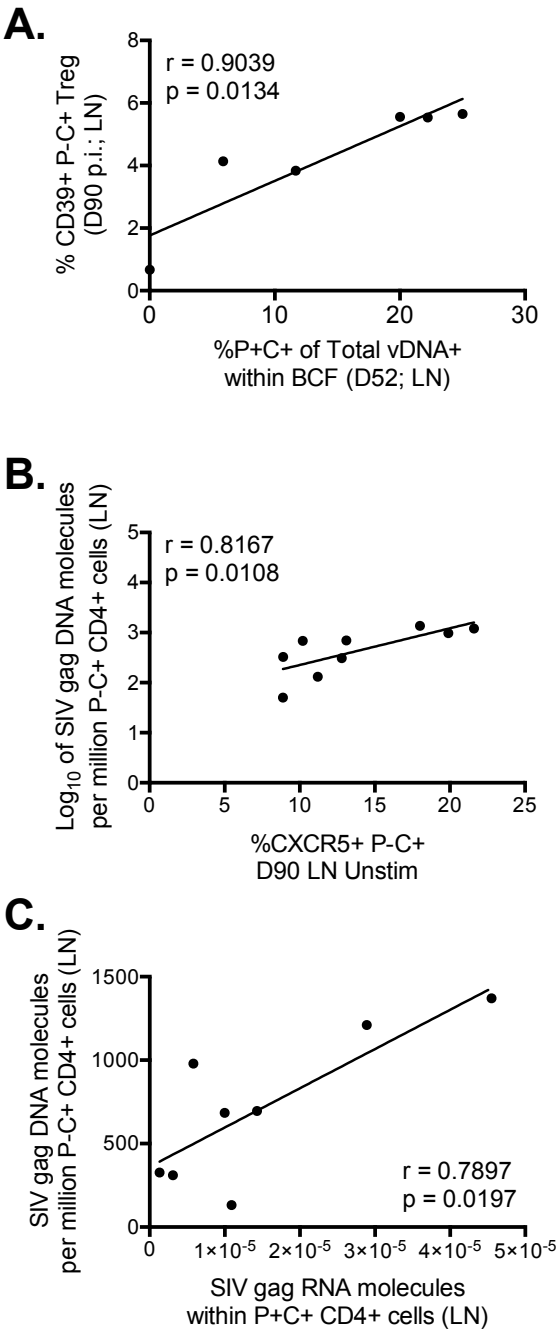


Figure 5.1. Interactions between PD-1+CTLA-4+ TFH and CTLA-4+PD-1- Treg cells promote viral seeding and persistence. (A) The correlation between the fraction of SIV DNA levels in PD-1+CTLA-4+ (P+C+) in the BCF preART initiation (D52) and the levels of activated CTLA-4+PD-1- CD4+ T cells (CD39+ P-C+) in the LN D90 p.i. is shown. n=6. The correlation

between the levels of CXCR5 on CTLA-4+PD-1- CD4+ T cells in the LN D90 p.i. (B), as well as the levels of SIV RNA within PD-1+CTLA-4+ memory CD4+ T cells in the LN at necropsy (C), were related to the frequencies of memory CTLA-4+PD-1- CD4+ T cells harboring SIV DNA in the LN at necropsy. SIV DNA levels were log_{10} transformed prior to plotting in (B). n=9. All statistical analyses were performed using Spearmen correlation tests.

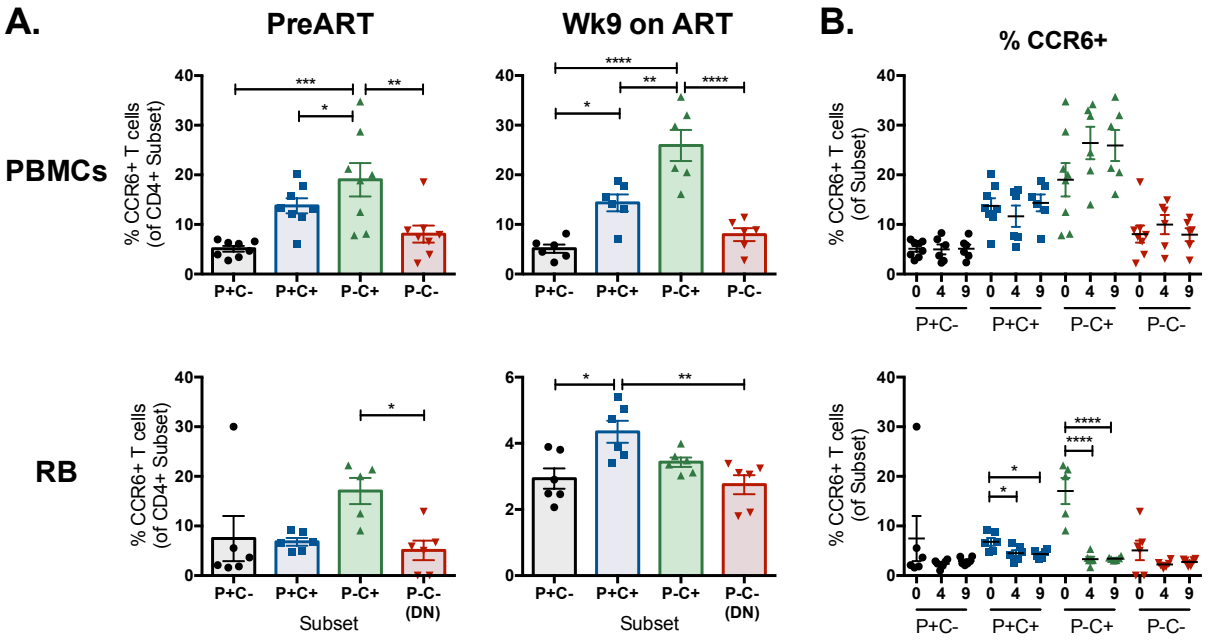


Figure 5.2. CTLA-4+PD-1- memory CD4+ T cells express CCR6 to coordinate their migration during active viral infection. (A) Frequencies of CCR6+ cells were quantified within each CTLA-4 and PD-1-expressing subset in the PBMCs and RB of ART-treated, SIV-infected RMs prior to ART initiation and 9 weeks on ART therapy (n=6, except for PBMCs pre-ART, where n=8; 2 RMs were sacrificed prior to the week 9 collection). (B) Frequencies of CCR6+ cells within CTLA-4 and PD-1-expressing memory CD4+ T cell subsets were measured and compared longitudinally. Averaged data are presented as the mean ± SEM, and ANOVAs using Tukey's adjustment for multiple comparisons were used to compare differences between subsets. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

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