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Investigating the role of CD4+ and CD8+ T cells in SIV persistence and virus production during antiretroviral therapy

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By

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B.A., College of the Holy Cross, 2011

Advisor: Guido Silvestri, MD

An abstract of
A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory
University in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Graduate Division of Biological and Biomedical Science
Immunology and Molecular Pathogenesis
2016

Abstract

Human immunodeficiency virus 1 (HIV-1) infection is a global pandemic affecting over 30 million people worldwide. HIV-1 infection leads to a compromised immune system and, if left untreated, patients will succumb to opportunistic infections and acquired immunodeficiency syndrome (AIDS). Treatment with antiretroviral therapy (ART) can control virus replication and prolong the life of infected individuals. However, current regimens cannot eradicate the virus and any treatment interruption leads to viral rebound due to a small pool of latently infected cells invisible to both ART and the immune system. This "HIV reservoir" is the major barrier to HIV eradication efforts. In order to better understand the mechanisms and precise cellular subsets involved in HIV reservoir maintenance, we utilized the well-characterized animal model of HIV infection, simian immunodeficiency virus (SIV) infection in non-human primates (NHP).

First, we examined the dynamics of a subset of CD4+ memory T-cells with stem cell-like properties, the CD4+ T stem cell memory (CD4+ T_{SCM}), during experimental pathogenic infection of rhesus macaques (RM). We found these long-lived, multipotent memory T-cells were significantly disrupted in pathogenic SIV infection. Next, we investigated if initiation of ART during pathogenic SIV infection of RM would alter the homeostasis of CD4+ T_{SCM}. We found that ART initiation restored some balance to the CD4+ T_{SCM} compartment. Considering their enhanced longevity and susceptibility to infection, CD4+ T_{SCM} are of intense interest as a site of SIV persistence during ART. We found no reduction in the fraction of infected CD4+ T_{SCM} after initiation of ART, confirming a potentially critical role for CD4+ T_{SCM} during SIV persistence.

Finally, to further assess the mechanisms of SIV persistence during ART, we performed a CD8+ T-cell depletion study in SIV-infected ART-treated RM. We found that depletion of CD8+ T-cells during continuous ART resulted in a measurable increase in plasma viremia and reconstitution of CD8+ T-cells was associated with re-establishment of viral control. These data suggest a previously underappreciated role for CD8+ T-cells cooperating with ART to suppress viremia in SIV-infected RM.

Overall this dissertation provides novel insights into cellular subsets involved in SIV pathogenesis and persistence and demonstrates for the first time a role for CD8+ T-cells in maintaining virus suppression during ART. These results support the design of novel therapeutic strategies that specifically target long-lived CD4+ memory T-cells and enhance the CD8+ T-cell response to HIV/SIV during ART.

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Acknowledgements

First and foremost I would like to thank my mentor, Guido Silvestri, for providing me with the opportunity to work in the lab. His guidance allowed me to develop into an independent scientist. I would also like to thank the members of my lab, particularly Diane and Maud, for their unwavering support and encouragement over the past 5 years. I would also like to thank my committee members for their advice and helpful suggestions during this process. I would never have made it through grad school without the love and support of my friends. Whether they are in a PhD program and could always provide me with new insights into my project, or they work out with me every morning and listen to me complain, I will be forever grateful.

I would like to thank my family, I wouldn't be where I am without you. They may not always understand what I am complaining about but they are always there to listen to me. Their consistent support of me, from undergraduate research through this PhD process means everything to me.

Lastly, I would like to thank Ryan my ever supportive, ever encouraging, always loving boyfriend. Throughout this process he has always been a shoulder to cry on and a sounding board for my experimental plans. I couldn't have made it through this process without you and our newly acquired dog, Addie whose unconditional affection has really brightened up my life.

Thank you to you all for everything you have done for me, I will never be able to fully express how much your love and support have meant to me.

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Chapter 1:

Introduction

Discovery and Origin of HIV

In the early 1980s, cases of *Pneumocystis carinii* (now renamed jirovecii) pneumonia were diagnosed in five young, previously healthy homosexual men (1, 2). The presentation of these opportunistic infections in otherwise healthy individuals was the first indication of a severe immunodeficiency syndrome. Until the first female case in a Haitian woman, the associated immunodeficiency syndrome was called Gay-Related-Immunodeficiency (GRID) (3, 4), which was later renamed acquired immunodeficiency syndrome (AIDS). The virus now known as human immunodeficiency virus (HIV) was isolated from AIDS and "pre-AIDS" patients and named human T-cell leukemia-lymphoma virus (HTLV)-III based on its homology to HTLV-I and II (5-8). Renamed in 1986, HIV is a member of the *Retroviridae* family of viruses; specifically, it is a lentivirus. To date, mammals are the only known hosts for lentiviruses (9). They have a single stranded, positive sense, RNA genome. Currently, HIV-1 and HIV-2 are the two major groups of human lentiviruses, the result of two separate cross-species transmission events of two different simian immunodeficiency viruses (SIV) (10). SIV is endemic in old world monkeys, such as chimpanzees (CPZ), sooty mangabeys (SM), and African green monkeys (AGM). HIV-1 originated from a cross-species transmission event from chimpanzees (SIVcpz) to humans (11) whereas HIV-2 is phylogenetically closer to SIV from SM (SIVsm) (12). Interestingly, SIVsm and SIVagm do not cause disease in their natural hosts, the SM and AGM, respectively. HIV-1 accounts for the majority of infections in the global pandemic, whereas HIV-2 infection is geographically restricted to West Africa (13). In addition to being more restricted geographically, HIV-2 has a lower transmission rate and slower disease progression, compared to HIV-1 (14, 15). Because

of its global presence and greater pathogenicity, most of the research to date focuses on HIV-1. Transmission of HIV occurs primarily through direct fluid to fluid contact at mucosal surfaces, such as the rectum and genital tract. It can also be transmitted through direct injection into the blood stream by a contaminated needle or infected blood transfusion. Before routine screening for HIV, hemophiliacs were at incredibly high risk of HIV infection. It can also be vertically transmitted from mother to infant either *in utero*, during delivery, or after birth through breastfeeding (16-18).

There are four subgroups of HIV-1: M (main), N (new), O (outlier), and P. Genomic studies of the M group have identified nine subtypes, or clades, which cause over ninety percent of HIV-1 infection and disease (19). These studies have revealed that each clade originated in Africa, where the cross-species transmission event first occurred, and each strain spread out to establish infections in their respective geographical areas (20, 21). Clade C makes up the majority of HIV-1 infections in sub-Saharan Africa and, with that region accounting for sixty percent of the total HIV infections, Clade C is the predominant HIV-1 subtype worldwide. Clade B is the predominant subtype in North America and Europe (19). There is no clear evidence that one clade is more intrinsically virulent than another (22). However, antiretrovirals were originally designed in Europe and North America to target Clade B virus, they are less effective against non-clade B viruses (21). This caveat was overlooked during drug development, thus highlighting the need for a better understanding of viral diversity and the differential effects it can have on treatment efficacy.

Virology and Life Cycle

As mentioned previously, HIV is a retrovirus which reverse transcribes its single stranded RNA genome into double stranded DNA and integrates into host chromosomes (23). The enzyme which enables HIV to violate the central dogma DNA→RNA→protein

is reverse transcriptase (RT). It is encoded in the *pol* gene of HIV, and is one of several proteins packaged in the mature virion. It is an essential viral enzyme with three critical functions. First, it is an RNA dependent, DNA polymerase that reverse transcribes the (+) single stranded RNA (ssRNA) into the first (-) ssDNA. Second, it has an RNase H activity, which digests the original RNA template. Its third and final function is a DNA dependent DNA polymerase, which transcribes the complimentary DNA (cDNA) strand. As a DNA polymerase, RT is error prone and lacks proof-reading mechanisms that cellular DNA polymerase has (24). This lack of fidelity aids HIV in generating extreme viral diversity.

Initiation of reverse transcription begins in the virion, before fusion with the next cell (25). In the target cell, elongation occurs with cytosolic nucleotides and the single stranded RNA genome is reverse transcribed into double stranded cDNA. The HIV viral protein R (Vpr) and the enzyme integrase remain associated with the cDNA and, along with the matrix protein, compose the pre-integration complex (PIC). Like many lentiviruses, HIV can infect non-dividing cells. Therefore, in order to integrate into the host cell genome, the PIC must be actively transported to the nucleus (26). Once in the nucleus, integrase catalyzes the reaction that allows cDNA to insert into the host cell genome. Once integrated into the cellular genome the HIV provirus is subject to regulation by the host cell's transcriptional and translational machinery. To initiate a new round of infection, full-length unspliced mRNA must be transcribed from the proviral template, and each new virion encapsulates two full-length RNA genomes. These unspliced mRNAs are trafficked to the assembling Gag and Env at the plasma membrane. Maturation of the virion begins after budding and release by protease cleavage of the Gag polyprotein (27).

All retroviruses, including HIV, encode three major genes: *gag* contains the core structural proteins which encapsulate the genome, *pol* encodes the enzymes required for replication and integration into the host cell, and *env* encodes the envelope glycoproteins. HIV is distinguished from simple retroviruses by the encoding of six accessory genes: *tat, rev, vif, vpr, vpu, nef*. These genes evolved to give the virus advantages over the host by subverting immune defense mechanisms (27). These accessory proteins are the quintessential example of the evolutionary battle between host and pathogen. For example, the human restriction factor apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G (APOBEC3G) is efficiently targeted for degradation by HIV-1 viral infectivity factor (Vif) (28, 29). Viruses lacking Vif have extremely poor replication capacity *in vitro* and are rarely, if ever seen in an *in vivo* infection (30). Degradation of APOBEC3G is the only known function of Vif. Another example is HIV-Nef, which down regulates MHC-I from the cell surface to prevent recognition by the host CD8+ T cell response (31). HIV-nef also down regulates CD4 to prevent unnecessary super-infection of a cell (32). The accessory proteins of HIV and SIV ensure the virus is extremely well equipped to evade the immune response and survive within its host.

Target cells and viral tropism

The primary target cell of HIV infection is the CD4 T cell. The quintessential sign of clinical HIV disease progression is an increase in viral load and the progressive loss of the CD4 T cell compartment. To infect a cell, HIV envelope glycoprotein gp120 binds to CD4, the Ig-superfamily member expressed on T helper cells. While the CD4 molecule is necessary, alone it is not sufficient to allow virus entry. Once bound to CD4, a conformational change occurs in gp120, allowing binding of a co-receptor (27). CCR5 and CXCR4, the primary co-receptors for HIV entry, are both members of the chemokine

family of receptors (33, 34). CCR5 is a β-chemokine receptor expressed mostly on a small subset of peripheral memory CD4+ T cells (35, 36). β-chemokines, the ligand for CCR5, are produced in response to inflammation and cells that express CCR5 are trafficked to areas of inflammation. Of note, CCR5 is highly expressed in the rectal mucosa, a primary site of HIV transmission (36, 37). Conversely, CXCR4 is a chemokine receptor found on most CD4 T cells and is highly expressed on naïve T cells. CXCR4 expressing cells tend to be more quiescent than those expressing CCR5 (36, 38). Because CCR5 is highly expressed in the rectal mucosa, most transmitted founder viruses are CCR5-tropic (39). Late in disease course, viruses within an individual may switch from R5- to X4-tropic. Emergence of CXCR4 tropic viruses late in infection is associated with faster disease progression and accelerated loss of CD4 T cells (40, 41). In addition to CCR5 and CXCR4, numerous other chemokine receptors have been implicated in HIV transmission such as CXCR3, CXCR6, CCR6, and GPR1 (42).

Clinical Disease Course

The clinical disease course of HIV-1 infection can be divided into three stages, a primary, chronic and end stage disease. During primary infection, about 50-75% percent of patients will develop acute influenza-like symptoms such as fever, gastrointestinal distress, and lymphadenopathy which resolves within a few weeks (43). These symptoms, collectively known as acute retroviral syndrome (ARS), are rarely severe enough for consultation with a physician and thus, primary infection in patients goes undiagnosed. Additionally, 25% of individuals do not present with any clinical symptoms during acute infection. Primary infection is divided into six stages, depending on the laboratory assay necessary to confirm HIV infection. Collectively these stages are referred to as Fiebig stages (44, 45). Stage I is detection of only HIV RNA in plasma, without any p24 antigen or antibody response. This stage begins anywhere from seven

to twenty-one days post infection, and lasts about a week. Stage II begins when individuals become positive for p24, on average, three weeks post-infection. Stage III patients have enough antibody in the blood to test HIV positive by ELISA, on average about 5 days after p24 antigen appears. Stage IV is the period post-ELISA conversion when the Western blot can come up positive or negative. Fiebig stage V is a period where Western blots are positive but p31 antigen, HIV-integrase, is not detected. The final stage of primary HIV infection and seroconverstion is Fiebig stage VI, when all samples are positive by Western blot and p31 antigen. This period lasts for an indeterminate amount of time (44, 45). Primary infection is accompanied by a high peak in virus titers in the blood about two weeks post infection, followed by a depletion of peripheral and mucosal CD4 T cells (46).

Resolution of the acute phase is followed by a period known as “clinical latency”, not to be confused with viral latency, which will be discussed later. During clinical latency, the immune system is highly active. Robust humoral and cellular immune responses are induced, CD8+ T cells expand, peripheral CD4 T cells return to near normal levels, and plasma viral load declines (46). During this period, virus replication can proceed unchecked in lymphoid organs causing progressive damage (47). Though humoral and cellular immune responses develop, they are rarely sufficient to control the virus. The viral load declines until it stabilizes about 4-6 months post-infection at a level called the virus set point. The viral set point is a major determinant of disease progression and clinical outcome (48-50). An HIV-infected individual can live anywhere from one to twenty years in the clinically latent stage without diagnosis of infection. As mentioned above, individuals infected with CXCR4-tropic strains will progress to disease faster than those infected with CCR5-tropic strains, as CD4 T cell decline is more rapid . Once CD4 T cells have declined below a threshold level, patients will display outward signs of

immunosuppression such as opportunistic infections and cancers. If left untreated, this leads to end stage disease, when a patient will progress to AIDS, with the immune system overwhelmed by opportunistic infections.

There are a small percentage of individuals who, upon infection with HIV do not progress to disease. These individuals can be broken up into three groups, broadly termed non-progressors. The rarest of these cohorts, the elite controllers (EC), generally have a CD4 T cell count above 350 cell/μL, and, in the absence of ART, maintain a viral load below the clinical limit of detection of 20 copies/mL. Another such cohort, viremic controllers (VC) also maintains a stable CD4 count and controls viremia between 50 and 200 copies/mL, in the absence of therapy. Finally, individuals that maintain a high CD4 count despite viral loads of greater than 2000 copies/mL, for a period longer than seven years are called long-term non-progressors (LTNPs) (51). In recent years, the CD4 T cell count has become a less important distinction, and these designations are given primarily based on viral loads. The mechanisms of control of HIV disease progression in these cohorts are varied and not necessarily consistent, even within cohorts. Some of these mechanisms will be discussed later in the dissertation.

Non-human Primate Models

As mentioned previously, HIV-1 arose from a cross-species transmission event from an SIV infected chimpanzee to humans, while HIV-2 probably arose from SIVsm (11, 12). SIV infection of Asian macaques is one of the most useful models ever developed to study HIV disease progression and pathogenesis. If left untreated, macaques infected with SIV will develop simian AIDS, a syndrome that closely resembles human AIDS. The most common NHP used for SIV pathogenesis studies is the Indian rhesus macaque (RM). Macaques are more closely related physiologically and immunologically to humans than either mice or rats. Additionally, use of the non-

human primate (NHP) model allows extensive tissues sampling of areas that are difficult to access in humans such as the central nervous system (CNS), rectal mucosa, and lymph node biopsies. The NHP model also allows for elective necropsies to study virus effects of multiple organs before end stage disease. Using experimental techniques such as antibody-mediated depletions to manipulate certain lymphocyte subsets, we are able to directly study the effect of these subsets on disease progression and pathogenesis. Finally, NHP serve as one of the first large animal tests of experimental immunological and therapeutic interventions, such as recombinant proteins and checkpoint blockade inhibitors. Using the NHP model, researchers are able to control for most variables not accounted for in natural HIV infection including dose, route, and timing of infection. Knowing the precise timing of infection has proven extremely useful, and the NHP model has given extensive insights into the early immunologic and virologic events of acute HIV/SIV infection. However, as with any model system there are limitations. Disease progression in macaques is faster than humans, and the set point viral loads trend to be higher, 10^4 in humans versus 10^5 in macaques (52 2002). Also, though they are similar physiologically and immunologically, monkeys are not humans, and just as importantly, SIV is not HIV. SIV has some unique genetic characteristics, such as the presence of the viral protein Vpx. Vpx targets the host protein SAMHD1, allowing for more efficient infection of dendritic cells and macrophages (53). This particular difference means the importance of infection of dendritic cells in HIV infection cannot be extrapolated from the SIV infection model.

In addition to pathogenic infection in Asian macaques, some species of African monkeys are utilized for NHP studies. Mentioned previously, sooty mangabeys (SM) and African green monkeys (AGM) are two species of “old world” monkeys referred to as “natural hosts” of SIV. These NHPs are model systems for the study of non-pathogenic

SIV infection. We have gained extensive insights into the mechanisms of pathogenesis and disease progression through comparative studies of pathogenic infection in Asian macaques and non-pathogenic infection of the natural hosts (54). Similar to infection in the RM, SIV infection of the natural host results in high viral titers and robust innate immune activation. However, there are several important differences. The immune activation seen during acute infection of the natural host is quickly resolved, with no lingering chronic immune activation characteristic of pathogenic SIV infection (55). Significant depletion of mucosal CD4 T cells occurs during acute infection of both the natural host and RM. However, gut mucosal barrier integrity is maintained with little to no microbial translocation in the natural host (56). Additionally, T cell homeostasis is maintained in the natural host, without progressive loss of peripheral CD4 T cells. The mechanism of CD4+ T cell preservation varies between SM and AGM, SM specifically protect their central memory CD4 T cells by lower levels of CCR5 expression and AGM downregulate CD4 on T helper cells. Both result in preservation of CD4+ T cell homeostasis and lack of disease progression (57). In addition to *in vitro* studies with human peripheral lymphocytes, lymph nodes and rectal biopsies, NHP models are the most widely used to understand HIV pathogenesis.

Immune Response to HIV:

Innate

Despite its name, the human immunodeficiency virus is an extremely potent activator of both the innate and adaptive immune system. Unfortunately, HIV targets precisely those cells that are needed to destroy it, which leads to immunodeficiency during chronic infection. Since primary HIV infection often goes unnoticed in newly infected patients, catching this window in acutely infected HIV patients is difficult and requires constant, longitudinal monitoring of at-risk populations. Most of our knowledge

of the innate immune response to HIV comes from *in vitro* experiments and *in vivo* studies utilizing the NHP models of SIV infection. Initiation of the innate immune response is triggered when the innate immune system recognizes a pathogen based on certain danger associated molecular patterns (DAMPs) and triggers a signaling cascade to create a hostile environment for the invading virus, including secretion of pro-inflammatory cytokines, chemokines, and recruitment of innate and adaptive immune cells.

Specifically, for HIV there are many pattern recognition receptors (PRRs) which recognize some form of the virus. This includes the classical Toll-like receptors (TLR) 7/8, that recognize intracellular RNA (58). Triggering of TLR7/8 pathway will activate the type-1 interferon (IFN) response pathway. Interferon-inducible protein 16 (IFI-16) and cyclic GMP-AMP synthase (cGAS) were identified as two interferon-stimulated genes (ISGs) that are potent regulators of HIV infection. IFI16 is a DNA-binding protein shown to co-localize with HIV-DNA and further stimulates the type 1 IFN-signaling pathway (59). It also drives inflammasome activation and cell death by pyroptosis in cells non-permissive to HIV infection (60). In response to HIV-infection, cGAS induces the production of cyclic-GMP and AMP which activates the IRF3 pathway, also leading to production of IFN-α/β (Type 1 IFN) and innate restriction factors such as APOBEC3G and SAMHD1 (61-64). APOBEC3G, mentioned briefly earlier, is a cytidine deaminase that targets cytosine residues on first strand DNA. During second strand synthesis, adenosine/adenine will pair with the deaminated cytosine (which now resembles uracil) causing G→A hypermutations in the proviral DNA (65). SAMHD1, another innate restriction factor, depletes deoxynucleotides from the cytosol inhibiting reverse transcription of HIV-1. However, SAMHD1 has a dual role in HIV infection. SAMHD1 activation prevents complete activation of dendritic cells (DCs) during acute infection. By

inhibiting formation of cDNA in the cytosol, SAMHD1 prevents recognition by other innate sensors such as IFI16 and cGAS, thus increasing the pathogenesis of HIV. SIV and HIV-2 encode a viral protein to inhibit SAMHD1, Vpx. Upon knockdown of Vpx, activation of dendritic cells is significantly reduced, and transfection of exogenous Vpx during *in vitro* HIV-1 infection will enhance infection and activation of DCs (66). IFI16, cGAS, APOBEC3G, and SAMHD1 are found in plasmacytoid dendritic cells (pDCs) as well as CD4 T cells. Recognition of HIV by these PRRs is essential for initiating the innate immune response. In addition to direct infection by HIV, migratory DCs serve as conduits for HIV entry into the lymph nodes. DCs express the molecule DC-SIGN, which can bind to HIV. In this way, dendritic cell trafficking is co-opted by the virus to bring it to a site rich with activated target cells (67).

As mentioned above, signaling through PRRs induces proinflammatory cytokines and chemokines. These soluble molecules activate innate immune effector cells such as conventional DCs, macrophages, and natural killer (NK) cells. NK cell activation depends on robust activation of dendritic cells and macrophages, referred to as NK cell licensing, or education (68-70). NK cells are important innate immune cells that are able to directly kill virally infected cells. They accomplish this through various mechanisms including (i) direct recognition of stress ligands (positive signal), (ii) lack of inhibitory signals, and (iii) antibody-dependent cell-mediated cytotoxicity (ADCC). HIV/SIV induces stress ligands on infected cells through activation of the host restriction factor APOBEC3G. It induces upregulation of the DNA-damage response pathway and, consequently, upregulation of NKG2D activating ligands, sensitizing the infected cells to NK cell mediated killing (71). NK cells integrate the signals from both activating and inhibitory receptors, in healthy individuals maintaining a delicate balance. The balance can be tipped towards killing by an increased presence of activating ligands, or a

decreased presence of inhibitory ligands. NK cells express inhibitory receptors that recognize MHC I on the cell surface. Upon recognition of MHC I, the NK cell receives an inhibitory signal to ignore that cell. As mentioned briefly, HIV-nef downregulates MHC I from the surface of an infected cell to hide that cell from the CD8+ T cell response. However, MHC I down regulation sensitizes cells to NK cell mediated death by removing the inhibitory ligand. NK cells also express Fc-receptors that recognize the constant regions of antibodies. Activation of these Fc-receptors will activate an NK cell to degranulate and kill an antibody coated cell. Additionally, NK cells are proposed to have functions modulating the adaptive immune response both by direct cell killing and modulation of dendritic cell priming to T cells (72, 73).

Immune Response to HIV:

Adaptive: Humoral

The adaptive immune response is initiated in the lymph node where antigens are presented to B and T cells. The generation of a potent B cell response and subsequent antibody production is critical to control of HIV. Briefly, B cells take up antigen, and with the help of follicular T helper cells (T_{FH}), B cells are primed to enter the germinal center and the B cell receptors undergo class-switching and somatic hypermutation. During HIV infection, most patients develop detectable antibodies within two months of infection (74), with some developing as early as two weeks post infection (75). The first antibodies to develop during HIV infection are binding, non-neutralizing antibodies. These antibodies are generally to HIV envelope, the most exposed epitopes of the virus (76). Though they develop early in infection, binding antibodies have no effect on levels of plasma viremia (76). Antibodies responsible for reducing or controlling viremia are called neutralizing antibodies. Early data suggested neutralizing antibodies were only generated late in infection, and at very low titers (77, 78). More recent data has shown neutralizing

antibodies (nAbs) to HIV develop very early during acute infection but the virus, with its high mutation rate, rapidly escapes detection by these antibodies (79). Thus, if you take serum from an infected patient around 3 months post-infection, they will have neutralizing antibodies to the transmitted founder virus, but the virus has already escaped that initial immune pressure, and the transmitted founder virus is no longer the dominant variant (79). The most elusive and highly coveted antibodies are the broadly neutralizing antibodies (bnAbs). These antibodies target conserved regions of HIV envelope such as the CD4 binding site, the membrane proximal region, and N-linked glycans (80-82). They generally have long complementarity determining regions (CDRs) and have undergone many rounds of somatic hypermutation, making them vastly different than the germline counterparts (83). BnAbs are highly potent and able to mediate neutralization of Tier 2 and even Tier 3 viruses, as well as neutralize viruses from different clades. Tier 2 viruses are isolates that are matched to genetic subtype of the virus, while tier 3 viruses are mismatched in subtype making them more difficult to neutralize (84). One important caveat to bnAb induction is in the increased likelihood these Abs will be self-reactive (85). In this case, tolerance mechanisms such as anergy and deletion of self-reactive B cells may be delaying and preventing the generation of bnAbs in order to avoid self-reactive antibodies (86). Whatever the mechanism, broadly neutralizing antibodies are difficult to elicit in most patients, and only arise years later in infection, too late to be of any significant therapeutic benefit.

Immune response to HIV:

Adaptive: CD4 T cells

When migratory dendritic cells bring HIV antigen to the lymph nodes to initiate the B cell response, they are also responsible for activating the CD4 and CD8+ T cell response. Understanding the CD4 T cell response to HIV is complicated by the

knowledge that HIV infects and depletes CD4 T cells. Most importantly, HIV specifically targets HIV specific CD4 T cells, likely due to their increased activation status during infection. (87). During a normal immune response, CD4 T cells scan DCs in the lymph node looking for their cognate peptide:MHC-II. Because naïve CD4 T cells need to be activated by DC priming, HIV-specific CD4 T cells will spend a relatively long time in close proximity to the DCs during HIV infection, compared to non-HIV specific CD4 T cells (47, 88, 89). Since these DCs are likely to be infected, or harboring HIV, proximity of HIV-specific CD4 T cells increases the likelihood they will be infected.

CD4 T cells are critical helpers in the development of lasting and efficient adaptive immune responses. They are necessary for development of memory CD8+ T cells and B cells. T follicular helper cells (T_{FH}) are the specific subset responsible for helping B cells. T_{FH} are necessary for formation of germinal centers, B cell class-switching and somatic hypermutation. These cells have come under intense investigation in the field of HIV/SIV research as sites of preferential infection and replication, both in treated and untreated infection (90, 91). Though most memory T cell subsets are depleted during infection, T_{FH} cells are spared from depletion, and actually accumulate. One hypothesis for their accumulation is the localization of T_{FH} to the B cell follicle, specifically the germinal center, protects them from CD8+ T cell mediated killing. There is substantial evidence that most CD8+ T cells are excluded from the germinal center (92). Another hypothesis is T_{FH} accumulation is due to a depletion of follicular regulatory cells (T_{FR}) (93). T_{FR} cells regulate the expansion of T_{FH} and enhance the production of antigen-specific B cells. Whatever the mechanism, this aberrant accumulation of T_{FH} results in expansion of non-HIV/SIV specific B cell responses and hypergammaglobulonemia.

As mentioned above, CCR5+CD4 T cells are the major targets of HIV infection. CCR5+CD4 T cells present at mucosal sites are not all HIV specific, but by virtue of proximity to this primary site of transmission are major targets for HIV/SIV infection. CCR5 expressing CD4 T cells are the most effector-like T helper cells that preferentially reside in peripheral, non-lymphoid tissues, and are primed to respond to incoming antigen (36, 94). These cells are rapidly depleted from the mucosa in primary infection. By infecting and depleting the more differentiated subsets of T cells during acute infection, HIV/SIV forces the immune system to differentiate new CCR5+ cells from less-differentiated precursors, presumably stem cell-like (T_{SCM}) and central memory T cells (T_{CM}). The ability of precursor memory T cells to generate more differentiated effector subsets will be discussed in depth later in this chapter. It is important to note that from our studies of non-pathogenic infection, the compensatory mechanism of homeostatic differentiation of long-lived precursor cells to effector memory (T_{EM}) T cell depletion should prevent progression to disease. However, in pathogenic infection, the precursor cells are also infected and depleted, leading to disruption of the homeostatic balance of the memory T cell compartment (95, 96).

In addition to their role as helper cells, several groups have shown CD4 T cells play a direct role killing virally infected cells. Cytotoxic CD4 T cells have been identified in Epstein-Barr Virus (EBV) infection and viral hepatitis, as well as in HIV-infections (97-99). In HIV infection, 50% of Gag-specific CD4 T cells could up-regulate CD107a upon stimulation, indicating a degranulation event characteristic of cytotoxic T cells. Interestingly, in this study of *in vitro* stimulated cells, there was no upregulation of CD154 (CD40L), a classical T helper cell molecule (98). The cytotoxic ability of CD4 T cells was shown, in mice, to be MHC-II dependent (100). Therefore, in addition to abolishing

classical T helper responses, HIV infection may also inhibit the cytotoxic CD4 T cell responses.

Immune Response to HIV:

Adaptive: CD8+ T cells

CD8+ T cells, sometimes referred to as cytolytic or cytotoxic T cells are responsible for immune defense against viruses, intracellular bacteria and cancers. The CD8+ T cell response is a critical mediator of viral control in HIV/SIV infection. There is a temporal association between the appearance of HIV-specific CD8+ T cells and the decline of plasma viremia (101). As mentioned previously, HIV/SIV viremia peaks during the first few weeks of acute infection and declines to the set point viral load. The appearance of HIV-specific T cells is associated with this post-peak decline. In one study of five patients, looking at the appearance of HIV-specific CD8+ T cells, all but one patient mounted a robust CD8+ T cell response. This patient in whom the CD8+ T cell response was delayed, had poor virus clearance and faster disease progression, supporting the hypothesis that antigen specific CD8+ T cells are necessary for this phenomenon (101). Another study found similar results in patients with acute retroviral syndrome (ARS). Individuals that mounted strong CD8+ T cell responses were the best at controlling acute viremia (102). This temporal association is also found in the NHP model. Virus-specific CD8+ T cell responses are detected as early as day 7 in the blood and lymph nodes of SIV infected macaques, well before the appearance of SIV-specific antibodies at day 14 (103, 104). Peak plasma viremia in SIV-infected rhesus macaques occurs anywhere from day 7-14 post infection, with CD8+ T cell responses peaking around day 14-21 post-infection (105). The appearance and expansion of virus specific CD8+ T cells prior to and concurrently with the decline of plasma viremia is highly suggestive of the importance of these cells in controlling plasma viremia.

Further evidence supporting CD8+ T cell mediated control of viremia is the appearance of specific mutations in the virus preventing recognition by CD8+ T cells. Mutations in HIV-*gag* that render the virus unrecognizable to autologous CD8+ T cells suggest these cells are exerting some immune pressure on the virus (106). By mutating away from the CD8+ T cell response, the virus would gain a fitness advantage over other variants, thus becoming the dominant variant. In one particular case, a single amino acid substitution in the immunodominant CD8+ epitope showed a selective advantage over the transmitted founder virus, which was susceptible to CD8+ T cell recognition (107). Studies in the rhesus macaque model show these escape mutations originate from ongoing virus replication in lymph nodes and migration of these variants to peripheral plasma virus (108). Recent studies suggest the latent HIV reservoir is predominantly composed of viruses that have escaped the CD8+ T cell response (109).

As mentioned previously, a small percentage of HIV infected individuals maintain high CD4 counts and have slower progression to disease. Two of these cohorts, the elite controllers (EC) and the viremic controllers (VC), maintain low viral loads in the absence of ART. These individuals have a more polyfunctional CD8+ T cell response, meaning they are able to produce multiple cytokines (110). More importantly, they are able to maintain these responses without the canonical CD8+ T cell exhaustion seen in normal progressors. CD8+ T cells from controllers also have greater proliferative capacity when stimulated, compared to those CD8+ T cells from normal progressors. While there is no correlation between the frequency nor magnitude of the CD8+ T cell response and control of viral load, there is a significant inverse correlation between the functionality, or quality, of the response and viral loads (110). Therefore, one explanation for control of viremia in these cohorts is the enhanced polyfunctionality of the CD8+ T cell response.

The mechanism of CD8+ T cell antiviral function is known and unknown. There are two primary mechanisms, but their individual contributions to the total CD8+ antiviral activity is unknown. One mechanism is cytolytic: T cell receptor (TCR) recognition of peptides presented by MHC I and subsequent release of cytotoxic granules directed at the infected cell. In HIV/SIV infection, there are significant associations between certain MHC alleles and either accelerated or delayed disease progression (111). For example, *HLA-B*57 and –B*27* are both associated with slower disease progression in humans infected with HIV. However, *HLA-B*35* is associated with rapid progression. In rhesus macaques, several alleles are associated with slower disease progression. *Mamu-A*01 and- B*17* are associated with lower viral set point during chronic infection (112, 113). Animals with the *Mamu-B*08* allele are overrepresented in elite controller macaques (114). Importantly, not all controllers or rapid progressors have these alleles and not all individuals with these alleles are controllers or rapid progressors (110). One explanation for lack of concordance between individuals with a given HLA lies in the fundamental differences of the TCR repertoire of the CD8+ T cells. The CD8+ repertoire in *HLA-B*27* controllers is clonotypically distinct from *HLA-B*27* progressors (115). CD8+ T cells isolated from the controllers are better able to recognize viral peptides and kill infected cells *in vitro.* Though they all express the same HLA, controllers have more breadth in their responses than progressors. A greater understanding of the generation of TCR repertoire could lend insight into control of HIV/SIV infection.

The second mechanism of antiviral CD8+ T cells is a non-lytic secretion of soluble factors, both general immune defense factors and specific HIV/SIV factors. These secreted factors have multiple functions and mechanisms of action, such as to promote antigen presentation and cell death, prevent infection of new cells, and possibly suppress transcription. The most commonly measured antiviral cytokine is interferon-γ

(IFN-γ). This is a non-specific but essential cytokine that upregulates genes involved in antigen presentation (both MHC I and II), anti-proliferative pro-apoptotic genes such as Fas/Fas Ligand. IFN-γ also induces expression of IL-12, a cytokine responsible for activating NK cells and macrophages and enhancing a hostile, pro-inflammatory environment (116). Activated CD8+ T cells can also secrete RNases, which can degrade viral RNA within an infected cell (117). Antiviral soluble factors secreted by CD8+ T cells that are specific to HIV/SIV are the β-chemokine family: Mip-1α/β (CCL3/4) and RANTES (CCL5). These molecules are the ligands for CCR5, the primary HIV/SIV co-receptor, and are only effective at blocking R5-tropic virus. They exert their antiviral effect in a competitive way, primarily through blocking HIV entry (118). β-chemokines bind to CCR5, causing receptor internalization, therefore removing CCR5 from the surface and helping to prevent infection of new cells (119).

Another soluble factor secreted specifically by CD8+ T cells has been shown to have significant antiviral effects *in vitro* (120). This molecule, CD8+ antiviral factor (CAF), is secreted by activated CD8+ T cells with a central memory-like (T_{CM}) phenotype. This antiviral activity cannot be suppressed using blocking antibodies to any known anti-HIV/SIV molecule including Type I or Type II interferons, β-chemokines, IL-10, RNase, or α-defensins (121). CAF is able to suppress HIV replication at the level of transcription through the LTR, a post-entry phase of infection. However, studies have been guided by the assertion that CAF is a completely secreted molecule. Some data, however, suggests “CAF” functions best when there is cell-cell contact (122). One interesting study showed the CAF activity is from exosomes secreted from the endosomal compartments of CD8+ T cells that directly interact with the surface of infected cells and initiate a STAT-1 dependent signaling pathway (122). This study showed no internalization of the exosomal contents, along with no CD8+ detected in the exosome

(though CD3 was found). Recently, a study utilizing serial progressive deletions of the HIV-LTR showed the antiviral activity of CAF can be abolished by deleting the CATA-box (analogous to the TATA-box), indicating CAF can suppress transcription anywhere upstream of this site (123). Because of the stage at which CAF acts, it is able to exert cross-clade inhibition of HIV-1 and inhibition of SIV. CD8+ T cells from SIVmac251 infected cynomolgous macaques also exhibit this suppressive function (124). However, the identity and impact of CAF on the *in vivo* antiviral function of CD8+ T cells is still an active area of research.

Direct evidence for CD8+ control of plasma viremia comes from CD8+ depletion studies in the RM. One way to study direct effects of a lymphocyte population is to remove the population and monitor the effects, and the NHP model allows us to utilize this method. In rodent models, it is possible to generate mice lacking a specific gene of interest, but this is not experimentally possible in humans or non-human primates, and spontaneous lack of the gene of interest is generally rare. In the NHP model, we use antibody mediated depletion studies. Briefly, an antibody directed at the cell or protein of interest is injected into the monkey. By various mechanisms, including complement and antibody mediated phagocytosis, the cells coated with the antibody are depleted.

The first CD8+ depletion study in SIV-infected RM was conducted by administration of the CD8+ depleting antibody during acute infection. This antibody depletes both CD8+ T cells and CD8α-expressing NK cells (125). Upon administration during acute infection, the animals did not have the canonical post-peak viral load decline. Instead, plasma viral loads remained high and the animals progressed more rapidly to simian AIDS (125). CD8+ lymphocyte depletion results in an increase in the frequency of activated CD4+ T cells. Okoye, et al elegantly showed that this increase in target cells does not fully account for the progression to disease in CD8+ depleted

animals (126). Another group administered the same antibody to macaques chronically infected with SIV and saw a dramatic rise in the levels of plasma viremia, which was reversed upon reconstitution of the CD8+ T cell pool (127). Another important CD8+ depletion study comes in the context of vaccination to prevent SIV acquisition. Live attenuated vaccines, specifically SIVmac239Δnef are the most effective preventative vaccines studied to date. Since one of the functions of the HIV/SIV protein Nef is to downregulate MHC-I to hide infected cells from recognition by CD8+ T cells, Δnef viruses are sensitive to CD8+ T cell recognition and control. In one study of SIVmac239Δnef vaccinated animals, all vaccinated animals were protected from a heterologous challenge with SIVmac251. Depletion of CD8+ lymphocytes in challenged macaques abrogated protection. However, once the CD8+ lymphocytes returned, plasma viral load returned to undetectable levels (128). Interestingly, a study using CD8+ lymphocyte depletion in SIV-infected SM also revealed a modest, but significant increase in plasma viremia following antibody administration (129).

To try and understand the mechanism of CD8+ lymphocyte mediated suppression, two independent studies utilized antibody mediated CD8+ lymphocyte depletion in SIV-infected RM treated with ART after depletion and found that the rate of decay of plasma viremia was the same between CD8+ depleted and mock-depleted animals (130, 131). The treatment with ART following CD8+ lymphocyte depletion was to assure that no new rounds of replication were occurring, therefore viremia should only be coming from cells infected prior to depletion. Surprisingly, these studies showed there was no difference in the decay of plasma viremia between CD8+ depleted and mock depleted animals. Further mathematical modeling and statistical analysis suggests that CD8+ T cells act on productively infected cells primarily by preventing new infection, not cytolysis (132). Importantly, the idea of a non-cytolytic mechanism of CD8+ T cell

mediated antiviral activity does not conflict with the appearance of viral escape mutants. In other words, two independent studies of mathematical modeling confirmed that (i) the rate of decay of CD8+ escape virus was the same as the rate of decay of wild-type virus (133), and (ii) it was possible for non-lytic mechanisms to drive HIV escape variants (134).

Immune Dysfunction and exhaustion

In addition to the immune dysfunction caused by depletion of peripheral and mucosal CD4 T cells, chronic HIV infection causes significant changes in innate cells, B cells and CD8+ T cells. Microbial translocation across the damaged gut epithelium can lead to persistent signaling through PRRs, production of pro-inflammatory cytokines, and activation of innate immune cells such as macrophages and NK cells (135). Interestingly, direct binding of HIV envelope to NK cells inhibits their proliferation, secretion of IFN-γ, and cytotoxic function. *In vitro* exposure of NK cells to gp120 resulted in apoptosis of the NK cells, adding an additional layer of innate immune dysfunction caused by HIV infection (136). B cell dysfunction was one of the first observations in HIV infected individuals (137). B cells from chronically infected individuals have decreased proliferative responses, increased activation and differentiation state, as well as substantially increased levels of antibody production (hypergammaglobulinemia) (137). This immune dysfunction is a result of both T dependent and T independent mechanisms. The HIV-specific CD8+ T cells of chronically infected individuals lack much of the antiviral functions described above. The inhibitory receptor programmed death-1 (PD-1) is highly expressed on HIV/SIV-specific CD8+ T cells during chronic infection and is associated with disease progression (138, 139). CD8+ T cell dysfunction includes loss

of perforin and granzyme expression, as well as decreased proliferative capacity. Importantly, CD8+ T cells of controllers lack this characteristic T cell dysfunction.

Antiretrovirals: How they changed the course of HIV

AIDS associated morbidity and mortality has significantly decreased with the advent of antiretroviral therapy (ART). However, there are no drugs which can excise an integrated provirus from a latently infected cell, or drive the cell into permanent latency. As such, any interruption in treatment will result in rapid viral rebound, and treatment must be maintained for the life of the patient. Due to the infidelity of reverse transcriptase and poor adherence to ART regimens due to toxicity, drug resistance mutations can develop during long-term ART (140). Multiple drugs must be available that target different stages of the viral life cycle to prevent the virus from mutating away from a monotherapy, even in a perfectly adherent patient. Fortunately, there are several stages unique to the viral life cycle to serve as targets for ART that will not interfere with normal cellular processes.

The first drug to be used against HIV was azidothymidine (AZT). It is a thymidine analog with a high affinity for HIV reverse transcriptase. The thymidine analog gets incorporated into the proviral genome and causes chain termination, due to the lack of a 3'-hydroxyl. There are two classes of reverse transcriptase inhibitors, nucleoside analog reverse transcriptase inhibitors (NRTIs), like AZT, which mimic nucleosides in the cytoplasm of an infected cell and cause chain termination, and non-nucleoside reverse transcriptase inhibitors (NNRTIs) that directly bind to and inhibit the enzyme reverse transcriptase. Another process unique to the HIV life cycle is the integration of proviral DNA into the host genome, catalyzed by the enzyme integrase. Integrase inhibitors block the strand transfer reaction, thus preventing the provirus from incorporating into

the host cell. We know that viral proteins encoded in *gag*, *pol,* and *env* are translated as polyproteins by host ribosomes, which need to be cleaved by the viral protease in order to become fully functional. Protease inhibitors are an important class of drugs which prevent this process. This targets post-integration stages of the viral life cycle such as virion assembly, budding and maturation. There are also entry and fusion inhibitors, which block CCR5 and gp41 respectively, preventing infection of new target cells.

As mentioned previously, antiretroviral therapy has dramatically changed the disease course for HIV infected individuals (141-144). Through the mechanisms described above, ART reduces the level of plasma viremia to below the clinical limit of detection (20 copies/mL). With lower levels of plasma HIV RNA the likelihood of transmission is significantly decreased (49, 145). Reduction in viral load also partially decreases immune activation, though levels still remain elevated compared to uninfected individuals (146). The most widely accepted hypothesis for residual levels of immune activation is incomplete restoration of gut mucosal integrity and elevated levels of lipopolysaccharide (LPS) in plasma (135). We know chronic immune activation is a critical factor in pathogenesis of HIV/SIV infection based on our studies of the comparative model of non-pathogenic SIV infection of SM and AGM. These species mount a robust innate immune response that is quickly resolved, with no microbial translocation, preservation of gut mucosal integrity, and no chronic immune activation (147).

ART also restores functionality of CD8+ T cell responses (148). After prolonged ART, CD8+ T cell phenotype is markedly changed from chronic HIV infection. CD8+ T cells from ART treated individuals have a less differentiated phenotype, expressing more IL-7Rα and CD28 than CD8+ T cells in viremic patients (148). They express less PD-1 and have increased polyfunctionality. However, their ability to immediately kill virally

infected cells is reduced, compared to viremic patients, as evidenced by decreased levels of perforin within cytotoxic granules (148). However, decreased cytotoxic potential does not mean the cells are functionally impaired as they may have returned to a more resting, central memory-like phenotype in the absence of antigen. Interestingly, the clonality of the CD8+ T cell repertoire dramatically changes under prolonged ART (149). Briefly, certain clonotypes persist, or are generated, under long-term ART and these clonotypes have superior functional abilities compared to those found in chronic infection. It has yet to be shown whether upon antigen decline, the persistent clonotypes improve their functionality, or if particular clonotypes, with greater functional capacity, have a selective advantage in limited antigen situations (149). Despite these observed changes in CD8+ T cells during antiretroviral therapy, no one has directly assessed the role for CD8+ T cells in maintaining virus suppression under ART. This will be discussed in Chapter 4 of the dissertation.

Patients on ART partially reconstitute their CD4 T cell compartment, restoring some balance to the immune system. The restoration of the CD4 T cell compartment depends on initial CD4 T cell depletion and numbers of CD4 T cells at time of ART initiation. However, incomplete CD4 T cell reconstitution is especially evident in the gut mucosa (150). Lessons from non-human primates suggest the quality of the CD4 T cell subsets that are restored is as important as the quantity. In particular, the Th17 subset, which is responsible for maintaining gut mucosal integrity, is depleted from the gut early in infection. This particular subset is not reconstituted, even under long-term suppressive ART. Based on studies in SM and AGM, preservation of Th17 cells is a critical factor in preventing pathogenesis. Recent studies in NHP have suggested cytokine therapy to specifically restore Th17 cells to rectal mucosa and reduce chronic immune activation (151).

Challenges of ART

Immune reconstitution inflammatory syndrome (IRIS) occurs in 10-20% of patients initiating a potent HAART regimen. This inflammatory syndrome can sometimes present as an "unmasking" of the opportunistic infections associated with AIDS. Incidence of IRIS declines with time during continuous ART (152). In addition to IRIS, there are significant challenges associated with establishing universal antiretroviral therapy. As mentioned above, this drug regimen can be harsh on patients, with many side effects, including loss of appetite, nausea, lipodystrophy, lipohypertrophy, and even renal complications, though newer generation regimens have dramatically reduced occurrences of these symptoms (153). Despite side effects and some early inflammatory complications upon initiation of treatment, the benefits of ART far outweigh the costs. Yet, side effects are not the major barrier to adherence and universal treatment. The largest barrier is resource availability, with the majority of HIV infections concentrated in resource poor areas like sub-Saharan Africa. According to the World Health Organization (WHO), in 2013, an estimated 35 million people are living with HIV, and over 60 percent of those people live in sub-Saharan Africa. Adults and adolescents are "eligible" for ART when their CD4 counts go below 500 cell/μL, with the recommendation that all children under 5, pregnant women, and individuals co-infected with *Mycobacterium tuberculosis* (MTb), or Hepatitis B (HBV) receive treatment. Of those 35 million people, about 28 million people are eligible to receive antiretroviral therapy, while only 11 million are currently on treatment. So, while ART is effective for those who receive proper treatment, universal access is still out of reach.

HIV Persistence: Virus replication versus production

In addition to cost and accessibility concerns, ART is unable to eradicate HIV from infected cells post-integration. This is perhaps the most significant limitation of

current therapy. In addition to viral rebound upon treatment interruption, about 75-80% of individuals on therapy with viral loads below the clinical detection limit (20 copies/ml,) have residual plasma viremia of, on average, 3 copies/ml, with an assay limit of detection of 1 copy/ml (154). There are two working hypotheses for the origin of this viremia. The first hypothesis is residual viremia is evidence of ongoing virus replication. The second hypothesis is that patients on suppressive treatment for many years harbor a small pool of quiescent, infected cells ready to produce virus given the opportunity (155-157). This pool of cells is called the HIV reservoir. The precise location and mechanism of maintenance of this HIV reservoir is an active area of research.

To understand the distinction between the two hypotheses we must address the distinction between virus production and virus replication. Production of virus requires full genome transcription and virion budding from an already infected cell, without regard to what that virion does once it is out of the cell. Replication includes production of virus and then infection of new target cell. The ongoing replication hypothesis assumes there are sites where ART is not fully penetrant or 100% effective, and therefore, new cells can be infected. To support this hypothesis, it is well established that while concentrations of ART in the blood are high, penetration into tissues is markedly reduced. Certain classes of drugs have differential ability to penetrate different tissues, and some tissues are considered sanctuaries from ART, such as the CNS and female genital tract. Though not completely excluded, ART penetrance into lymphoid tissues is also less than peripheral blood. NRTIs and NNRTIs have the highest penetration capacity and protease inhibitors have the lowest (158-160). These sites, especially lymphoid tissue, are rich with infected cells and a prime location for migration of new target cells. Therefore, low drug penetrance could allow for ongoing replication and subsequent escape of these variants into the plasma.

There is significant evidence against the ongoing replication hypothesis. First, reverse transcriptase is extremely error prone, making a mistake almost every round of replication (140). If residual viremia were a result of ongoing virus replication, there should be accumulation of mutations and virus evolution during long-term ART. In patients on ART for many years, there is evidence of drug resistant mutations in residual plasma viremia. If replication was occurring, the prediction would be an increase in viremia in these patients due to outgrowth of drug resistant variants. However, these drug resistant viruses do not result in sustained accumulation of virus in the plasma, suggesting no active selective pressure, and refuting the ongoing replication hypothesis (161, 162). Further evidence against this hypothesis comes from treatment intensification studies, where, in addition to the traditional ART regimen, an integrase inhibitor is added. If residual viremia was the result of ongoing virus replication, preventing integration should result in a decrease in viremia and a decay in the viral reservoir over time. In one particular study, patients were given Raltegravir, an integrase inhibitor, in addition to their ART regimen. This treatment had no effect on levels of residual plasma viremia or decay of the HIV reservoir (163, 164). This study also looked at the formation of 2-LTR circles. 2-LTR circles occur when the 5' and 3' LTRs ligate together after reverse transcription. Since 2 LTR circles are a relatively unstable form of proviral DNA they are used as a marker for recent infection. Using this measurement, 29% of those receiving Raltegravir intensification had an increase in 2 LTR circles, compared to 5% of placebo. These data suggests a possible role of ongoing replication, but only in a subset of individuals. In another study looking only at the replication competent reservoir, they saw an overall reduction with Raltegravir intensification (165). These divergent results could be do to the different methods used to measure the reservoir, to be discussed more in depth below. Based on these conflicting data, there is

still much research and debate on the question of ongoing virus replication under ART, particularly in areas with sub-optimal drug penetration.

HIV Persistence: Latency

The second, prevailing hypothesis is that residual viremia is a product of reactivation from a small pool of long-lived, latently infected cells established prior to ART initiation. Viral latency is a transcriptionally silent, quiescent stage of the viral life cycle, where the genomic material is still replication competent, but no proteins or transcripts are being made. Other viruses, such as those of the Herpesviridae family have a latent stage of their life cycle. Latency provides several advantages to the virus, including evading the immune system and preventing cell death due to virus cytopathology. In the absence of treatment, HIV causes rapid virus and CD4 T cell turnover, making the frequency of latent HIV infection difficult to estimate (166, 167). ART allows a distinctly unique opportunity to understand HIV latency, as the frequency of new and productively infected cells dramatically decreases with length of treatment, eventually nearing zero.

The establishment of HIV latency *in vivo* is not completely understood. One hypothesis is an activated CD4 T cell is infected with HIV and, upon reversion to a quiescent, resting memory state, the provirus, now dependent on host cell machinery also enters a latent, transcriptionally silent state as well. There is ample evidence supporting this hypothesis. Resting memory CD4 T cells express levels of CCR5 similar to naïve CD4 T cells, and significantly less than activated CD4 T cells, making direct infection of these cells inefficient and a rare event (168, 169). Another barrier to direct infection of resting memory CD4 T cells is the epigenetic changes that occur upon reversion to resting memory. These changes include recruitment of histone deacetylase (HDACs) to alter the chromatin structure to be less permissive for transcription (170,

171). *In vitro* it has been shown that activated cells can revert to a resting state while harboring integrated provirus (172). Additionally, quiescent cells lack the necessary transcription factors to induce HIV-1 transcription, such as NFκB, NFAT and PTEFb (171, 173-175). Together, these data support the hypothesis that the HIV reservoir is a pool of latently infected cells, but suggests HIV latency is an accidental consequence of T cell activation state.

Other mechanisms for the establishment of latency have been proposed. For example, after co-culture with CCL19, the ligand for CCR7, there is preferential establishment of latent infection, rather than productive infection. In *vitro* co-culture with CCL19 shows an enhanced efficiency of nuclear localization of RNA. This effect is transient and reversible, showing a limited pretreatment window of three hours before infection. Interestingly, levels of CCL19 are extremely high in T cell zones of the lymph node, so the lymphoid tissue resident central memory T cells infected with HIV are constantly exposed to this cytokine. This suggests latent infection is preferentially established in T_{CM}. In this model of latency, levels of integrated DNA correlated with actin polymerization, independent of cellular activation (measured by CD69, HLA-DR, and CD25) (172). While this method of latent infection is independent of cellular activation state, it still suggests viral latency is an accidental consequence of the host-cell environment.

Recently, the hypothesis that latent HIV infection is an active, virus intrinsic mechanism of immune evasion has gained supporting evidence. The HIV accessory protein transcription transactivator (Tat) is essential to virus replication. Blocking of this protein *in vitro* leads to significant inhibition of virus replication (176). Despite it's essential function during virus replication, this protein is not packaged into the budding virion. The gene is located extremely 3' of the 5' LTR. It is the first protein to be

translated from the rare, multiply spliced RNA produced from a newly integrated provirus (177). Once translated, Tat binds to TAR elements and significantly enhances the efficiency of transcription. Importantly, viruses with mutant Tat transition into latency much faster than those with wild-type Tat, though wild-type Tat viruses can still establish latent infection (171, 178). One study in particular showed that Tat variants accumulate early in infection, and do not all appear to be driven by MHC restriction. Additionally, virus isolated from ART suppressed co-cultures harbored more Tat sequences with impaired function, compared to pre-treatment plasma virus (178). This suggests that viruses with mutant Tat are selected to persist long-term because of inefficient transcription. Additionally, latency can be "rescued" or reversed by addition of exogenous Tat. Tat can, independently of cellular activation state, activate NFκB and recruit PTEFb, both transcription factors necessary for replication. It will also recruit modulators of histone acetylation, causing epigenetic changes to a more transcriptionally permissive state (179). It is worth noting, not all replication competent virus is induced during the viral outgrowth assay (180). Investigators find full-length, intact, replication competent HIV proviruses in cells that did not produce virus despite stimulation with potent activation reagents. Another study recently showed *in vitro* relaxation from activated to resting state in primary CD4 T cells had little no effect on virus transcription, despite confirmed down regulation of classical activation markers (181). These data suggest establishment and maintenance of latency is a complex process that is not an accidental consequence of cellular activation state, but something that is intrinsic to the evolution of HIV. This hypothesis is consistent with latent stages of other viruses as particular evolutionary adaptations.

There are many ways to measure the latent viral reservoir. The viral outgrowth assay is the standard for measuring the size of the replication competent reservoir.

Briefly, cells isolated from ART suppressed individuals are stimulated with a potent mitogen and cultured for several weeks. The amount of virus is measured either by ELISA of supernatant or intracellular p24 staining. Based on these measurements, the frequency of replication competent, latently infected cells in ART treated patients was estimated to be about 1 in 10^6 (182). Recent evidence suggests this is a minimal estimate, as the viral outgrowth assay only measures the inducible latent reservoir. In fact, if the non-responding wells of a viral outgrowth assay are re-stimulated a percentage of the non-responding wells will now make virus (154). The HIV reservoir estimate by viral outgrowth is approximately 2 logs lower than the frequency determined by measuring total cell associated HIV-DNA. Measuring cell-associated HIV*gag* by PCR, however, significantly overestimates the replication competent HIV reservoir. Most cell-associated DNA is replication incompetent with large deletions and hyper-mutations. Currently, the most accurate measurement of the entire, replication competent, latent reservoir includes sequencing the virus found in infected cells from ART suppressed individuals and cloning these genomes into vectors to assess replication competency. This particular method requires substantial amounts of material, and thus the degree to which this can be done is limited. Using this combined sequencing and cloning technology, the frequency of latent, replication competent virus is estimated to be closer to 50-100 cells per million in long-term ART suppressed patients (180).

HIV Persistence: The problem of T cell memory

The immune system is responsible for in recognizing foreign invading pathogens and clearing them from the body. The adaptive immune system learns from every encounter and can respond more rapidly to a pathogen it has seen before due to the presence of a subset of cells which remain after the infection is cleared. These memory cells are a critical line of defense against invading pathogens. Unfortunately, HIV has co-

opted this very important adaptive immune response for its own benefit. Based on their longevity and susceptibility to infection, memory CD4 T cells are the most well characterized site of HIV persistence. Understanding the generation and maintenance of the various memory subsets is critical to understanding HIV persistence.

Originally, two subsets of CD4 and CD8+ memory T cells were described with different homing potentials and effector functions (183). The first of these subsets was named central memory (T_{CM}) based on their expression of the lymphoid homing molecules CCR7 and CD62L and their primary residence in secondary lymphoid organs. They make mostly IL-2 promoting T cell survival, and only small amounts of interferon-γ (IFN-γ). The second subset, lacking CCR7 expression, has a more effector-like phenotype (effector memory, or T_{EM}). These effector memory cells are able to produce high levels of IFN-γ, as well as cytolytic molecules such as perforin and granzyme. These cells reside in peripheral tissues and circulate through the blood. Interestingly, in humans, CD8+ T cells are almost exclusively CCR7-, with a small subset of CCR7+CD8+ T_{CM} residing in lymphoid tissues (184). Both T_{CM} and T_{EM} cells can proliferate robustly in response to antigenic stimulation (183). Further phenotypic and functional analysis has delineated several more subsets of memory T cells (185). One such subset important to this dissertation is a naïve-like subset with stem cell-like properties. Expression of the Fas death receptor (CD95) and the IL-2Rβ (CD122) on otherwise naïve-like cells, delineates these memory T cells with stem cell-like properties (T_{SCM}). These cells were originally described in mice, and have since been found in humans and non-human primates (186-188). T_{SCM} are enriched in secondary lymphoid tissues, compared to peripheral tissues, and have enhanced proliferative potential and self-renewal capacity compared to other memory subsets. T_{SCM} are better able to retain

their phenotype upon antigenic stimulation, while still producing more differentiated cells such as T_{CM} and T_{EM}.

While the heterogeneity of memory CD4 and CD8+ T cell subsets is well established, the precise mechanism for generation and maintenance of these memory T cells is an active area of research. One model of memory T cell generation is the linear contraction model. This model postulates that all activated naïve T cells go through an effector phase (E) and the cells that survive contraction become T_{EM}. These T_{EM} have the capacity to become T_{CM}. This model calls the T_{CM} the "true" memory T cells, endowed with all the proliferative capacity and ability to generate effector cells lacking in the T_{EM} population (189). This model places the T_{EM} population as a transient state on the way from activated effector to T_{CM} and, presumably, T_{SCM}. This linear contraction model of memory generation is the most consistent with the establishment of latent HIV infection as infected cells return to a quiescent, resting memory state. Eventually, this would allow T_{CM} to comprise the entire HIV reservoir.

The progressive differentiation model of memory T cell generation predicts the nature of the interaction between the TCR and peptide:MHC (pMHC) determines the differentiation state of the cell. In this case, a naïve T cell gets activated, and based on the strength of the interaction between pMHC and TCR, the length of the interaction, and additional co-stimulatory signals, the cell differentiates into one of several memory T cell subsets (190). Based on the combination of these interactions, the activated cell will become T_{SCM}, T_{CM}, or T_{EM}. In this model, the subsets are hierarchical in nature, with the preceding subset having the capability to generate each subsequent subset. The progressive model is supported by the observation that a single naïve T cell, specific for a single antigen can give rise to a heterogeneous T cell population. (191).

T cell Memory: Maintenance

Because CD4 memory T cells are the most well characterized HIV/SIV reservoir, the molecules and interactions required for maintaining that pool are also factors involved in maintaining HIV persistence. Original studies delineating factors necessary to maintain memory T cells were done in cytokine and MHC deficient mice and found differential requirements for CD4 and CD8+ T cell maintenance. Whereas CD8+ T cells could survive on IL-7 and IL-15 alone, CD4 T cells required tonic signaling through TCR:MHC-II, in addition to cytokine signals. IL-7 in particular has been shown to induce proliferation and accumulation of memory CD4 T cells in ART treated macaques (192). Based on the data that IL-7 promotes virus production in productively infected cells, a clinical trial was conducted trying to use IL-7 to reactivate latently infected cells under ART. IL-7 administration led to rapid proliferation of memory CD4 T cells and a 70% increased in CD4 T cells harboring HIV-DNA, with no reactivation events (193). This study has shown that IL-7 promotes homeostatic proliferation and HIV persistence, not reactivation, during ART. *In vitro* studies examined the potential for homeostatic proliferation to reactivate latent virus and found cells will divide, but virus transcription does not occur (194).

Studies in humans using deuterated, or heavy, water showed the half-life of memory T cells is 1-12 months (185). Memory T cells also have shorter telomeres, indicating they are maintained by homeostatic proliferation and have undergone several rounds of cell division. Recent studies looking at the newly described T_{SCM} subset showed these cells are able to persist in humans for up to 12 years and in addition to IL-7 and IL-15, are heavily reliant on the Wnt/β-catenin signaling pathway (188), (195). Wnt signaling is highly prevalent in hematopoietic stem cells (HSCs), which are extremely multipotent and self-renewing. Canonical Wnt signaling requires binding of a ligand to the Wnt receptor. In the absence of this binding, a destruction complex including GSK3β,

recruits and phosphorylates β-catenin, targeting it for degradation. β-catenin is the master transcription factor for the cell survival gene program initiated by Wnt signaling (196). Wnt signaling prevents GSK3β from associating with the destruction complex, thus allowing β-catenin to translocate to the nucleus. Along with TCF1 and LEF1, β-catenin translocates to the nucleus and promotes cell survival. Studies have shown naïve T cells express higher levels of TCF1 and LEF1 than activated or memory T cells (197). T_{SCM} express levels slightly lower than naïve, but greater than any other memory subset (188). Using a pharmacological inhibitor of GSK3β on stimulated naïve T cells, Gattinoni et al were able to generate a large population of T_{SCM}, highlighting the importance of this signaling pathway in the differentiation of T cells and maintenance of T_{SCM}. There is therapeutic potential for inhibitors of β-catenin (GSK3β agonists) in cancer and HIV persistence.

Memory Subsets and HIV/SIV persistence

We know memory T cell homeostasis to be a critical factor in regulating pathogenesis in HIV/SIV infection based on our comparative studies of the NHP model of pathogenic and non-pathogenic infection. As mentioned briefly, SIV is endemic in African monkeys and several species are infected in the wild, yet never develop progressive disease. Two species that have undergone the most extensive study are the sooty mangabey (SM) and the African green monkey (AGM). There are several factors that are critically different in non-pathogenic SIV infection. One, discussed briefly earlier, is the resolution of acute immune activation and lack of microbial translocation (147).

The pattern of infected cells is also believed to be a critical determinant for pathogenicity in HIV and SIV infection. It is well established that pathogenic and non-pathogenic HIV/SIV infection rapidly depletes effector memory-like T cells highly expressing the co-receptor CCR5 during acute infection (38, 95). These cells are

replenished through differentiation of $T_{CM,}$ as evidence by a dramatic increase in proliferation during acute infection (198). This production of short-lived effector cells temporarily restores balance to the CD4 T cell pool. In pathogenic infection, this increase in proliferation is maintained during chronic infection until overt disease. In non-pathogenic infection, this proliferation is quickly resolved along with further signs of chronic immune activation (199). A distinct difference between pathogenic and non-pathogenic infection is the infection and depletion of the less-differentiated T_{CM} (95, 96). In non-pathogenic infection these cells are relatively protected from infection (57, 95, 96), allowing natural hosts, such as SM and AGM, a preservation of the homeostasis of their CD4 memory T cell compartment.

Several groups have shown the long-lived, central and transitional memory CD4 T cells are the major sites of virus residence during both treated and untreated HIV/SIV infection (95, 96, 200, 201). Based on their increased longevity compared to T_{CM}, a role for CD4 T_{SCM} has been proposed in HIV persistence. One elegant study showed T_{SCM} harbor higher levels of HIV DNA in HAART treated patients than other memory subsets (T_{CM}, T_{TM}, T_{EM}) and much higher than naïve CD4 T cells (202). This study shows the amount of HIV-DNA in T_{SCM} is stable over time, but the contribution to the total reservoir increases, based on the decline of HIV-DNA in T_{TM} and T_{EM} (202). In contrast, another study found very low levels of infection of T_{SCM} in HAART treated patients compared to other memory subsets. However, they also indicated this frequency of infection was stable, and therefore the contribution to the total HIV-DNA reservoir increased over time (203). While the frequency of infection in HAART treated patients is inconsistent between these two studies, the stability of the HIV-DNA within these cells makes them an important site of HIV persistence. The role of CD4 T_{SCM} in SIV pathogenesis and their

potential as an HIV/SIV reservoir will be discussed in Chapters 2 and 3 of this dissertation.

Summary

Over the more than 30 years of HIV/SIV research, we have gained extensive insights into the mechanisms of HIV pathogenesis and successful treatment leads to lower transmission and decreased morbidity and mortality. We have learned the immune response to HIV/SIV infection is complex with many factors influencing disease progression. However, much is still unknown about why the mechanisms of immune homeostasis in CD4+ T cells fails to protect the host from disease progression. Elucidating the role of the recently described CD4+ T_{SCM} in pathogenic and non-pathogenic SIV infection allows us to postulate a role for these cells in protecting the CD4+ T cell compartment from dysregulation. Though CD4+ T_{SCM} may be important in maintaining immune homeostasis, based on their longevity compared to other memory subsets and their level of infection in pathogenic SIV infection, CD4+ T_{SCM} are a potentially critical site of HIV/SIV persistence during ART. While understanding the precise cellular nature of the HIV/SIV reservoir may be important for future interventions, equally as important is understanding the interactions of the host immune response during ART. Specifically, what role CD8+ lymphocytes play in promoting virus suppression and maintaining the HIV/SIV reservoir. We know CD8+ lymphocytes phenotypically and functionally undergo dynamic changes during ART, as antigen is withdrawn from the system. However, whether or not the cells that remain are necessary for viral control is unknown. Overall, the goal of this dissertation is to understand what role CD4+ T cell memory plays in pathogenesis and SIV persistence, as well as CD8+ lymphocyte antiviral function during ART.

Chapter 2:

Title: Divergent CD4+ T memory stem cell dynamics in pathogenic and nonpathogenic SIV infections

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Abstract

Recent studies have identified a subset of memory T cells with stem cell-like properties (T_{SCM}) that include increased longevity and proliferative potential. Here, we examined the dynamics of CD4+ T_{SCM} during pathogenic SIV infection of rhesus macaques (RM) and nonpathogenic SIV infection of sooty mangabeys (SM). While SIV-infected RM show selective numeric preservation of CD4+ T_{SCM}, SIV infection induced a complex perturbation of these cells defined by depletion of CD4+CCR5+ T_{SCM}, increased rates of CD4+ T_{SCM} proliferation, and high levels of direct virus infection. The increased rates of CD4+ T_{SCM} proliferation in SIV-infected RM correlated inversely with the levels of central memory CD4+ T cells (T_{CM}). In contrast, nonpathogenic SIV infection of SM evidenced preservation of both CD4+ T_{SCM} and CD4+ T_{CM}, with normal levels of CD4+ T_{SCM} proliferation, and lack of selective depletion of CD4+CCR5+ T_{SCM}. Importantly, SIV DNA was below the detectable limit in CD4+ T_{SCM} from eight out of ten SIV-infected SM. We propose that increased proliferation and infection of CD4+ T_{SCM} may contribute to the pathogenesis of SIV infection in RM.

Introduction

Pathogenic HIV infection of humans and SIV infection of rhesus macaques (RM) are characterized by progressive depletion of CD4+ T cells and development of a lethal state of immunodeficiency termed AIDS (204). In contrast, SIV infection of African nonhuman primate species that are natural hosts for the virus, such as the sooty mangabeys (SM) and the African green monkeys (AGM), are typically nonpathogenic despite high virus replication (54) While the mechanism responsible for the development of AIDS remains incompletely understood, a series of recent studies have emphasized the role played by chronic immune activation and the direct infection of CD4+ central memory T cells (CD4+ T_{CM}), with these two phenomena being significantly reduced in SIV-infected SM as compared to RM (96). During pathogenic HIV and SIV infections, high levels of direct virus infection of CD4+ T_{CM} are associated with the depletion of CD4+ T cells in blood, lymph nodes, and mucosal tissues, thus suggesting a direct link between virus-mediated CD4+ T_{CM} killing, CD4+ T_{CM} depletion, and onset of a clinically relevant immunodeficiency (95, 198, 205-207). According to this model, CD4+ T_{CM} are essential to maintain the overall CD4+ T cell homeostasis due to their in vivo longevity and high proliferative potential resulting in the ability to maintain their pool as well as the more differentiated pool of CD4+ effector memory (T_{EM}) T cells (183, 208). Interestingly, infection of CD4+ T_{CM} seems to play a significant role in the persistence of reservoirs of latently infected cells in HIV-infected individuals treated with antiretroviral therapy (201), and low levels of latent CD4+ T_{CM} infection are present in HIV-infected individuals that control virus replication either spontaneously (206) or after ART (207).

A series of recent studies has identified a phenotypically and functionally novel subset of memory T cells with stem cell-like properties that were termed “T memory stem cells” or T_{SCM} (187, 188, 209, 210). These cells represent the “stem cells” of the memory T cell compartment, as they are uniquely able to self-renew as well as differentiate into all other memory T cell subsets (i.e., T_{CM}, T_{EM}, and transitional memory T_{TM}) (188). Additional properties of T_{SCM} include increased longevity and higher proliferative potential when compared to other T cell memory subsets (188). Phenotypically, T_{SCM} are defined in humans as CD45RA+CD45RO-CD62L+CCR7+CD27+CD28+CD127+CD95+CD122+, and in RM and pigtailed macaques as CD45RA+CCR7+CD27+CD28+CD127+CD95+ (187, 188). In both humans and RM, T_{SCM} express levels of CXCR3, Bcl-2, and LFA-1 intermediate between naive and T_{CM} (187, 188). T_{SCM} are found predominantly in peripheral blood and secondary lymphoid tissues, but not in mucosal tissues (187). In the context of SIV infection, CD8+ T_{SCM} are involved in the long-term maintenance of virus-specific CD8+ T cell-mediated responses (187). At this time, however, the contribution of CD4+ T_{SCM} to HIV or SIV pathogenesis remains unknown.

In this study, we examined for the first time CD4+ T_{SCM} in both healthy and SIV-infected RM and SM. We found that the absolute number of CD4+ T_{SCM} is preserved during both pathogenic and nonpathogenic SIV infections, but SIV-infected RM showed a selective depletion of CD4+CCR5+ T_{SCM}. We also found that, in SIV-infected RM, but not in SIV-infected SM, CD4+ T_{SCM} display significantly higher levels of proliferation that correlate inversely with both percentage and absolute number of CD4+ T_{CM}. Importantly, substantial levels of direct virus infection of CD4+ T_{SCM} were seen only in SIV-infected RM, with the majority of SIV-infected SM lacking SIV DNA within CD4+ T_{SCM}. Based on these data we propose that increased proliferation and infection rates of CD4+ T_{SCM} may

play a role in the pathogenesis of SIV infection in RM. Based on their longevity and high levels of direct virus infection in pathogenic SIV-infection, we postulate CD4+T_{SCM} may be an important site for the HIV/SIV reservoir as well as for maintaining memory T cell homeostasis.

Materials and Methods

Animals. Twenty-seven SIV-uninfected RM and 13 SIV-uninfected SM, plus 39 chronically SIV-infected RM and 19 chronically SIV-infected sooty SM were included in this study. All SIV-infected RM had been previously infected intravenously (i.v.) with SIV_{mac239} or SIV_{mac251}. To obtain frequency of infection data from SM, blood was collected from six experimentally infected and four naturally infected SM. Four experimentally infected SM were infected i.v. with 0.5 ml of plasma (titrated to 10^7 SIV RNA copies/ml) from a naturally SIV_{smm}-infected SM and two experimentally infected SM were inoculated i.v. with 25 ng p27 equivalent of SIVsmE041 (primary isolate derived from a naturally SIV_{smm}-infected SM, 31). Of the SIV-infected SM, eight were heterozygous for the wild type CCR5 allele and the previously described delta 2 or delta 24 alleles that are not associated with reduced susceptibility to infection in the heterozygous state (29). In RM, acute infection was defined as day 7 to 14 post infection, early stage chronic infection was defined as day 42 to 84 post infection, and late chronic infection was defined as day 128 to 365 post infection. We obtained complete blood counts (CBC) for 16 SIV-uninfected, 18 acutely infected, 11 early chronic, and 10 late chronic RM, thus absolute numbers could only be calculated from these animals. All animals were anesthetized prior to the performance of any procedure, and proper steps were taken to ensure the welfare and to minimize the suffering of all animals in these studies. The animals were housed at the Yerkes National Primate Research Center of Emory University and maintained in accordance with US National Institutes of Health guidelines under IACUC approved protocols. Anesthesia was used for all blood collections.

Tissue processing. Peripheral blood mononuclear cells (PBMCs) were freshly isolated from whole blood by density centrifugation or sodium citrate CPT tubes. Frozen PBMCs were thawed in 37 degree water bath and used immediately.

Immunophenotyping. Immunophenotyping was performed according to standard procedures and monoclonal antibodies cross-reactive in both SM and RM were used. The following antibodies were used for immunophenotyping of CD4+T_{SCM} in SM and RM PBMCs: Live/Dead Fixable Aqua from Invitrogen, CD14-V500 (M5E2)**,** CD16-V500 (3G8)**,** CD3-APC-Cy7 (SP34-2), CD45RA-APC (5H9), CCR7-PE-Cy7 (3D12), Ki67-Alexa 700 (B56), CCR5-PE (3A9), CD95-PE-Cy5 (DX2), CXCR3-PerCP-Cy5.5 (1C6/CXCR3), CD11a-FITC (HI111), CD122-Biotin (Mik-β3**)**, streptavidin-PE**,** all from Becton Dickinson. CD4-BV650 (OKT4), CD8-BV711 (RPA-T8), CD27-BV570 or -BV605 (O323), streptavidin-605 all from Biolegend, CD28-ECD (CD28.2) from Beckman Coulter. Flow cytometric acquisition was carried out on an LSRII flow cytometer driven by the FACS DiVa software package (Becton Dickinson). Analysis of the acquired data was carried out using FlowJo (Tree Star) and PRISM (Graph Pad) software.

Cell Sorting. Sorting of CD4+ T_N, T_{SCM}, T_{CM}, and T_{EM} cells from SIV-infected rhesus macaques and sooty mangabeys was performed on a FACS Aria II flow cytometer (Becton Dickinson). Cells were initially gated on the basis of light scatter, followed by positive staining for CD3 and CD4 and negative staining for Live/Dead Aqua. CD4+ T_N (CD28+CD95-CCR7+), T_{CM} (CD45RA-CD28+CD95+CCR7+) and T_{EM} (CD45RA-CD95+CCR7-) cell subsets were gated on characteristic patterns of CD28, CD95, CCR7, and CD45RA. CD4+ T_{SCM} cells were sorted as CD45RA+CCR7+CD95+CD122+. This population was determined to be uniformly positive for CD27, CD28 (Figure 2.1A) and CD127 (data not shown).

Plasma viral load. Plasma viral RNA quantification was determined as previously described (211).

***Quantitative PCR for SIV gag DNA*.** Quantification of SIV_{mac} *gag* or SIV_{smm} *utr* DNA was performed as described in (130, 212). For cell number quantification, quantitative PCR was performed simultaneously for monkey albumin gene copy number. Albumin primers and probe along with qPCR conditions were previously described in (95). The sensitivity of the assay is 5 SIV DNA copies per 10^5 cells. Samples with undetectable SIV DNA were assigned a level of half of the lower limit of detection for graphical purposes and statistical analysis.

Statistical Analyses*.* Comparisons between frequencies in RM and SM (Figure 2.1B-E and Figure 2.2) and SIV-uninfected and SIV-infected SM (Figure 2.6) were carried out using a non-parametric Mann Whitney test. Comparisons between frequencies of CD4+ T cell subsets over time during pathogenic infection of RM (Figures 2.3-2.5) were carried out using a Kruskal Wallis test. Comparisons between frequency of infection in RM and SM (Figure 2.7) were carried out using non-parametric Mann-Whitney test. Correlations were determined using the non-Gaussian Spearman correlation. Significance was attributed at $p < 0.05$. All analyses were conducted using GraphPad Prism 5.0.

Results

Identification of CD4+ T_{SCM} in healthy rhesus macaques and sooty mangabeys

CD4+ and CD8+ T_{SCM} have been phenotypically identified in humans as CD45RA+CD45RO-CD62L+CCR7+CD27+CD28+CD127+CD95+CD122+, and in RM and pigtailed macaques are defined as CD45RA+CCR7+CD27+CD28+CD127+CD95+ (187, 188). We have first confirmed this immunophenotypic definition in healthy RM (see

Figure 2.1A for gating strategy) and SM (data not shown). As expected, CD4+ T_{SCM} isolated from both RM and SM expressed intermediate levels of CXCR3 and LFA-1 that were between those of naive and central memory CD4+ T cells (Figure 2.2). We also confirmed in healthy RM that CD4+ T_{SCM} can be readily identified in the blood, lymph nodes, bone marrow, and spleen, but are present at a lower frequency in the intestinal mucosa (data not shown). We next compared the levels of circulating CD4+ T_{SCM} in healthy SIV-uninfected RM and SM as percentage of total CD4+ T cells. As shown in Figure 2.1B, the percentage of CD4+ T_{SCM} ranged between 1-8% in RM and 0.5-3% in SM, with the levels observed in RM being significantly higher ($p = 0.0004$). Interestingly, CD4+ T_{SCM} from SIV-uninfected SM show higher levels of proliferation (measured as expression of Ki67) as compared to RM ($p = 0.0034$, Figure 2.1C), perhaps suggesting that a relatively smaller pool of CD4+ T_{SCM} maintains CD4+ memory T cell homeostasis through higher baseline rates of proliferation in SM. We also found that CD4+ T_{SCM} from RM also express slightly higher levels of the inhibitory marker PD-1 as compared to CD4+ T_{SCM} from SM, though this trend was not statistically significant (Figure 2.1D). Several previous studies have shown that CD4+ T cells of both SIV-infected and uninfected SM express lower levels of the SIV coreceptor CCR5 than CD4+ T cells of humans and RM, and that this difference is particularly evident for CD4+ T_{CM} (96, 213). We next examined the levels of CCR5 expression on CD4+ T_{SCM} from healthy RM and SM and, consistent with previous findings, we found significantly higher percentages of CCR5+CD4+ T_{SCM} from RM compared to SM ($p = 0.0009$, Figure 2.1E). Three representative examples of CCR5 staining on CD4+ T_{SCM} are shown in Figure 2.1F, which emphasize the almost complete absence of CCR5 on CD4+ T_{SCM} of SM.

Numeric preservation of CD4+ T_{SCM} during pathogenic SIV infection of RM

Pathogenic SIV infection of RM is characterized by a progressive depletion of CD4+ T cells from blood and mucosal tissues, which is typically associated with the loss of CD4+ T_{CM} homeostasis (198). To examine the dynamics of CD4+ T_{SCM} during pathogenic SIV infection of RM, we examined a total of 51 RM, including healthy SIV-uninfected animals and SIV-infected animals at different stages of infection. Consistent with many previous studies (214-217), the animals studied here exhibited the well-characterized progressive depletion of circulating CD4+ T cells associated with SIV infection, measured as the fraction of CD3+ T lymphocytes (Figure 2.3A) or absolute number of cells per micro liter of blood (Figure 2.3B). We next examined, in the same animals, the relative distribution of four key CD4+ T cell subsets, i.e., naive (CD28+CD95-); CD4+ T_{SCM} (CD45RA+CCR7+CD27+CD28+CD95+CD122+); CD4+ T_{CM} (CD28+CD95+CCR7+); and CD4+ T_{EM} (CD95+CCR7-). As expected, levels of both CD4+ T_{CM} and T_{EM} (primary targets for SIV infection) were altered by SIV infection, with a significant decline of the fraction and absolute number of CD4+ T_{CM} in both early and late chronic SIV infection ($p < 0.01$ and $p < 0.001$, respectively, Figure 2.3C-D), and a significant decline of the absolute number of CD4+ T_{EM} in late chronic SIV infection ($p < 0.001$, Figure 2.3D). Interestingly, neither the fraction nor the absolute number of CD4+ T_{SCM} was decreased in either acute or chronic SIV infection of RM (Figure 2.3C-D). As such, these data indicate that pathogenic SIV infection of RM is not associated with a significant numerical decline of circulating CD4+ T_{SCM}.

Pathogenic SIV infection of RM is associated with a significant depletion of CCR5+CD4+ T_{SCM}

CCR5 is the main coreceptor for both HIV and SIV, and depletion of CD4+CCR5+ T cells, particularly in mucosal tissues, is a well-known hallmark of pathogenic HIV and SIV infections (215, 218, 219). In this study, we first confirmed the depletion of

circulating CD4+CCR5+ T cells that begins in acute infection and continues during early and late chronic SIV infection of RM (Figure 2.4A). We next measured, in our four groups of animals, the levels of CCR5 expression on the four studied subsets of CD4+ T cells (T_N, T_{SCM}, T_{CM}, and T_{EM}). As shown in Figure 2.4B, we found that the fraction of CCR5+ cells is significantly decreased during late chronic SIV infection of RM in both CD4+ T_{CM} and T_{EM} ($p < 0.05$ and $p < 0.01$, respectively). Interestingly, we observed that the fraction of CCR5+CD4+ T_{SCM} was significantly decreased in SIV-infected RM examined during early chronic SIV infection compared to uninfected animals ($p < 0.05$). Although the median level of CCR5+CD4+T_{SCM} was also decreased in late chronic SIV infection, this difference was not statistically significant when compared to SIV-uninfected animals, likely due to a wide range of values. Importantly however, we found a significant depletion of the absolute number of CCR5+CD4+T_{SCM} (Figure 2.4C) during both early and late chronic SIV-infection. Together with the data shown in Figure 2.3, these results indicate that pathogenic SIV infection of RM is associated with a depletion of CCR5+CD4+ T_{SCM} that occurs in the context of an overall preservation of the CD4+ T_{SCM} pool.

Pathogenic SIV infection of RM is associated with increased proliferation of CD4+ T_{SCM} that correlates inversely with circulating level of CD4+ T_{CM}

To further investigate whether and to what extent pathogenic SIV infection of RM perturbs the homeostasis of CD4+ T_{SCM} we next measured, in our four groups of animals, the expression of the proliferation marker Ki67 in the studied subsets of CD4+ T cells (T_N, T_{SCM}, T_{CM}, and T_{EM}). Consistent with previous studies (198), we observed that early and late chronic SIV infection is associated with increased proliferation of both T_{CM} and T_{EM} (Figure 2.5A). Interestingly, the percentage of Ki67+CD4+ T_{SCM} was also significantly

increased in SIV-infected RM examined during both early ($p < 0.05$) and late ($p < 0.001$) stages of infection as compared to healthy uninfected animals (Figure 2.5A). The observed increase in the fraction of cycling CD4+ T_{SCM} could be the result of homeostatic proliferation in response to the overall depletion of memory CD4+ T cells, due to chronic immune activation, or both. In an attempt to assess whether homeostatic proliferation may be responsible for the increased proliferation of CD4+ T_{SCM} in SIV-infected RM, we investigated the relationship between the fraction of Ki67+CD4+ T_{SCM} and the levels of the four studied CD4+ T cell subsets. As shown in Figure 2.5B-D, we found a significant inverse correlation between the percentage of Ki67+CD4+ T_{SCM} and the percentage of circulating CD4+ T_{CM} ($p = 0.02$), but not with the level of any of the other memory CD4+ T cell subsets. These data suggest that the increased proliferation of CD4+ T_{SCM} observed in SIV-infected RM represents, at least in part, a compensatory response to the CD4+ T_{CM} depletion induced by SIV infection.

Dynamics of CD4+ T_{SCM} during nonpathogenic SIV infection of SM

Previous studies have shown that nonpathogenic SIV infection of SM is typically associated with preserved CD4+ T cell counts and low levels of immune activation (211, 220-222). Here we examined, for the first time, how SIV infection impacts the levels of CD4+ T_{SCM} in SM. As shown in Figure 5A, we found no difference in the levels of any of the four studied subsets of CD4+ T cells (T_N, T_{SCM}, T_{CM}, and T_{EM}) in SIV-infected SM as compared to uninfected animals, in contrast to the depletion of T_{CM} and T_{EM} seen in chronic SIV infection of RM (Figure 2.3C). Similarly, no differences were found between SIV-infected and uninfected SM with respect to the absolute number of CD4+ T_{SCM} per micro liter of blood (Figure 2.6B). We next investigated whether SIV infection of SM is associated with a selective depletion of CD4+CCR5+ T_{SCM}, and unlike the loss of these

cells in SIV-infected RM (Figure 2.4B), we found similar levels of CD4+CCR5+ T_{SCM} in SIV-infected and uninfected SM (Figure 2.6C). To determine whether SIV infection of SM is associated with increased proliferation of CD4+ T_{SCM}, we measured the fraction of these cells expressing Ki67. As shown in Figure 2.6D, we found no difference in the fraction of CD4+ T_{SCM} expressing Ki67 among SIV-infected and uninfected SM. Taken together, these data indicate the that nonpathogenic SIV infection of SM is characterized by overall preservation of the CD4+ T_{SCM} compartment, involving both CD4+CCR5- and CD4+CCR5+ cells, and does not result in increased turnover of this memory cell subset, consistent with the previously described maintenance of peripheral CD4+ T cell homeostasis in SIV-infected SM (96).

Robust levels of CD4+ T_{SCM} infection in vivo are observed in SIV-infected RM but not in SIV-infected SM

In two prior studies we have shown that pathogenic SIV infection of RM is associated with higher levels of SIV DNA in both circulating CD4+ T_{CM} as well as lymph node-based CD4+ T cells as compared to SIV-infected SM (95, 96). These results led us to hypothesize that preservation of the CD4+ T_{CM} compartment is a key determinant of the nonpathogenic nature of SIV infection of SM. To expand upon these observations, here we measured the levels of SIV DNA in flow-cytometrically sorted samples of the four studied subsets of CD4+ T cells (T_N, T_{SCM}, T_{CM}, and T_{EM}) in nine SIV_{mac251}-infected RM and ten SIV_{smm}-infected SM. Please note that for sorting we have phenotypically defined T_{CM} as CD45RA-CD28+CD95+CCR7+ and T_{EM} as CD45RA-CD95+CCR7-, which differs slightly from the markers used previously in Paiardini et al, and Brenchley et al (96, 204). The reason for this choice was to incorporate the definition of T_{SCM} in nonhuman primates established by Lugli et al (187). As shown in Figure 2.7A, we

observed a robust frequency of infection (i.e., greater than 1/1000 cells) in CD4+ T_{SCM} isolated from nine out of nine SIV-infected RM. In contrast, SIV DNA levels in CD4+ T_{SCM} were undetectable in eight out of ten SIV-infected SM. As previously reported (96), the level of SIV DNA was higher in CD4+ T_N and CD4+ T_{CM} of RM as compared to SM. Shown in Figure 2.7B, plasma viral loads of SIV-infected RM tended to be higher than those of SM, although this trend was not statistically significant. Overall, these results indicate that in vivo infection of CD4+ T_{SCM} is frequent during pathogenic SIV infection of RM, but is absent or rare during nonpathogenic SIV infection of SM.

Discussion

In the past several years, the mechanisms responsible for AIDS pathogenesis have been extensively investigated in the pathogenic model of SIV infection of RM and the nonpathogenic model of SIV infection of SM (54, 223). These comparative studies led to the definition of a model in which chronic immune activation and disrupted homeostasis of central memory CD4+ T cells are the key mechanisms responsible for the lentivirus-associated immunodeficiency (96, 198, 205, 224). More recently, a novel subset of memory CD4+ and CD8+ T cells has been identified, and named “memory stem cell” (T_{SCM}) based on their unique ability to generate all other memory T cell subsets de novo (187, 188). In this study, we sought to determine how CD4+ T_{SCM} are affected by pathogenic and nonpathogenic SIV infections of RM and SM, respectively. To the best of our knowledge this study represents the first systematic investigation of the dynamics of CD4+ T_{SCM} during SIV infection.

The main results of this study are that: (i) CD4+ T_{SCM} are numerically preserved during both pathogenic and nonpathogenic SIV infections, with SIV-infected RM showing a selective depletion of CD4+CCR5+ T_{SCM}; (ii) CD4+ T_{SCM} show significantly higher levels

of proliferation that correlate inversely with the percentage of CD4+ T_{CM} in SIV- infected RM, but not SM; (iii) robust levels of direct virus infection of CD4+ T_{SCM} are found only in SIV-infected RM, with the majority of SIV-infected SM showing no evidence of CD4+ T_{SCM} infection. The observation that CD4+ T_{SCM} of healthy, SIV- uninfected RM express higher levels of CCR5 as compared to CD4+ T_{SCM} of healthy SM is consistent with previous findings in T_{CM} and a potential inherent resistance to direct infection at the virus entry level in SM (3).

Taken together, these data allow us to delineate a model for the role of CD4+ T_{SCM} in SIV pathogenesis. In SIV-infected RM, the we observe significant perturbation of the homeostasis of CD4+ T_{SCM} in three ways, as these cells can be directly infected by the virus, are depleted in the percentage of cells expressing CCR5, and manifest increased proliferation. In contrast, none of these perturbations in the T_{SCM} pool are present in SIV-infected SM. The significant inverse correlation between CD4+ T_{SCM} proliferation and CD4+ T_{CM} depletion we observed in SIV-infected RM suggests that CD4+ T_{SCM} proliferate at least in part to compensate for the progressive loss of CD4+ T_{CM}. While the overall numeric homeostasis of CD4+ T_{SCM} is not altered in SIV-infected RM, it is possible that this cellular compartment loses, in time, the ability to effectively support the maintenance of CD4+ T_{CM}. Whether and to what extent the deficit of CD4+ T_{CM} that is associated with progression to AIDS is related to a functional exhaustion of CD4+ T_{SCM} as opposed to the direct depleting effects of virus infection and/or bystander apoptosis remains to be determined.

A striking difference between SIV-infected RM and SIV-infected SM is the level of virus infection of these cells as measured by fraction of SIV DNA positive cells. While all SIV-infected RM showed a calculated percentage of CD4+ T_{SCM} infection between 0.3 and 10%, eight out of ten SIV-infected SM show undetectable levels of CD4+ T_{SCM}

infection (i.e., less than 0.005%). Given the lower levels of CCR5 expression on CD4+ T_{SCM} of SM as compared to RM, one possibility is that these cells are resistant to virus infection at the entry level, analogous to what has been observed for CD4+ T_{CM} (96). It should be noted, however, that other co-receptors, in addition to CCR5 (225, 226), as well as post-entry factors may be involved in determining the different levels of SIV infection in CD4+ T_{SCM} of RM and SM. Unfortunately, due to the relatively small number of CD4+ T_{SCM} that can be isolated from SM, we were not able to directly confirm in vitro that these cells are intrinsically more resistant to in vitro SIV infection then CD4+ T_{SCM} of RM.

A recent compelling study indicates that CD4+ T_{SCM} represent an increasingly important component of the persistent reservoir of latently infected cells in HIV-infected individuals treated with antiretroviral therapy (ART) (202). The observation that CD4+ T_{SCM} are infected at high levels during pathogenic SIV infection of RM is consistent with the possibility that, once virus replication is suppressed by ART, a subset of these cells remain latently infected and may seed the previously described persistent reservoir in T_{CM} (201). Under these circumstances, the contribution of CD4+ T_{SCM} to the persistent reservoir may increase over time simply as a consequence of their enhanced proliferative ability. An intriguing corollary of this hypothesis is that, in SIV-infected SM, the absence of virus infection in CD4+ T_{SCM} may result in an inability to maintain a persistent reservoir of latently infected CD4+ T cells when virus replication is suppressed by ART. An experiment in which SIV-infected SM are treated with ART for increasing periods of time is currently ongoing in our laboratory. We hope that the results of these studies will further elucidate the role of direct CD4+ T_{SCM} infection as an obstacle to a functional cure for HIV infection.

In conclusion, this study provides evidence that pathogenic SIV infection of RM, but not nonpathogenic SIV infection of SM, is associated with significant infection and homeostatic perturbation of CD4+ T_{SCM}. We therefore propose that CD4+ T_{SCM} play an important role both in the pathogenesis of disease progression as well as the persistence of infection under ART.

Figures

Figure 1.

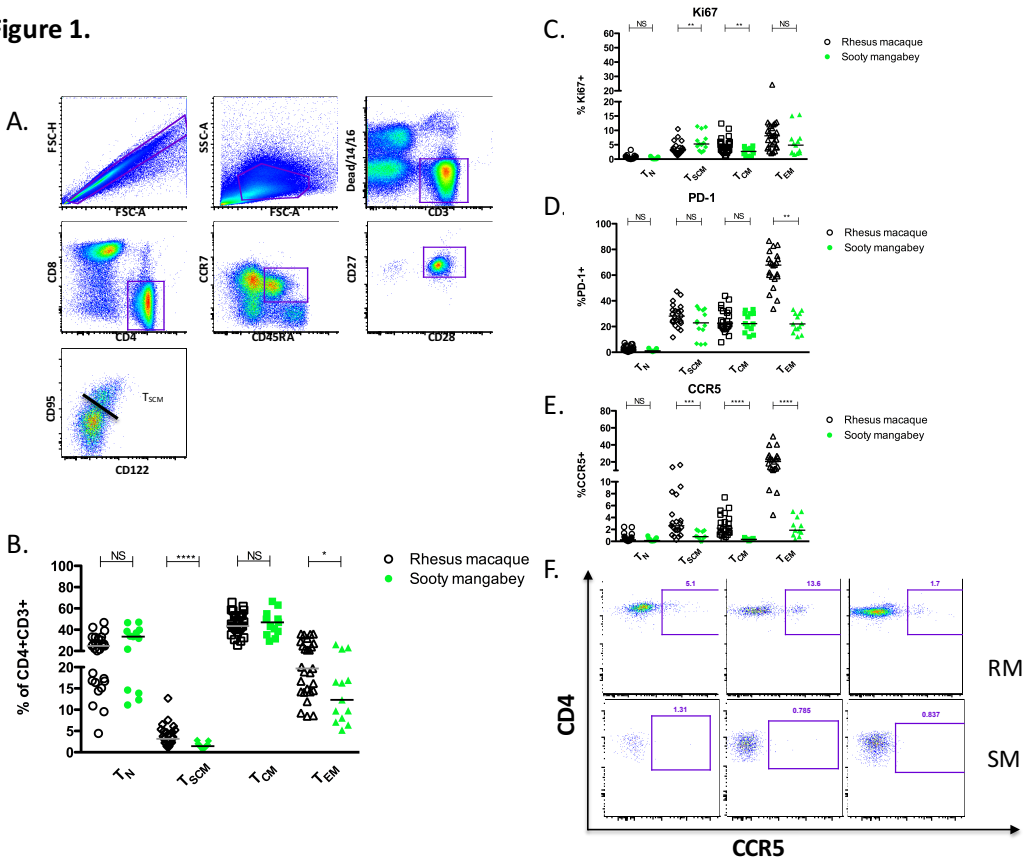

Figure 2.1 . Identification of CD4+ T_{SCM} in healthy RM and SM. (A) Flow cytometric analysis of PBMC from a representative SIV-uninfected RM. CD4+ T_{SCM} were defined as shown by expression of CD45RA+CCR7+CD27+CD28+CD95+CD122+. (B) Frequencies of circulating CD4+ T cell subsets (T_N, T_{SCM}, T_{CM}, T_{EM}) in 27 SIV-uninfected RM and 13 SIV-uninfected SM along with the fraction of each subset expressing Ki67 (C), PD-1 (D), and CCR5 (E). T_N, T_{CM}, T_{EM} were defined using CD95, CD28 and CCR7: T_N (CD28+CD95-CCR7+), T_{CM} (CD45RA-CD28+CD95+CCR7+), T_{EM}(CD95+CCR7-). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, NS = not significant (Mann-Whitney). Bars are drawn at the median. (F) Representative CCR5 staining on CD4+ T_{SCM} cells from 3 SIV-uninfected RM and SIV-uninfected SM.

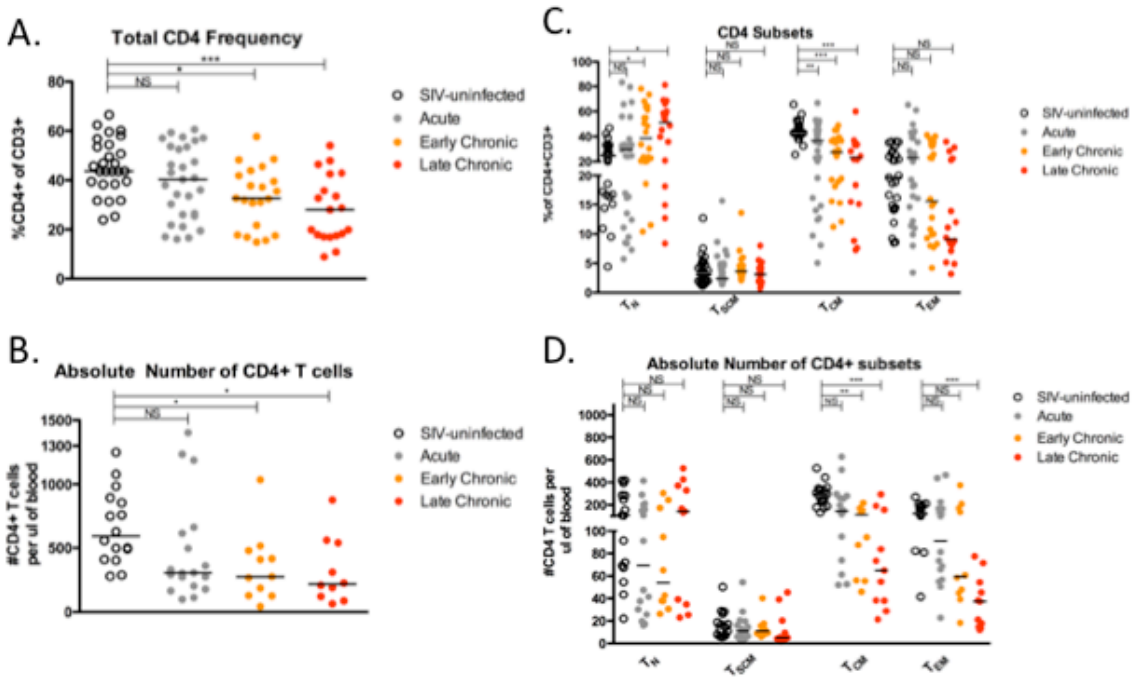

Figure 2.2. Selective preservation of CD4+ T_{SCM} cells during pathogenic SIV infection of RM. Frequency (A) and absolute number (B) of total CD3+CD4+ lymphocytes in PBMC of RM during pathogenic SIV infection. Frequency (C) and absolute number (D) of T_N, T_{SCM}, T_{CM}, T_{EM} subsets in PBMC of RM during pathogenic SIV infection. Data in (A) and (C) represent the following RM: 27 SIV-uninfected, 29 acutely infected (day 7-14) 22 early chronic infection (day 65-84), and 19 late chronic infection (day 128-365). Data in (B) and (D) represent the following RM: 16 SIV-uninfected, 18 acutely infected (day 7-14), 11 early chronic (day 65), and 10 late chronic (day 128-365). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, NS = not significant (Kruskal-Wallis test, compared to SIV-uninfected). Bars are drawn at the median.

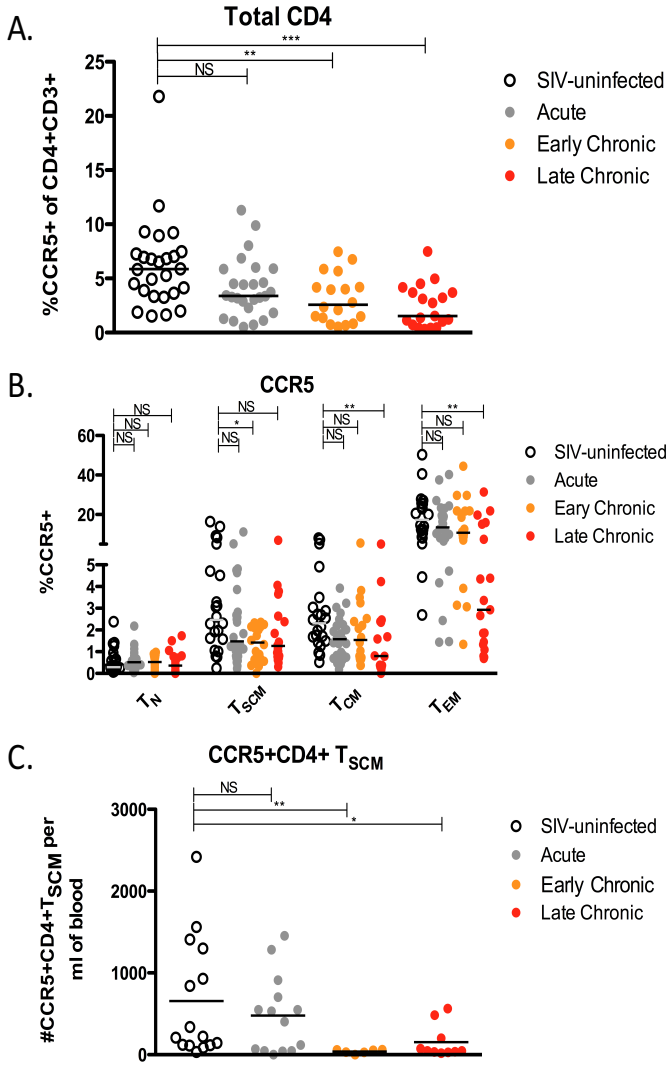

Figure 2.3. Pathogenic SIV infection of RM is associated with significant depletion of CCR5+CD4+ T_{SCM}. (A) Frequency of total CCR5+CD4+ T cells as a frequency of CD3+ lymphocytes during pathogenic SIV infection of RM. (B) Frequency of CCR5+ cells found in each of the four subsets (T_N, T_{SCM}, T_{CM}, and T_{EM}). Data in (A) and (B) represent the following RM: 26 SIV-uninfected, 26 acutely infected (day 7-14), 18 early chronic infection (day 65-84), and 19 late chronic infection (day 128-D365). (C) Absolute number of CCR5+CD4+ T_{SCM} during pathogenic SIV infection of RM per ml of peripheral blood. Data in (C) represent the following RM: 15 SIV-uninfected, 15 acutely infected, 6 early chronic SIV-infection, and 10 late chronic SIV-infection. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, NS = not significant (Kruskal-Wallis, compared to SIV-uninfected). Bars are drawn at the median.

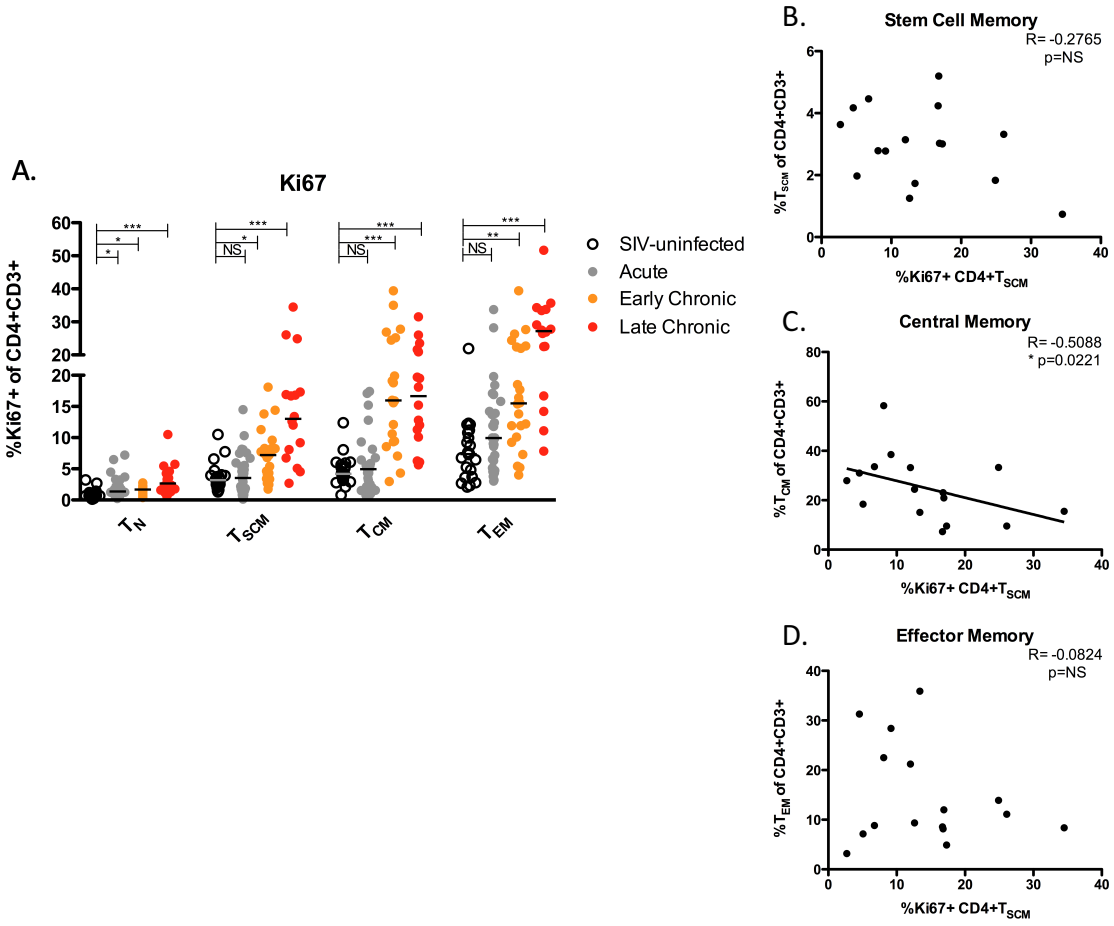

Figure 2.4. Pathogenic SIV infection of RM is associated with increased proliferation of CD4+ T_{SCM} that inversely correlates with the level of CD4+ T_{CM}. (A) Frequency of Ki67+ T_N, T_{SCM}, T_{CM}, T_{EM} in PBMC during pathogenic SIV infection. Data represents the following RM: 22 SIV-uninfected, 29 acutely infected (day 7-14), 22 early chronic infection (day 65-84), and 18 late chronic infection (day 128-365). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, NS = not significant (Kruskal-Wallis test, compared to SIV-uninfected). Bars are drawn at the median. (B-D) Correlations of the fraction of Ki67+CD4+ T_{SCM} and fraction of circulating CD4+ T_{SCM}, CD4+ T_{CM}, and CD4+ T_{EM} during late chronic infection. R and p values were determined by Spearman correlation.

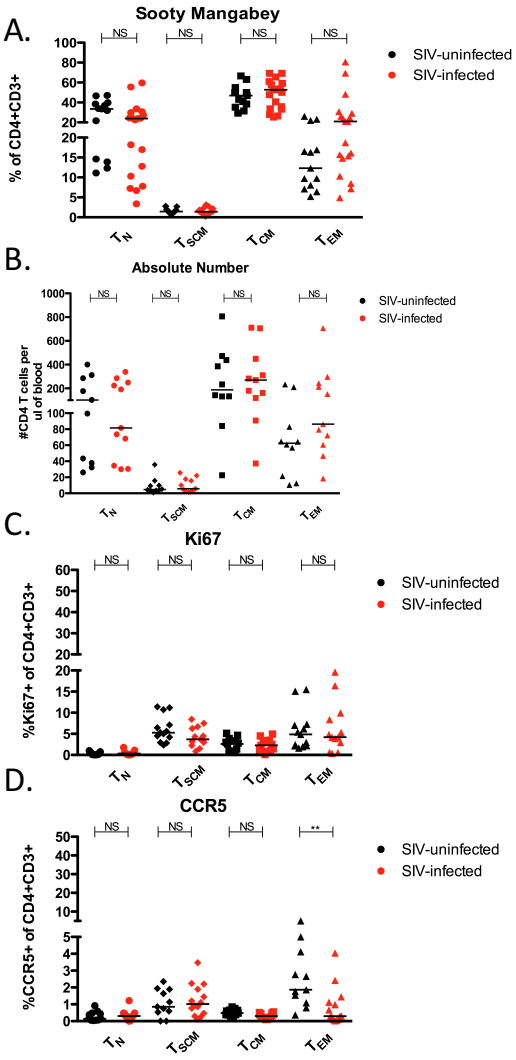

Figure 2.5. CD4+ T_{SCM} are unperturbed during non-pathogenic SIV infection of SM. Comparison of frequency (A) and absolute number (B) of CD4+ T_N, T_{SCM}, T_{CM}, T_{EM} in SIV-uninfected and chronically SIV-infected SM. (C) Frequency of CCR5+ T cells in each CD4+ T cell subset in SIV-uninfected and chronically SIV-infected SM. (D) Frequency of proliferating CD4+ T cell subsets, as measured by Ki67 expression in both uninfected and chronically SIV-infected SM. ** $p < 0.01$, NS = not significant (Mann-Whitney). Bars are drawn at the median.

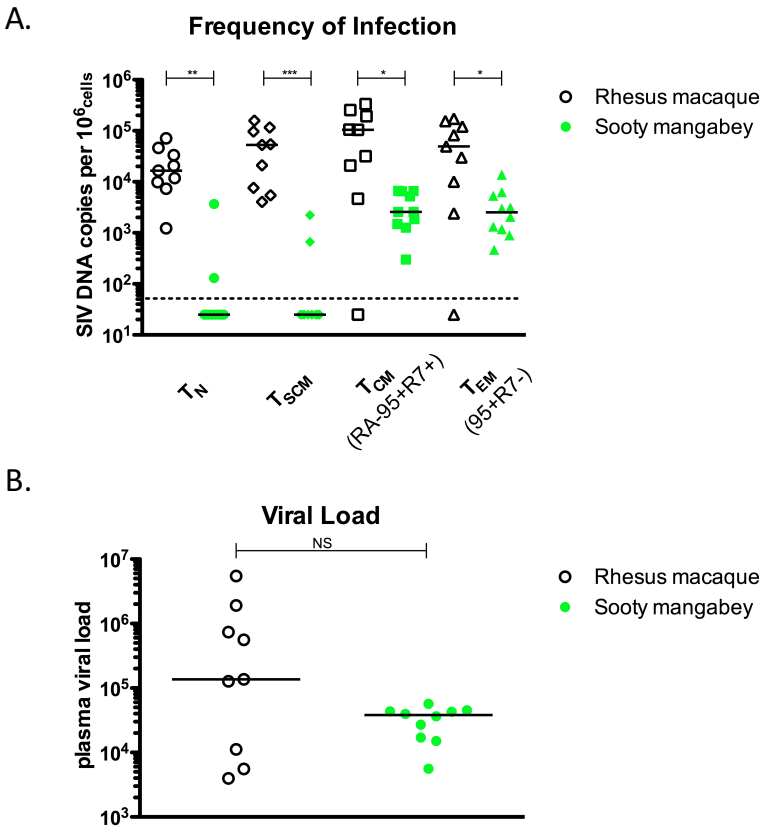

Figure 2.6. Robust levels of CD4+ T_{SCM} infection *in vivo* are observed in SIV-infected RM but not in SIV-infected SM. (A) Fraction of SIV-infected CD4+ T_N, T_{SCM}, $T_{CM,}$ and T_{EM} cells, as determined by quantitative PCR for the number of SIV *gag* (RM) or SIV *utr* (SM) DNA copies per cell in 9 SIV_{mac251}-infected RM, 6 experimentally SIV_{smm}-infected SM, and 4 naturally SIV_{smm}-infected SM. Cell number was determined using simultaneous PCR for albumin gene copy number. (B) Plasma viral load of RM and SM shown in (A) as determined by RT-PCR. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS = not significant (Mann-Whitney test). Bars are drawn at the median.

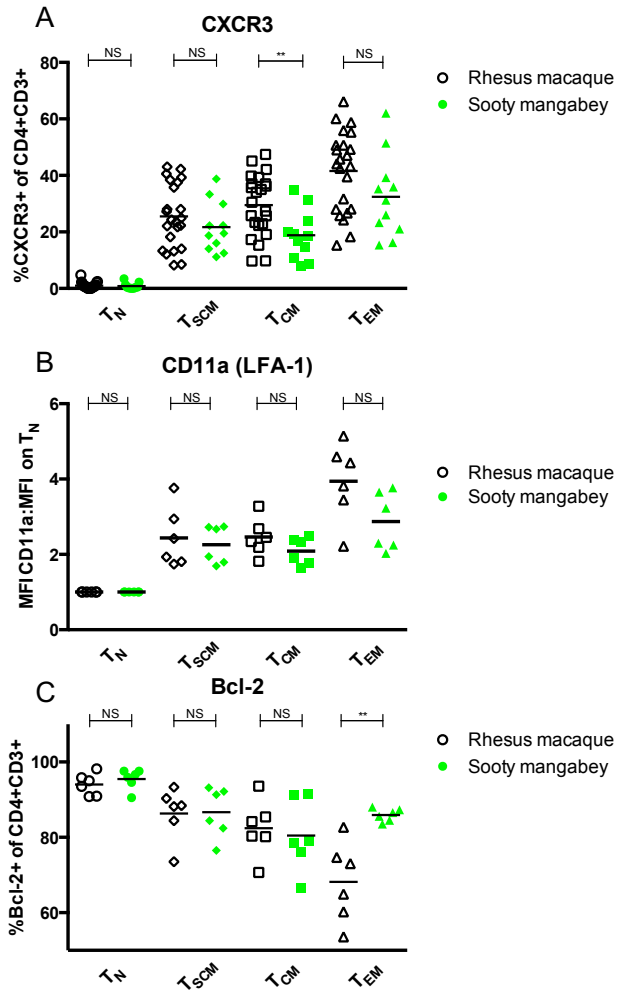

Figure 2.S1. Expression of CXCR3, LFA-1, and Bcl-2 in SIV-uninfected RM and SM. (A) Frequency of circulating CXCR3+ T_N, T_{SCM}, T_{CM}, and T_{EM} in 22 SIV-uninfected RM and 11 SIV-uninfected SM. (B) Expression of LFA-1 on circulating T_N, T_{SCM}, T_{CM}, and T_{EM} expressed as a ratio of MFI on each subset compared to T_N from 6 SIV-uninfected RM and 6 SIV-uninfected SM. (C) Frequency of circulating Bcl-2+ T_N, T_{SCM}, T_{CM}, and T_{EM} in 6 SIV-uninfected RM and 6 SIV-uninfected SM. ** $p<0.01$ (Mann-Whitney test). Bars are drawn at the median.

Chapter 3

Title: Initiation of antiretroviral therapy restores CD4+T_{SCM} homeostasis in SIV-infected macaques

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Abstract (250 words): Treatment of human immunodeficiency virus (HIV) infection with antiretroviral therapy (ART) has significantly improved the prognosis of this infection. Unfortunately, interruption of ART almost invariably results in viral rebound, which is attributed to a pool of long-lived, latently-infected cells. Based on their longevity and proliferative potential, CD4+ T memory stem cells (T_{SCM}) have been proposed as an important site of HIV persistence. In a previous study, we found that, in SIV-infected rhesus macaques (RMs), CD4+T_{SCM} are preserved in number but show (i) a decrease in the frequency of CCR5+ cells, (ii) an expansion of the fraction of proliferating Ki-67+ cells, and (iii) high levels of SIV-DNA. To understand the impact of ART on both CD4+ T_{SCM} homeostasis and direct virus infection, we conducted a longitudinal analysis of these cells in the blood and lymph nodes of twenty-five SIV-infected RMs. We found that ART induced a significant restoration of CD4+CCR5+ T_{SCM} in both blood and lymph nodes, and a reduction in the fraction of proliferating CD4+Ki-67+ T_{SCM} in blood (but not lymph nodes). Importantly, we found that the level of SIV-DNA in CD4+ transitional memory (T_{TM}) and effector memory (T_{EM}) T cells declined ~100-fold after ART in both blood and lymph nodes, while the level of SIV-DNA in CD4+T_{SCM} and central memory T cells (T_{CM}) remained constant. These data suggest that ART is effective at partially restoring CD4+T_{SCM} homeostasis and, by showing stable levels of virus in T_{SCM}, support the hypothesis that these cells are a critical contributor to SIV persistence.

Importance: Understanding the role of various CD4+ T cell memory subsets in immune homeostasis and HIV/SIV persistence during antiretroviral therapy (ART) is critical to effectively treat and cure HIV infection. T memory stem cells (T_{SCM}) are a unique memory T cell subset with enhanced self-renewal capacity and the ability to differentiate into other memory T cell subsets, such as central and transitional memory T cells (T_{CM} and T_{TM}, respectively). CD4+ T_{SCM} are disrupted, but not depleted, during pathogenic SIV infection. We find ART is partially effective at restoring CD4+ T_{SCM} homeostasis and that SIV-DNA harbored within this subset is stable compared to virus harbored in shorter-lived subsets, such as T_{TM} and effector memory (T_{EM}). Because of their ability to persist long-term in an individual, understanding the dynamics of virally infected CD4+ T_{SCM} during suppressive ART is important for future therapeutic interventions aimed at modulating immune activation and purging the HIV reservoir.

Introduction

Infection with pathogenic lentiviruses such as human and simian immunodeficiency viruses (HIV and SIV, respectively) significantly perturbs the homeostasis of the CD4+ T cell compartment through preferential infection and depletion of memory CD4+ T cells, which is the hallmark of progression to AIDS. While the availability of anti-retroviral therapy (ART) has significantly reduced the mortality and morbidity of HIV infection, this treatment cannot eradicate the virus, and even after suppression of viremia for many years, interruption of ART results in rapid viral rebound (227, 228). This rebound is most often attributed to the presence of a small pool of long-lived, largely resting, latently infected cells that harbor replication competent proviral DNA integrated in their genome (156, 157, 229). In addition, ART does not fully reverse the immunological abnormalities that are associated with this infection, including but not limited to CD4+ T cell depletion, chronic immune activation, premature immunological ageing, and mucosal immune dysfunction (158). It is now widely accepted that to reach a full understanding of the mechanisms responsible for virus persistence and residual immune dysfunction under ART it is essential to conduct studies in which various subsets of memory CD4+ T cells are investigated.

Numerous studies have shown that the memory CD4+ T-cell compartment is highly heterogeneous, and includes at least four specific cell subsets that are defined based on their differentiation status as follows: CD4+ memory stem cells (T_{SCM}, defined as CD45RA+CCR7+CD28+CD95+CD62L+), CD4+ central memory cells (T_{CM}, defined as CD45RA-CD95+CCR7+CD62L+), CD4+ transitional memory cells (T_{TM}, defined as CD45RA-CD95+CCR7+CD62L-), and CD4+ effector memory cells (T_{EM}, defined as CD95+CCR7-CD62L-) (230). In particular, T_{SCM} are a relatively small (~2-4%) subset of memory T cells that represent the “stem cells” of the memory T cell compartment, as

they are uniquely able to both self-renew and differentiate into all other memory T cell subsets (i.e., T_{CM}, T_{TM}, and T_{EM}) (188, 209). Additional properties of T_{SCM} include long *in vivo* lifespan, greater proliferative potential as compared to other T cell memory subsets, and preferential homing to secondary lymphoid tissues (188, 195). In the context of HIV and SIV infection, CD8+ T_{SCM} are thought to be involved in the long-term maintenance of virus-specific CD8+ T cell-mediated responses (187, 231, 232), while CD4+ T_{SCM} were shown to be important targets of HIV infection both in the natural history and under ART as they contain high levels of HIV-DNA (202, 233). The observation of high levels of HIV-DNA in T_{SCM} of HIV-infected individuals under ART, together with certain peculiar biological properties of these cells, such as high in vivo longevity, relative quiescence, and marked proliferative potential, led to the hypothesis that these cells represent a crucial contributor to virus persistence despite their relative low frequency (202). Indeed, two recent studies demonstrated that the length of ART is directly correlated with the relative contribution of CD4+ T_{SCM} to the total HIV DNA reservoir in HIV-infected individuals (202, 203).

In a previous study, we investigated CD4+ T_{SCM} during the natural history of experimental, pathogenic SIV infection of the non-natural hosts rhesus macaques (RMs) and the non-pathogenic SIV infection of the natural hosts sooty mangabeys (SMs) (234). We found that while the number of CD4+ T_{SCM} was overall preserved in both species upon infection, SIV-infected RMs show a significant decrease of the percentage of CD4+ T_{SCM} expressing the SIV co-receptor CCR5, and a significant expansion of the percentage of proliferating cells (based on the expression of the marker Ki-67) which correlated indirectly with the frequency of CD4+T_{CM} (234). In addition, SIV-infected RMs, but not SIV-infected SMs, show robust levels of direct virus infection (based on the measurement of the total cell-associated SIV-DNA (234). Given these results we

concluded that pathogenic SIV infection of RMs (but not the non-pathogenic SIV infection of SMs) is associated with a perturbation of the immunological homeostasis of CD4+ T_{SCM} and robust levels of virus infection in these cells. Of note, our previous study did not include any ART-treated SIV-infected RMs or SMs, and therefore did not address the potential role of these cells in contributing to the residual virological and immunological abnormalities that persist under ART.

To assess the impact of ART on both CD4+ T_{SCM} homeostasis and the level of direct virus infection during pathogenic SIV infection of RMs, we conducted a longitudinal analysis of these cells in the blood and lymph nodes of twenty-five animals that were treated for a period ranging between 2 and 6 months. The main findings of this study are that (i) ART induced a significant restoration of CD4+CCR5+ T_{SCM} in both blood and lymph nodes, (ii) ART was followed by a significant reduction in the fraction of proliferating CD4+Ki-67+ T_{SCM} in blood (but not lymph nodes), and (iii) ART did not induce a decline in the level of total cell-associated SIV-DNA in CD4+T_{SCM}. This latter finding is in stark contrast with the observation that the levels of SIV-DNA declined ~100-fold after ART in both CD4+ T_{TM} and T_{EM}. Overall these data (i) indicate that ART is effective at partially restoring CD4+T_{SCM} homeostasis disrupted during SIV infection in RMs, and (ii) support the hypothesis that T_{SCM} are a critical contributor to SIV persistence in this model of primate lentiviral infection.

Materials and Methods:

Animals

This study was conducted using 35 Indian-origin rhesus macaques. 12 infected intravenously with 3,000 tissue culture infectious dose ($TCID_{50}$) of SIV_{mac239};12 infected intravenously with 300 $TCID_{50}$ of SIV_{mac239}; 11 RM remained SIV-uninfected (used for SIV-uninfected lymph node time points) . All animals were housed at the Yerkes National Primate Research Center of Emory University and maintained in accordance with US National Institutes of Health guidelines. Anesthesia was used for all blood and tissue collections. All studies were approved by the Emory University Institutional Animal Care and Usage Committee (IACUC).

Antiretroviral therapy

12 RM were put on a four-drug ART regimen at 6 weeks post SIV infection (300 $TCID_{50}$): 20 mg/kg PMPA (Tenofovir), 40 mg/kg FTC (Emtricitabine), and 2.5 m/kg Dolutegravir administered once a day by subcutaneous (s.c) injection along with 400 mg Darunavir orally b.i.d. 12 RM (infected with 3000 $TCID_{50}$) were put on a 4-drug ART regimen at 8 weeks post-infection: 20 mg/kg PMPA (Tenofovir), 30 mg/kg FTC (Emtricitabine) administered once a day by subcutaneous (s.c) injection along with 100-150 mg Raltegravir and 400-800 mg Darunavir orally b.i.d. Animals were given oral antiretroviral treatment (Raltegravir and Darunavir) via orogastric tube on days where anesthesia was also administered, due to fasting and nausea.

Sample collection & tissue processing

Blood was collected in EDTA tubes. Plasma was obtained throughout the study by centrifugation. Peripheral blood mononuclear cells (PBMC) were obtained by density-

gradient centrifugation using Lymphocyte Separation Media (LSM) from Lonza. Lymph node biopsies were taken at 2 time points throughout the study (Day 56 pre-ART initiation, between 8-29 weeks post-ART initiation). Lymph node mononuclear cells (LMNC) were obtained by removing excess fat and connective tissue and grinding over a 70-µm cell-strainer.

Immunophenotype by flow cytometry

Multiparametric flow cytometry was performed according to a standard protocol on PBMC and LNMC using fluorescently labeled monoclonal antibodies cross-reactive in rhesus macaques. The following antibodies were used for immunophenotyping of CD4+ memory T cell subsets: CD3-APC/Cy7 (SP34-2), CD4-PE-CF594 (L200) CD8-BV711 (RPA-T8), CCR7-FITC (150503), CD45RA-PE/Cy7 (L48), CD95-PE/Cy5 (DX2), Ki67-AlexaFluor700 (B56), CD14-BV650 (M5E2), CD56-BV605 (NCAM16.2), CD62L-PE (SK11), HLA-DR-PerCPCy5.5 (G46.6), CCR5-APC (3A9) from BD Biosciences; CD28-ECD (CD28-2) from Beckman Coulter; CD16-BV421 (3G8), CD4-BV650 (OK-T4), PD-1-BV421 (EH12.2H7), from Biolegend. All flow cytometry specimens were acquired on an LSR II (BD Biosciences) equipped with FACS Diva software and analysis of the acquired data was performed using FlowJo software (Tree Star).

Cell sorting

After isolation, cells were resuspended in 30-50 ml PBS containing 2 mM EDTA and spun at 200 x g for 15-20 minutes (depending on cell volume) to remove contaminating platelets. Prior to sorting, CD4+ T cells in PBMC and LNMC were enriched using magnetic beads and column purification (Miltenyi Biotec). Enriched cells were then stained with previously determined volumes of CD3-APC/Cy7 (SP34-2), CD4-Brilliant

Violet 650 (OKT-4) CD8-Brilliant Violet 421 (RPA-T8), Live/Dead-Aqua, CD45RA-APC (5H9), CCR7-PE/Cy7 (3D12), CD95-PE/Cy5 (DX2), CD28-ECD (CD28-2), CD62L-PE (SK11). Populations for sorting were defined as follows: T_{SCM} (CD45RA+CCR7+CD95+CD62L+), T_{CM} (CD45RA-CD95+CD28+CCR7+CD62L+), T_{TM} (CD95+CCR7+CD62L-), and T_{EM} (CD95+CCR7-CD62L-). Sorting was performed on a FACSAria LSR II (BD Biosciences) equipped with FACS Diva software.

Plasma viral load and cell-associated SIVgag DNA

Plasma viral quantification was performed as described previously (211). DNA was extracted from sorted peripheral and lymph node CD4+ T-cells and CD4+ T-cell memory subsets using the Blood DNA Mini Kit (QIAGEN). Quantification of SIV_{mac} *gag* DNA was performed as previously described on the extracted cell-associated DNA by quantitative PCR using the 5′ nuclease (TaqMan) assay with an ABI7500 system (PerkinElmer Life Sciences) (235). The sequence of the forward primer for SIVmac *gag* was 5′-GCAGAGGAGGAAATTACCCAGTAC-3′; the reverse primer sequence was 5′-CAATTTTACCCAGGCATTTAATGTT-3′; and the probe sequence was 5′-6 FAM-TGTCCACCTGCCATTAAGCCCGA-TAMRA-3′. For cell number quantification, quantitative PCR was performed for monkey albumin gene copy number.

Results:

ART efficiently suppresses viremia but has no effect on frequency or absolute number of memory CD4+ CD45RA+CCR7+CD62L+(T_{SCM}) T cells

With our potent, four drug ART regimens, we were able to suppress viremia to below the limit of detection in 18 out of 25 animals (Figure 1A). Animals still viremic at "on ART" time point were excluded from "on ART" analysis, but included in SIV-uninfected and SIV+ where applicable. Time to suppression varied in each animal with one animal achieving full virus suppression prior to ART initiation and one animal requiring 23 weeks of therapy to achieve an undetectable time point (data not shown). When looking at total CD4+ T cells in PBMC and lymph nodes, we find ART unable to restore CD4+ T cell frequencies to uninfected levels (data not shown). However, when analyzed by count, there is a significant reconstitution of CD4+ T cells on ART compared to SIV-infected time point (SIV+ median= 275, on ART median= 578.5, $p<0.05$, Figure 1B), confirming the impact of ART on reducing SIV-associated mortality. To begin to understand the role ART has in restoring CD4+ T_{SCM} homeostasis, we examined the absolute count, and frequency of CD4+ T_{SCM} in PBMC and lymph node of SIV-uninfected (SIV-), after 6-8 weeks of SIV-infection (SIV+), and during ART (on ART) in RM. Consistent with previous observations that SIV-infection does not alter the frequency or number of total CD4+ T_{SCM} (234), we find ART has no impact on absolute number or fraction of memory CD45RA+CCR7+CD62L+ (T_{SCM}) in PBMC (Figure 1C, D). Additionally, both SIV-infection and ART treatment have no effect on frequency of memory CD45RA+CCR7+ (T_{SCM}) in lymph node (Figure 1E). Importantly, we find that ART is able to significantly restore numbers of memory CD45RA-CCR7+CD62L+(T_{CM}), CD45RA-CCR7+CD62L-(T_{TM}), and CCR7-CD62L-(T_{EM}) depleted during SIV-infection (data not shown).

ART significantly restores CCR5+CD4+ T cells in all tissues, though reconstitution is partial in memory CD45RA+CCR7+CD62L+(T_{SCM})

Depletion of CCR5+CD4+ T cells both in peripheral blood and in sites of transmission like the rectal mucosa is a hallmark of pathogenic SIV infection of RM (215, 218, 219). In our study, we find ART effectively increases the levels of total CCR5+CD4+ T cells in PBMC and lymph node, compared to chronic SIV-infection (Figure 2A, B). When examined by CD4+ T cell memory subset, ART is able to increase the frequency of CCR5+ memory CD45RA+CCR7+CD62L+ (T_{SCM}), CD45RA-CCR7+CD62L+(T_{CM}), and CD45RA-CCR7+CD62L-(T_{TM}) T cells in PBMC to levels comparable to SIV-uninfected PBMC (Figure 2C). However, the median frequency of circulating CCR5+ memory CD45RA+CCR7+CD62L+(T_{SCM}) on ART (1.44) remains statistically significantly lower than SIV-uninfected frequencies (T_{SCM}=4.14, $p<0.05$) (Figure 1C). This trend was consistent in CD4+T_{CM} and T_{TM} subsets in PBMC (data not shown). When we look at CCR5 expression on memory T cell subsets in lymph nodes during ART, we find a similar trend. The median frequency of CCR5+ memory CD45RA+CCR7+(T_{SCM}) on ART (2.43) remains statistically significantly lower than in SIV-uninfected lymph nodes (median= 5.14, $p<0.05$). However, ART was able to significantly restore CCR5+CD4+T_{CM}, T_{TM} and T_{EM} in lymph nodes. These data suggest that ART effectively restores the fraction of CCR5+CD4+ T cells depleted during SIV-infection, however this reconstitution was incomplete for CCR5+CD4+ T_{SCM}.

ART significantly decreases proliferation of CD4+ memory T cells in peripheral blood, but not in lymph nodes

To further assess impact of ART on CD4+ T_{SCM} homeostasis, we measured the level of proliferation in bulk CD4+ T cells and memory CD4+T cell subsets in SIV-

uninfected, SIV-infected and during during suppressive ART. Though the changes were not statistically significant, ART reduces proliferation of total CD4+ T cells as measured by Ki67 (on ART median=3.96, SIV+ median=9.7) (Figure 3A). In contrast, the frequency of Ki67+CD4+ T cells lymph nodes increases during SIV-infection and remains elevated during ART (Figure 3B). When measuring proliferation in memory T cell subsets in PBMCs, we find ART extremely potent and able to reduce the frequency of proliferating memory CD45RA+CCR7+CD62L+ (T_{SCM}) T cells compared to chronic SIV-infection to levels comparable to SIV-uninfected animals (Figure 3C). Again, this trend was consistent in other memory CD4+T cell subsets (data not shown). In contrast to PBMC, but consistent with elevated frequency of Ki67+ CD4+ T cells in total CD4+ T cells persisting during ART in lymph nodes, ART is unable to reduce the frequency of Ki67+ memory CD45RA+CCR7+ (T_{SCM}) T cells (Figure 3D). Additionally, the frequency of proliferating CD4+T_{CM} and T_{TM} remain elevated compared to SIV-uninfected baseline, though ART was able to significantly reduce proliferation compared to untreated SIV-infection for these subsets (data not shown).

Inverse relationship between frequency of proliferating memory CD45RA+CCR7+CD62L+(T_{SCM}) and fraction of circulating CD45RA-CCR7+CD62L+(T_{CM}) at all time points

Previous work showed an inverse relationship between the fraction of proliferating (Ki67+) CD4+T_{SCM} during chronic SIV-infection and the fraction of circulating CD4+ T_{CM}, suggesting increased proliferation as a homeostatic compensatory mechanism for CD4+ T_{CM} depletion (234). However, the previous work did not comment on the relationship between proliferating (Ki67+) T_{SCM} and fraction of T_{CM} in an an SIV-uninfected animal, which would lend insight into the homeostatic relationship between

these two subsets. In order to examine if the increased proliferation we see during SIV-infection in T_{SCM} could contribute to homeostatic disruption of that compartment, we examined the relationship of proliferating memory CD45RA+CCR7+CD62L+(T_{SCM}) and fraction of circulating memory CD45RA-CCR7+CD62L+ (T_{CM}) in SIV-uninfected, SIV+, and ART treated RM. We found there to be an inverse correlation between the frequency of proliferating memory CD45RA+CCR7+CD62L+(T_{SCM}) and the frequency of memory CD45RA-CCR7+CD62L+(T_{CM}) in the in SIV-uninfected PBMC, suggesting this relationship is present during homeostasis (Figure 4A). Consistent with previous observations, we also find an inverse relationship between frequency of Ki67+ memory CD45RA+CCR7+CD62L+(T_{SCM}) and frequency of memory CD45RA-CCR7+CD62L+(T_{CM}) in SIV+ PBMC (Figure 4B). Important for our conclusions, we also find this correlation exists during ART (Figure 4C), suggesting that despite changes in both proliferation of T_{SCM} and frequency of T_{CM}, the relationship between these two subsets is maintained. The observation that Ki67+T_{SCM} are always inversely correlated with frequency of T_{CM} in peripheral blood T cells suggests the increase in proliferation of T_{SCM} we see during SIV-infection could be a product of the depletion of T_{CM} in this compartment. Interestingly, we do not see the same trends in the lymph node, with no correlation between proliferating memory CD45RA+CCR7+(T_{SCM}) T cells and the frequency of memory CD45RA-CCR7+CD62L+(T_{CM}) T cells in SIV-uninfected RM at any time point (data not shown). Unsurprisingly, this suggests other factors in the lymph node microenvironment are important for maintenance of T_{CM} homeostasis.

No contraction of the SIV reservoir in memory CD45RA+CCR7+CD62L+ or CD45RA-CCR7+CD62L+ T cells in the lymph node after ART initiation

To examine the potential of CD4+T_{SCM} as a critical site of SIV persistence, we directly measure contribution of each memory CD4+ T cell subset to the total pool of SIV-DNA+ cells during ART. To accomplish this, we measured the frequency of infection of each memory subset in PBMC and lymph node of RM on ART. When comparing across subsets in either PBMC or lymph node, we find no differences in the frequency of infection (data not shown). To begin to understand the contribution of each memory subset to long-term SIV persistence, we measured the frequency of infection prior to ART initiation and after 8-29 weeks of therapy in sorted memory T cell subsets from PBMC and lymph nodes (Figure 5A-H). In PBMC, we find the frequency of infection in memory CD45RA+CCR7+CD62L+(T_{SCM}) decreases 1.5 logs (about 30-fold) from pre-ART to on ART (Figure 5A, p=0.07) and infection of memory CD45RA-CCR7+CD62L+(T_{CM}) decreases 1.8 logs (70-fold) (Figure 5B, p=0.06), though these trends were not statistically significant most likely due to small sample numbers. Similarly, the frequency of infection in the more differentiated memory CD45RA-CCR7+CD62L-(T_{TM}) and CCR7-CD62L-(T_{EM}) T cells in PBMC decreases 2 logs (2.1 log and 1.8 log, respectively; $p<0.001$) (Figure 5C, D). Most striking is the stability of SIV DNA in memory CD45RA+CCR7+CD62L+(T_{SCM}) T cells in the lymph nodes following ART initiation (Figure 5E). The median frequency of infection of memory CD45RA+CCR7+CD62L+(T_{SCM}) in lymph nodes prior to ART initiation is 1718 copies/10^6 cells compared to 1627 copies/10^6 cells on ART. Similarly, infection of CD45RA-CCR7+CD62L+(T_{CM}) does not significantly change, consistent with their role in SIV persistence (Figure 5F). When looking at the more differentiated T_{TM} and T_{EM}, like in PBMC, we see a significant contraction of the fraction of infected cells, though the contraction is slightly less dramatic (1.4 log CD45RA-CCR7+CD62L-(T_{TM}) and 1.6 log CCR7-CD62L-(T_{EM}); $p<0.01$) Figure 5G and H, respectively). These data are consistent

with the hypothesis that CD4+ T_{SCM} are an important contributor to the HIV/SIV persistence during ART, and that even though they are a smaller subset of the memory T cell compartment, the stability of infection and longevity of the subset suggest their contribution may increase with increased time on ART.

Discussion

Though ART has significantly decreased morbidity and mortality in HIV infected patients, individuals must remain on treatment for life, due to a small pool of long-lived infected cells. In addition, immune activation remains elevated in ART treated individuals compared to HIV uninfected individuals, despite suppression of viremia. Utilizing the powerful animal model of SIV-infection in rhesus macaques, we are able to more extensively examine the effects of ART on immune homeostasis and the potential for different subsets and anatomical compartments to contribute to viral persistence. To our knowledge, this is the first study to examine the phenotypic changes in CD4+ T_{SCM} during SIV-infection and ART in RM. We show that a potent 4 drug ART regimen is effective at suppressing viremia in 18 RM infected with SIV_{mac239}. ART is able to partially restore CD4+ T cell homeostasis disrupted during SIV-infection by restoring CCR5+ CD4+ T cells and restoring numbers of T_{TM} and T_{EM}. We also found an inverse relationship between the fraction of proliferating CD4+ T_{SCM} and the frequency of T_{CM} in SIV-uninfected animals. This suggests that T_{SCM} and T_{CM} occupy a specific niche within the peripheral CD4+ T cell compartment that is tightly regulated and consistently maintained. Importantly, this inverse relationship is present in SIV-infected RM, as well as RM on ART. These data are consistent with the observation that the increase in proliferating T_{SCM} seen during chronic SIV-infection is due to a decrease in peripheral T_{CM} (234).

However, during this relatively short course of ART, we see elevated activation in lymph nodes particularly within the memory CD45RA+CCR7+ T_{SCM} subset. It is important to point out the short duration of this ART regimen (8-28 weeks) and the varying time to suppression (0-21 weeks) in each animal. It is possible that longer duration of ART would eventually decrease levels of activation in memory

CD45RA+CCR7+ T_{SCM} in lymph nodes to SIV-uninfected baseline. However, it is also important to note that despite the short duration of ART, treatment was sufficient to reduce immune activation in all subsets in PBMC.

Of particular interest is the observation that despite elevated frequency of Ki67+ T_{SCM}, memory CD45RA+CCR7+T_{SCM} in the lymph nodes harbor equal proportions of SIV DNA on ART as their pre-ART frequency of infection. Previous observations would suggest CD4+ T cells harboring HIV/SIV DNA that are activated would be actively transcribing viral RNA and making viral proteins to be recognized by the immune system. Currently, we can only speculate on the apparent discrepancy. First, though we find SIV DNA within memory CD45RA+CCR7+CD62L+ (T_{SCM}) on ART, the virus may be replication incompetent. Several previous studies have shown that the majority of SIV DNA harbored during ART is replication incompetent (236, 237). Thus, activation status of memory CD45RA+CCR7+(T_{SCM}) would have no impact on production of viral proteins. However, this would also mean that T_{SCM} are not important contributors to long-term viral persistence. To address this, future studies should be conducted by sorting T_{SCM} from lymph nodes of ART treated RM and trying to isolate replication competent virus by quantitative viral outgrowth assay (QVOA) or single genome sequencing. Alternatively, is the hypothesis that viral latency is not tied exclusively to cellular activation state. Recent work has proposed that latency is an intrinsic viral property and not a consequence of cellular activation (181). Using the above described methods of sequencing and QVOA, this hypothesis can also be examined. Unfortunately, these assays are technically challenging to perform on small numbers of sorted T_{SCM} subsets, which are a relatively small proportion of CD4+ T cells. Future studies in which these questions are the primary aims and all possible resources go to answering these questions will be necessary.

An alternative hypothesis, also technically challenging to ascertain is the possibility that lymph node T_{SCM} are in an anatomically distinct part of the lymph node excluded from recognition by CD8+ T cells. Previous work has shown CD8+ T cells are largely excluded from the germinal center in progressive HIV/SIV infection (238). Due to the numerous phenotypic markers used to describe T_{SCM}, little work has been done to examine their precise anatomic locations within the lymph node. Additionally, expression of homing markers such as CXCR5 has not been examined in detail. Therefore, it is possible that T_{SCM} are residing in sanctuary sites during ART.

Just as important as the lack of contraction of SIV DNA in T_{SCM} and T_{CM}, we find our short-term ART regimen significantly impacted the SIV-DNA in both CD4+ T_{TM} and T_{EM} in PBMC and lymph nodes. This is consistent with previous observations by Buzon, et al and Jaafoura, et al that suggest the relative contribution of CD4+T_{SCM} increases as the total HIV DNA reservoir decreases (202, 203). It is also worth noting that the number of CD4+ T_{TM} and T_{EM} increases on ART from the number found in chronic SIV-infection. Supposing our ART regimen prevents new rounds of replication, the decrease in fraction of infected T_{TM} and T_{EM} at this early time point post-ART, may be due to an increase in cell number and not necessarily reflective of the half-life of the particular memory subset. One important caveat to this study is there was no long-term ART treated RM to directly compare the reservoir in each memory T cell subset over time, with a stable CD4+ T cell compartment. It will be important in future studies to allow ART treated RM to restore their CD4+ T cell compartment to equilibrium before attempting to estimate the contribution of each subset to long-term persistence.

Based on the observation these cells can survive in humans for decades, calculations on estimating the half-life of the HIV/SIV reservoir must be adjusted to include the half-life of infected CD4+ T_{SCM}. In fact, recent calculations estimate it will take

~100 years of ART to eradicate the CD4+ T_{EM} reservoir (203). This stark number to eradicate the shortest-lived reservoir brings into focus the need for combination approaches to therapy. These approaches may include latency reactivation agents (LRA), such as HDAC inhibitors or TLR-7 agonists, or check-point blockade inhibitors, such as PD-1 blockade. It may be important in the future to specifically modulate the longer-lived T_{SCM} population by using inhibitors of the Wnt/ β-catenin signaling pathway. Inhibition of the Wnt pathway will hopefully force T_{SCM} to differentiate into shorter-lived cells, thus shortening the half-life of this reservoir without the added risk of reactivating the virus. Studies are underway to understand the effect of these inhibitors on not only CD4+ T_{SCM} frequencies, but also CD8+ T cell function. The most likely scenario for future treatments will include combination of antiretroviral therapy and some cocktail of PD-1 blockade and LRAs, with a β-catenin inhibitor to specifically force T_{SCM} differentiation. We believe our study suggests CD4+ T_{SCM} are an important site of SIV persistence and investigation in how to safely and effectively manipulate this pool of cells is crucial to future HIV/SIV research.

Figures and legends

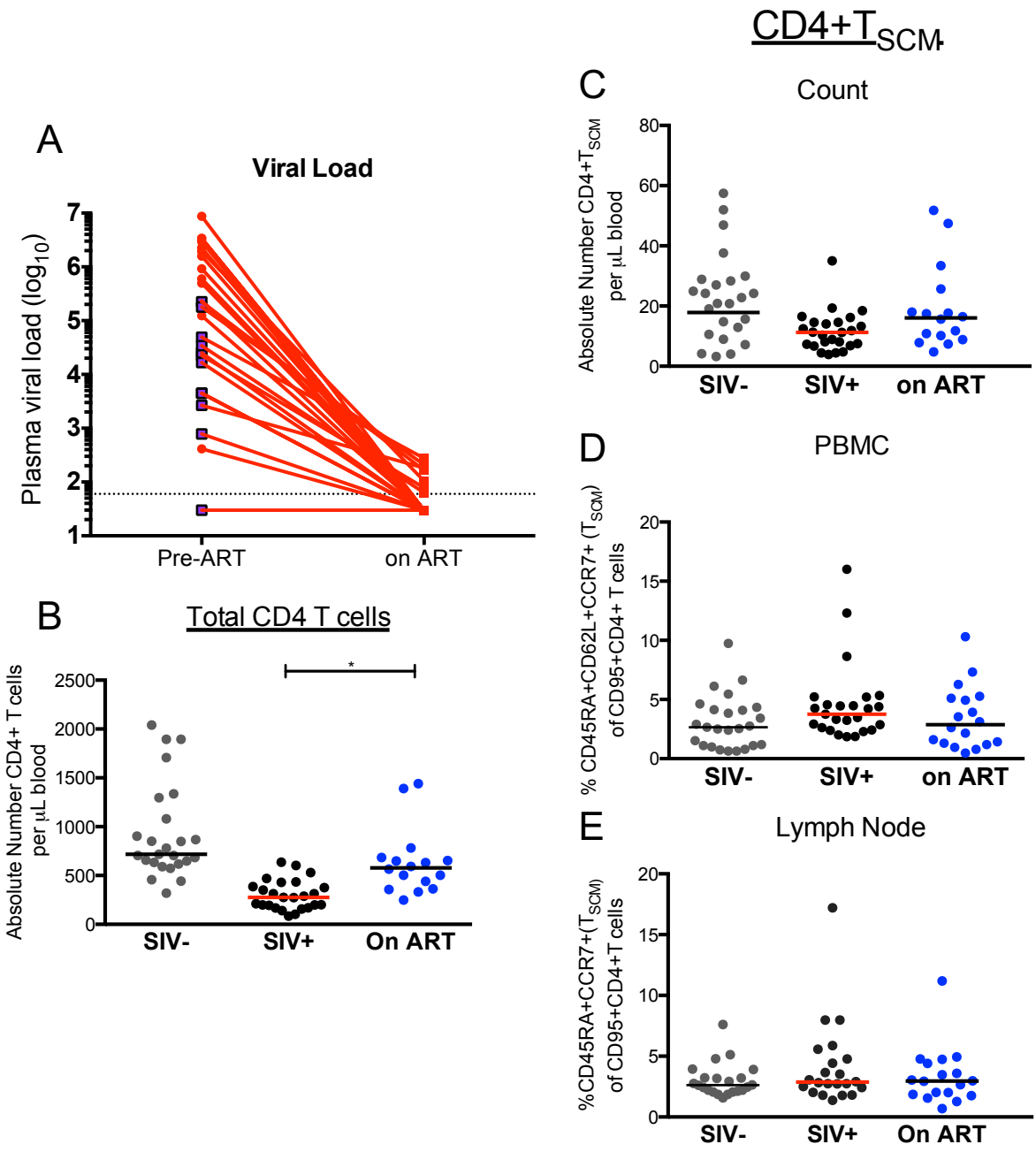

Figure 3.1. ART effectively reduces viremia and restores CD4+ T cells in PBMC. (A) Plasma viral load of 25 rhesus macaques prior to ART initiation (left side) and at "on ART" time point (8-28 weeks post-treatment initiation). Purple squares on left side are 12 animals infected with 300 $TCID_{50}$ and put on 4-drug regimen at 6 weeks SIV-infection. Animals with detectable viral loads were excluded from "on ART" time point in phenotype analyses. (B) Absolute number of CD4+ T cells in PBMC. (C) Absolute number of $CD4+T_{SCM}$. Fraction of $CD4+T_{SCM}$ in (D) PBMC and (E) lymph node. Bars are drawn at the median. Kruskal Wallis, two tailed, *$p<0.05$.

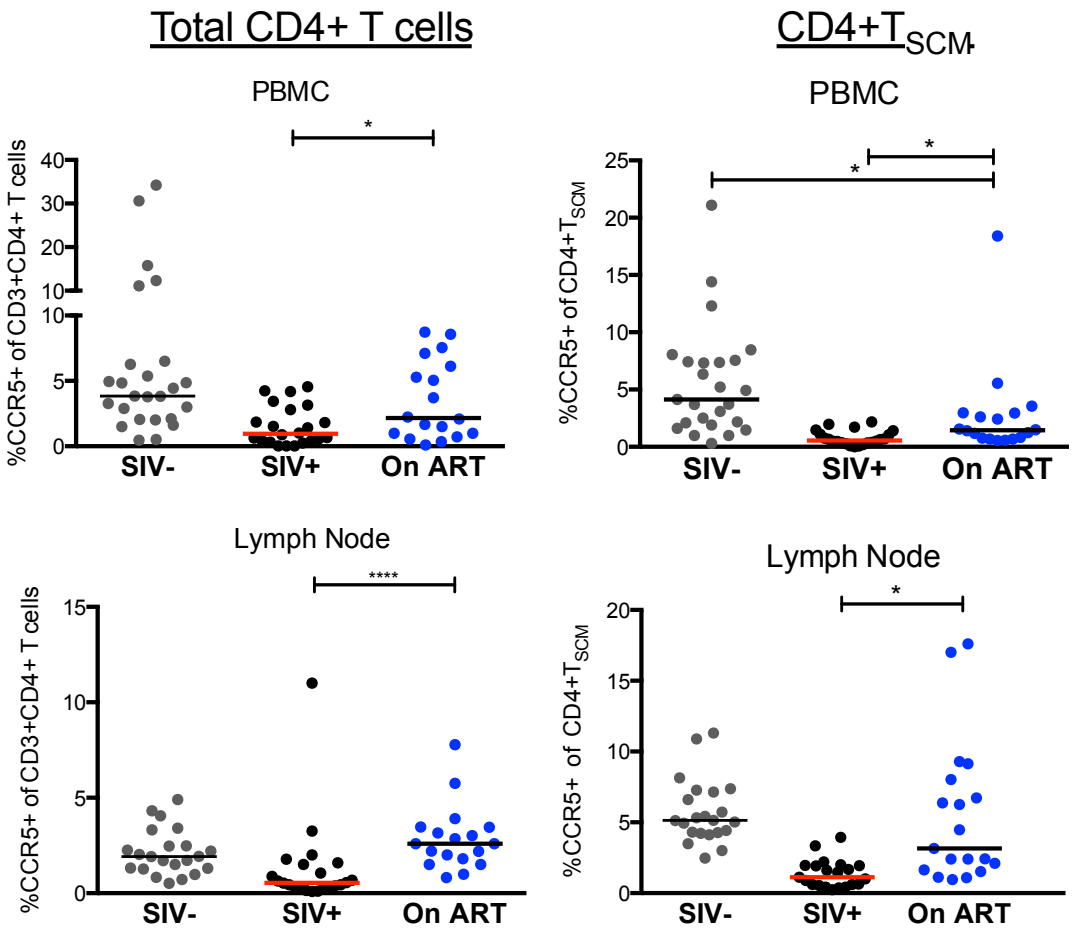

Figure 3.2. ART partially restores CCR5+CD4+ T_{SCM} in PBMC and lymph node. Fraction of CCR5+CD4+ T cells in (A) PBMC and (B) lymph node. Fraction of CCR5+ CD4+ T_{SCM} in (C) PBMC and (D) lymph node. Bars are drawn at the median. Kruskal Wallis, compared to "on ART", two tailed, *p<0.05, ****p<0.01.

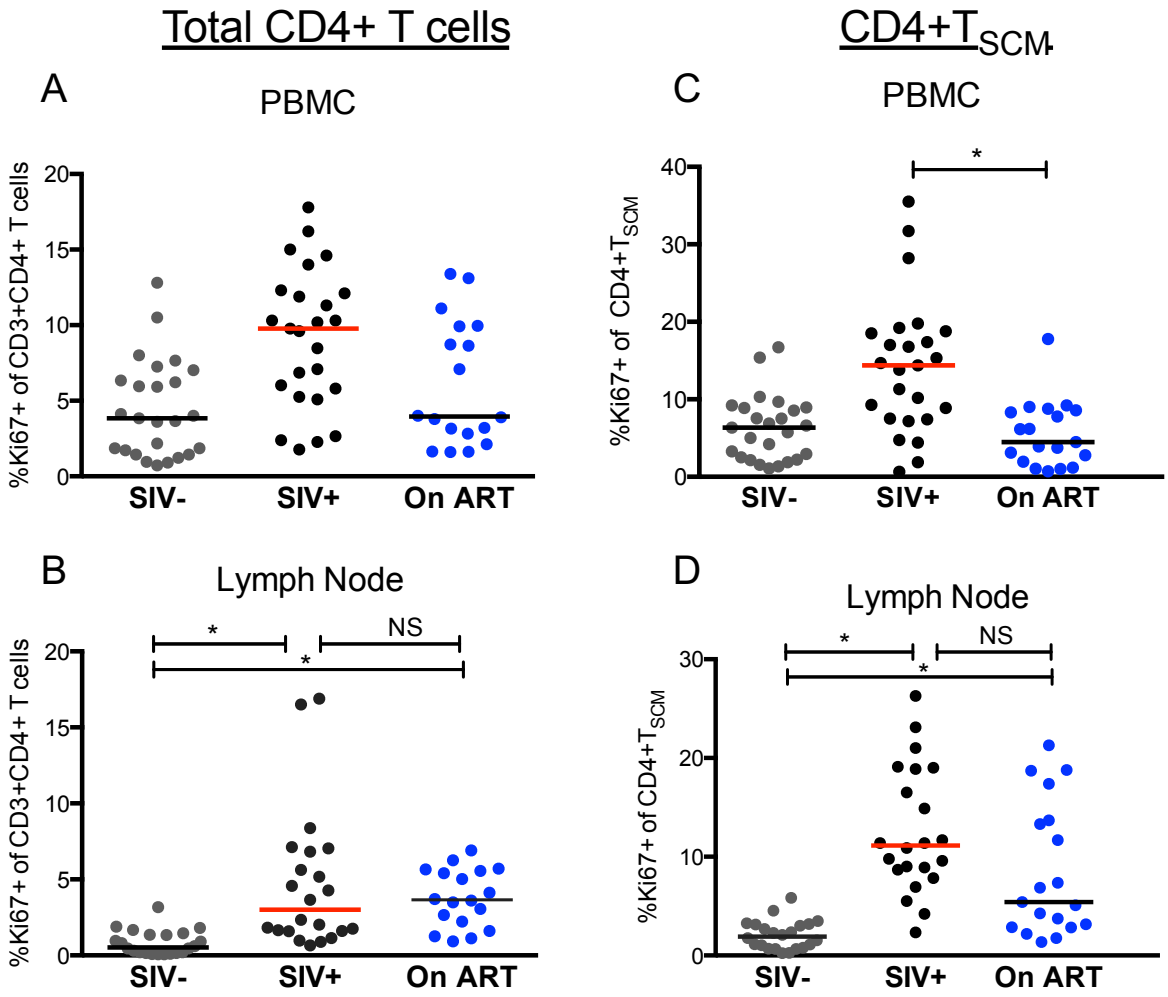

Figure 3.3. ART reduces the fraction of proliferating CD4+ T cells in PBMC but not in lymph nodes. Fraction of proliferating (measured by Ki67) CD4+ T cells in (A) PBMC and (B) lymph nodes. Fraction of proliferating CD4+ memory T_{SCM} in (C) PBMC and (D) lymph node. Kruskal Wallis, two tailed, *p<0.05.

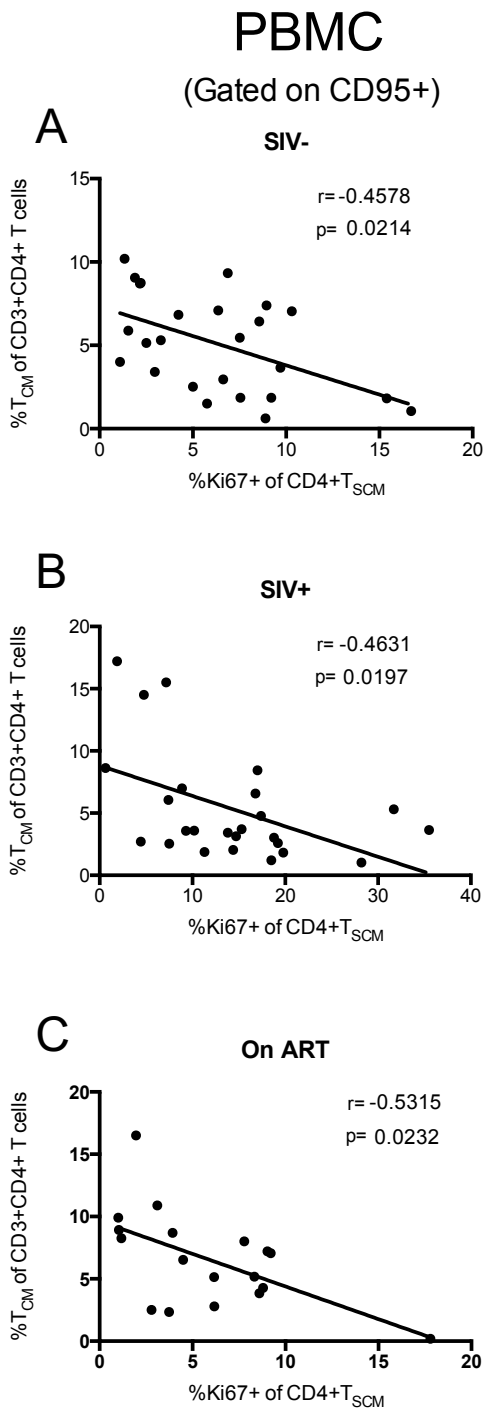

Figure 3.4. Inverse relationship between proliferating T_{SCM} and frequency of T_{CM} is maintained during SIV-infection and ART treatment. Correlation of Ki67+CD4+T_{SCM} and fraction of CD4+T_{CM} in PBMC of RM that are (A) SIV-uninfected, (B) SIV+, and (C) on ART. Spearman rank correlation, two tailed. P values shown.

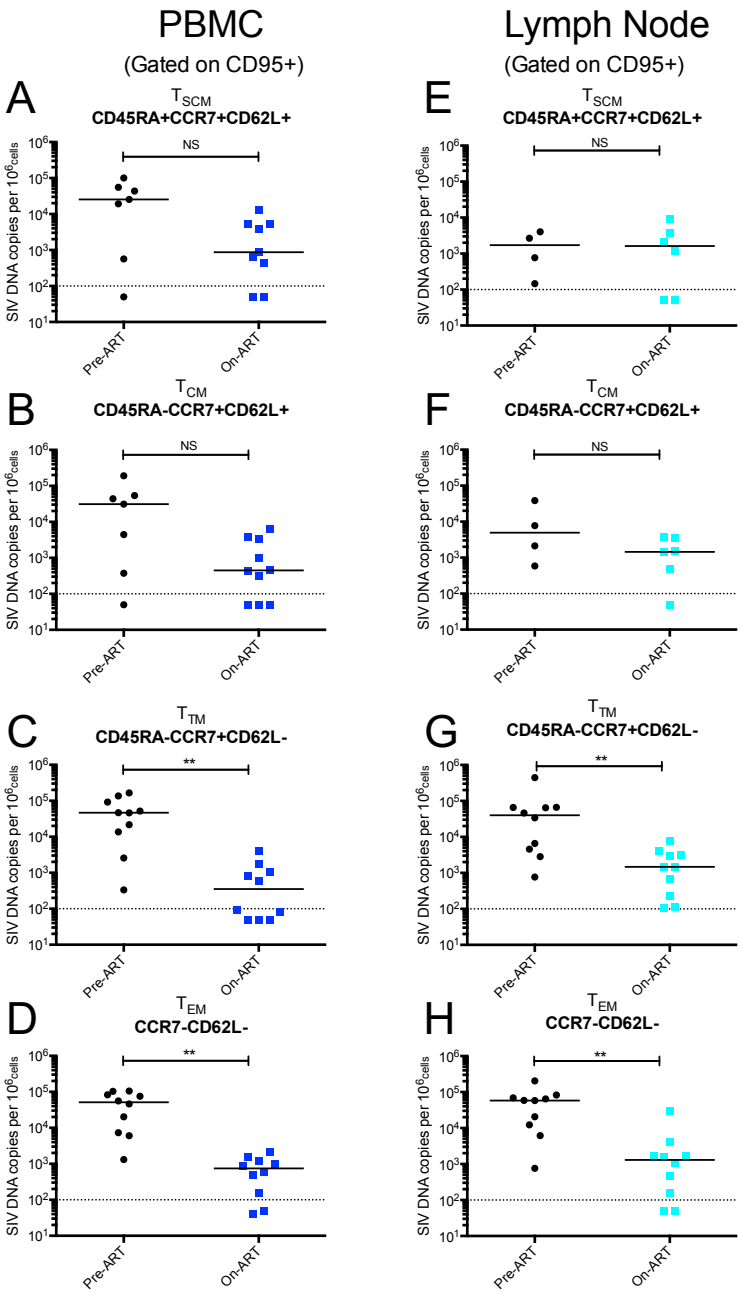

Figure 3.5. Fraction of SIV-infected CD4+T_{SCM} and T_{CM} is stable after ART initiation. Fraction of SIV-infected CD4+T_{SCM}, T_{CM}, T_{TM}, and T_{EM} cells as determined by quantitative PCR for the number of SIV*gag* DNA copies/cell in (A-D) PBMC and (E-H) lymph node. Data is shown for animals where >10,000 cells were sorted. PBMC pre-ART n=7 T_{SCM}, 7 T_{CM}, 10 T_{TM}, and 10 T_{EM}. Lymph node pre-ART n=4 T_{SCM}, 4 T_{CM}, 10 T_{TM}, and 10 T_{EM}, lymph node on-ART n=6 T_{SCM}, 6 T_{CM}, 10 T_{TM}, and 10 T_{EM}. Mann-Whitney *U*-test, two tailed **p<0.01, NS= not significant. Cell number was determined by using simultaneous PCR for albumin gene copy number. Bars are drawn at the median.

Chapter 3:

Title: CD8+ lymphocytes are required to maintain virus suppression in SIV-infected macaques treated with ART

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Abstract: Infection with human immunodeficiency virus (HIV) persists despite suppressive antiretroviral therapy (ART) and treatment interruption results in rapid viral rebound. Previous studies using antibody-mediated CD8+ lymphocyte depletion in simian immunodeficiency virus (SIV)-infected rhesus macaques (RM) showed that these cells contribute to control of viral replication in untreated animals. However, the contribution of CD8+ lymphocytes to maintaining virus suppression under ART remains unknown. We show that in ART-treated SIV-infected RMs, depletion of CD8+ lymphocytes results in increased virus levels in both plasma and lymphoid tissues in 100% of the animals, and that repopulation of CD8+ T cells (but not CD8+ NK cells) is associated with reestablishment of virus control. While the levels of SIV-DNA-positive cells remained unchanged after CD8+ lymphocyte depletion and reconstitution, we found that the frequency of SIV-infected CD4+ T-cells pre-depletion positively correlates with both peak and area-under-the-curve of viremia post-depletion. These results, demonstrating a role for CD8+ T cells in controlling virus production during ART, provide a rationale to explore immunotherapeutic approaches in ART-treated HIV-infected individuals.

Introduction

CD8+ lymphocytes are important for immune defense against viruses, intracellular pathogens and cancers. CD8+ T-cell responses to lentiviruses such as HIV and SIV can be detected as early as day 7 post infection (103, 104). Several lines of evidence indicate that CD8+ lymphocytes inhibit virus replication during HIV/SIV infection. First, there is a temporal association between the expansion of antigen-specific CD8+ lymphocytes and the post-peak decline of plasma viremia (101, 102). Second, there is a clear association between certain MHC class-I alleles (i.e., HLA-B*57 and Mamu-B*08 and –B*17) and disease progression during both HIV infection of humans and SIV infection of rhesus macaques (RM) (111-113, 239-242). Third, during both acute and chronic HIV/SIV infection, immunologic pressure mediated by virus-specific CD8+ T-cells is manifested by the emergence of viral escape mutations (107, 243, 244). Fourth, HIV-1-infected individuals with the “elite controller” phenotype exhibit CD8+ lymphocyte responses characterized by polyfunctionality, greater proliferative capacity, and stronger *in vitro* killing potential than those observed in normal progressors (110, 245, 246). However, this CD8+ T-cell response is unable to clear the infection and, eventually, the immune system is exhausted from chronic antigen exposure (247, 248).

In the experimental animal model of SIV infection of RMs, the most direct evidence for the role of CD8+ lymphocytes in viral control came from studies in which these cells were transiently depleted *in vivo* using CD8-specific monoclonal antibodies. *In vivo* experimental CD8+ depletion results in (i) abrogation of post-peak decline of viremia when performed during acute SIV infection (125, 249) and (ii) increased virus replication when performed during chronic SIV infection (93, 127, 128). The fact that viral loads rapidly return to pre-depletion levels upon reconstitution of the CD8+ lymphocyte

pool further confirms the antiviral role of these cells. However, the contribution of CD8+ lymphocytes in controlling virus replication and/or production during continuous, highly active ART is unknown. In this study, we directly assessed the function of CD8+ lymphocytes in a cohort of thirteen SIV-infected, ART treated RM and found that depletion of CD8+ lymphocytes resulted in increased virus levels in both plasma and lymphoid tissues in all treated animals.

Results

Administration of anti-CD8+ antibody MT-807R1 to SIV-infected ART-treated macaques

Sixteen Indian origin RM infected intravenously with the pathogenic strain SIV_{mac239} were initiated on a four-drug ART regimen at week 8 post infection and treated for a period ranging from 8 to 32 weeks (Fig. 1a and Supplementary Table 1). Three animals were euthanized prior to study completion due to rapid disease progression or side effects of ART, leaving 13 animals to complete the study. ART suppressed viremia to <60 copies/ml in 12 out of 13 RM that completed the protocol (see Online Methods). Seven RM showed at least four consecutive time-points with viremia below 60 copies/ml on ART (i.e., “persistent suppressors”) while five showed a mix of undetectable and very low but detectable levels (i.e., “intermittent suppressors”). The last animal never achieved undetectable viremia, even though ART decreased the viral load by >5 Logs. As expected, ART was effective at partially restoring CD4+ T-cells in PBMCs, lymph nodes, and rectal biopsies (data not shown). Despite variability in the duration of ART and kinetics of viremia suppression, at the time of CD8+ lymphocyte depletion viral load had declined >99.97% as compared to pre-ART levels in all animals (Supplementary Table 2). Once viral loads were consistently undetectable (persistent suppressors) or undetectable on at least 3 non-consecutive evaluations (intermittent suppressors), we

administered one dose of the α-CD8+ depleting antibody, MT-807R1, at 50 mg/kg iv. Animals were followed for 8 weeks after antibody administration, and ART was continued throughout this follow-up period (Fig. 1a). Using this method, we were able to deplete >95% of CD8+ T-cells in peripheral blood by day 1 post depletion (Fig. 1b). This depletion was rapid and sustained (mean 92% SD=9 and mean 94% SD=5 at weeks 1 and 3, respectively) until approximately 5 weeks post depletion, at which time a variable degree of CD8+ lymphocyte reconstitution in the periphery was observed (Fig. 1b, e). Since MT-807R1 is directed at the CD8α chain, CD3-CD8α+ NK cells were also depleted during this procedure (Supplementary Fig. 1). Of note, CD8+ lymphocyte depletion in lymph nodes was less complete than in peripheral blood with an average of ~70% depletion (SD=26) at week 1 and ~85% depletion (SD=11) at week 3 post depletion (Fig. 1c, f). CD8+ lymphocyte depletion was least efficient in the rectal mucosa, with an average of ~62% depletion (SD=27) at week 1 post MT-807R1 administration and a more rapid reconstitution starting by week 3 (mean 56% depletion, SD=30) (Fig. 1d, g). While MT-807R1 was less effective in depleting CD8+ lymphocytes in tissues as compared to blood, previous studies showed that CD8+ lymphocytes are functionally impaired following binding of the α-CD8+ antibody even if they are not physically depleted (250). CD8+ lymphocyte depletion is followed by homeostatic proliferation of CD4+ T-cells (92, 126), and we observed increased CD4+ T-cell proliferation starting at week 2 post depletion in PBMCs (Fig. 1H), week 3 in lymph node (Fig. 1i), and as early as week 1 in rectal mucosa (Fig. 1j). This increased CD4+ T-cell proliferation involved predominantly CD4+ effector memory (T_{EM}) cells, with smaller and delayed increases observed in CD4+ stem-cell memory (T_{SCM}) and central memory (T_{CM}) cells (Fig. 1k, l).

CD8+ lymphocyte depletion is followed by increase in plasma viral load and SIV RNA in lymph nodes in 100% of ART-treated macaques

As shown in Figure 2, all 13 SIV-infected RM showed a measureable increase in plasma viremia after CD8+ lymphocyte depletion and while still on ART, with several animals rebounding as early as day 1 post-depletion, and only one animal remaining below 60 copies/ml until week 3 post-depletion, at which point virus was detectable (RGb13). The seven persistent suppressors all showed at least one time-point with detectable SIV-RNA post-depletion, with 6 out of 7 showing at least three time-points with viremia >60 copies/ml (Fig. 2a). Similarly, the 5 intermittent suppressors had detectable viremia at all examined time-points post-depletion (Fig. 2b). The one animal that never achieved undetectable viremia despite 32 weeks of ART and a decrease of viral load of >5 logs (i.e., >99.99% decline from baseline) showed a remarkable increase in viremia after CD8+ lymphocyte depletion up to ~10^5 copies/ml (Fig. 2c). Of note, CD8+ T-cell reconstitution was consistently associated with a decrease in plasma viral loads to levels similar to those observed prior to CD8+ depletion. Interestingly, in 3 out of 4 cases where CD8+ T-cells did not reconstitute in peripheral blood during the 8 week follow up period post-depletion, plasma viral loads remained elevated above the limit of detection. To further analyze the impact of CD8+ lymphocyte depletion and reconstitution on SIV viremia we next divided the experimental timeline into three distinct periods that were prospectively defined as follows: period 1, the last 6 weeks of ART before CD8+ depletion; period 2, the time-points after CD8+ depletion in which the level of circulating CD8+ T-cells was <10% of baseline; and period 3, the time-points during CD8+ lymphocyte reconstitution in which circulating CD8+ T-cells were >20% of baseline (lines blue, green, and purple in Fig. 2, respectively, also Supplementary Table 2). Using a mixed linear effects model we compared viral loads in these three periods and found that

the geometric mean of period 2 (275; 95% CI: 116-653) was significantly higher than either period 1 (53; 95% CI: 33-83, $p<0.05$) or period 3 (90; 95% CI: 45-179, $p<0.001$) (Fig. 2d). Due to the many non-detectable time points, we also conducted a binary analysis (detectable/non-detectable) which does not assume normality, and found that period 2 had significantly more detectable time points than either period 1 or 3 (data not shown). When we applied the mixed linear effects model to only the 7 ART-treated SIV-infected RM with persistent suppression of viremia pre-depletion (Fig. 2a), the geometric mean of period 2 (135; 95% CI: 96-193) was again significantly higher than either period 1 (34; 95% CI: 29-41, $p<0.05$) or period 3 (44; 95% CI: 29 -65, $p<0.05$) (Fig. 2e).

To determine whether the trends in viremia observed in our cohort of SIV-infected RM were also present in lymphoid tissues, we next conducted a sequential analysis of the levels of SIV-RNA production in lymph nodes using the RNAscope technology at 3 time-points, i.e., before initiation of ART, during ART but before CD8+ depletion, and during ART after CD8+ depletion. As shown in Figure 3 for representative animals, we observed a dramatic decrease in the number of lymph node SIV-RNA-positive cells after ART and a rebound of SIV-RNA production after CD8+ lymphocyte depletion. Taken together, these data indicate that, in SIV-infected RMs, CD8+ lymphocytes contribute to maintenance of virus suppression during ART.

Ultrasensitive viral load assay reveals a 72 to 350 fold increase in virus production in the SIV-infected ART-treated RMs animals with residual viremia ≤25 copies/ml.

To better assess the level of residual viremia in the group of “persistently suppressed” SIV-infected ART-treated RMs at the time of CD8+ lymphocyte depletion, we repeated the measurement of viral load using an ultrasensitive viral load assay with limit of detection of 3 copies/ml, essentially as described (251). We found that viremia

was below 100 copies/ml in all these RMs, with 5 out of the 7 animals showing levels of residual viremia ≤25 copies/ml (Supplemental Table 2), thus in the range of levels of viremia measured with highly sensitive assays in many long-term ART-treated HIV-infected individuals (252),(253, 254). To more rigorously evaluate the impact of CD8+ lymphocyte depletion on residual viremia under ART, we next used the same ultrasensitive assay to measure viral load at all post-CD8+ depletion time points in the 5 RMs with the lowest residual viremia. As shown in Figure 4, these data largely recapitulate the results shown in Figure 2, and indicate that the level of viremia increased from pre-depletion ranging from <3-25 copies/ml (geometric mean=8.3; 95% CI: 2-31) to post-depletion peak viremia ranging from 700-4,900 copies/ml (geometric mean=1977; 95% CI: 822-4754, $p<0.05$), for a 72 to 350-fold increase. Overall, these data confirm that CD8+ lymphocytes contribute to the suppression of viremia observed under ART in SIV-infected individuals.

Pre-depletion SIV-specific CD8+ T cells but not CD8+ NK cells positively correlate with the magnitude of on-ART plasma virus rebound post-depletion.

The depleting antibody that was used in this study, MT-807R1, is directed at the α-chain of the CD8+ molecule and depletes CD8α+ NK cells as well as CD8+ T cells in RM. To determine the respective role of these two lymphoid populations in mediating immune control of virus production under ART we conducted a series of correlation analyses in our cohort of SIV-infected RM. As expected, the frequency and absolute number of SIV-specific CD8+ T cells decreased substantially after initiation of ART (data not shown). However, we were able to detect low frequencies of Gag-CM9 specific CD8+ T cells (as measured by tetramer staining) in the PBMCs of all RM at the time of CD8+ lymphocyte depletion. First, we determined that the number of pre-CD8+ depletion

circulating GAG-CM9 specific CD8+ T cells directly correlates with viral load and both 1 and 3 weeks post-depletion (Supplementary Figure 2a, b). However, we find no correlation between the numbers of circulating CD8+ NK cells pre-depletion and viral load post-depletion at either week 1 or 3 (Supplementary Figure 2c, d). Second, we conducted the same analysis described in Figure 2d-e by dividing the study into three periods based on the kinetics of CD8+ NK cell numbers: period 1, the last 6 weeks of ART before CD8+ depletion; period 2, the time-points after CD8+ depletion in which the level of circulating CD8+ NK cells was <20% of baseline; and period 3, the time-points during CD8+ lymphocyte reconstitution in which circulating CD8+ NK cells were >20% of baseline (blue, green, and purple respectively in Supplementary Figure 3). As shown in Supplementary Figure 3a for one representative RM with persistent suppression of viremia under ART, the CD8+ NK cells return to pre-depletion levels around week 3 post-depletion while CD8+ T cells are still significantly depleted. Using the same mixed linear effects model of Figure 2, we found that while the geometric mean of the viral load in period 2 (199; 95% CI: 136-321) is significantly higher than period 1 (53; 95% CI: 44-70, $p<0.05$), the geometric mean of the viral load in period 3 (221; 95% CI: 122-382) is unchanged when compared to period 2 (Supplementary Figure 3b). We found similar results when we analyzed only the cohort of 7 persistently suppressed RM (Fig. 2a), with viral loads that remain elevated as compared to pre-depletion levels despite reconstitution of the CD8+ NK cell pool (Supplementary Figure 3c). Finally, no correlation was observed between either the length of ART or the time to suppression (i.e., below 60 copies/ml) with the change in viremia observed following CD8+ depletion (data not shown). These data, together with those shown in Figure 2d-e, indicate that the depletion and repopulation of CD8+ T cells, but not the repopulation of CD8+ NK cells, is temporally associated with the control of viremia under ART.

Pre-depletion levels of cell-associated SIV-DNA are positively correlated with levels of plasma SIV RNA post depletion

To determine whether CD8+ lymphocyte depletion had an impact on the overall level of the SIV reservoir under ART we sequentially measured the level of total, cell-associated SIV-DNA by RT-PCR in bulk CD4+ T-cells as well as in subsets of CD4+ stem-cell memory (T_{SCM}), central-memory (T_{CM}), transitional memory (T_{TM}), and effector memory (T_{EM}) cells. As shown in Figure 4a and b, CD8+ depletion did not induce any significant changes in the level of SIV-DNA+ CD4+ T-cells in either peripheral blood or lymph nodes. Similarly, we did not find any significant change in the level of SIV-DNA in sorted CD4+ T_{SCM}, T_{CM}, T_{TM}, and T_{EM} in either blood or lymph nodes following CD8+ depletion (Supplementary Fig. 5a, b). Overall, these data do not support the hypothesis that the increased viremia observed following CD8+ depletion simply reflects an expansion of the pool of SIV-infected cells under ART caused by *de novo* rounds of virus replication. Interestingly, we observed a significant direct correlation between the level of cell-associated SIV-DNA in CD4+ T-cells before CD8+ depletion and both the peak and the area-under-the-curve of plasma viremia post CD8+ depletion (Fig. 5c, d), suggesting that the size of the reservoir under ART is a key determinant of the resultant level of virus production when CD8+ lymphocytes are removed from the system. Finally, we sought to determine whether the levels of virus production after CD8+ depletion (i.e., peak and/or area-under-the curve) correlated with observed changes in the level of CD4+ T-cell activation, as measured by Ki67 expression, in various cell subsets and tissues. As shown in Supplementary Table 3, we found no significant correlations between the level of activation in any of the examined CD4+ T-cell subsets after CD8+ depletion and the level of viremia at the same time-point. This lack of correlation is not

surprising as the CD8+ depletion-induced increase in plasma viremia occurred prior to any detectable increase in CD4+ T-cell activation (Fig. 1h-k and 2a-c). In aggregate, these observations suggest that the observed increase in viremia is not related to increased de novo infection of new target cells created by homeostatic proliferation of CD4+ T cells in response to depletion of CD8+ cells.

Single genome sequencing of post-depletion viremia reveals close relationship to peak viremia

In order to determine whether or not post-depletion viremia was occurring through *de novo* rounds of replication or is a result of reactivation of cells infected prior to CD8+ lymphocyte depletion we conducted single genome analysis (SGA) of the SIVmac239 envelope. SIV_{mac239} is known to be neutralization resistant (255, 256), therefore mutations found in *env* at 8 weeks post-infection are more likely to be stochastic or a result of CD8+ T cell mediated escape. Additionally, *env* is not the target of any ART in this regimen, so mutations found after initiation of ART, during CD8+ T cell depletion would not be attributable to drug resistance. For our analysis, we chose to sequence the virus at 3 time points: peak viremia (Day 10), prior to the peak of antigen-specific CD8+ T cell expansion, pre-ART (Day 56), post-antigen-specific CD8+ T cell expansion, and during CD8+ T cell depletion. In our small cohort of two animals (both persistently suppressed and *Mamu-A*01+*), we find that at peak viremia, the mutations in SIVenv are infrequent and randomly distributed throughout the gene, with one animal having 33% of and one animal having 6% sequences with a single amino acid change (Figure 6A). Not surprisingly, the virus at the pre-ART time point (56 days post SIV-infection) is highly mutated in both animals with ≥ 80% of sequences harboring 3 or more mutations (Figure 6B). Interestingly, viremia during CD8+ T cell depletion reveals a

switch back to a less diverse virus with only a small percentage of sequences showing more than one mutation (20% in one animal), and the majority showing one or no mutations (Figure 6C). These data suggest that viremia during CD8+ T cell depletion most closely resembles wild-type SIV_{mac239}.

Closer examination of the amino acid substitutions reveals one mutation R751G appears in both animals with a high frequency in the pre-ART plasma (Table 1). However, this mutation is absent from both peak viremia and is only present in 5% of total sequences post-depletion. Interestingly, each animal has at least one other mutation that is absent from peak viremia, abundant in pre-ART sequences, and again absent from viremia post-CD8+ lymphocyte depletion. V67M in RKq11 is present in 19 out of 24 sequences in pre-ART viremia, but not found in either peak viremia or post-depletion. Q739R in ROw8 is absent from peak viremia, found in 16 out of 22 sequences at the pre-ART time point, and found in only 1 out of 11 sequences post-depletion. We find a few other mutations that follow this trend, though none as dramatically (Table 1).

Discussion

This study demonstrates that CD8+ lymphocytes can contribute to maintaining suppression of plasma viremia in SIV-infected ART-treated RM. This observation is novel as to date the *in vivo* suppressive effect of CD8+ lymphocytes has primarily been characterized in studies involving untreated animals (93, 125, 127, 249) with only one report in three SIV-infected RM treated with a non-suppressive antiretroviral regimen consisting of tenofovir monotherapy (257). Indeed, the current work represents the first experiment in which SIV-infected RM underwent CD8+ lymphocyte depletion in the setting of highly active ART (i.e., >99.97% suppression of viremia). While the majority of the RM included in this study show some residual virus production under ART, these

levels of viremia were often within the range observed in long-term ART-treated HIV-infected individuals[28]. Importantly, the magnitude of the viral load increase after CD8+ lymphocyte depletion (i.e., 72 to 350 fold in the RM with the best suppression of viremia) and the fact that control of viremia is promptly reestablished upon CD8+ T-cell reconstitution strongly support the hypothesis of an important (and yet previously unrecognized) role of CD8+ lymphocytes in cooperating with ART to maintain virus suppression. As such, the current set of data provides an evidence base to explore immune-based interventions, such as therapeutic vaccines and check-point blockade inhibitors, in HIV-infected, ART-treated individuals. It should be noted, however, that interventions aimed at improving the antiviral CD8+ lymphocyte-mediated response under ART, like any antigen specific immune-based intervention, will be expected to be effective only against infected cells with sufficient expression of viral antigen, suggesting that combining viral induction and immune based approaches may enhance effectiveness of such interventions.

When using ultrasensitive viral load assays, residual viremia below clinical limit of detection is present in a high percentage of ART-treated HIV-infected individuals (253, 254, 258). Whether and to what extent this residual viremia is due to continuous, low-level *de novo* cycles of replication as opposed to either virus reactivation occurring in latently infected cells or the presence of persistently virus expressing cells remains an active area of research. Recent evidence suggests clonal expansion of virally infected cells during ART is another important contributor to HIV persistence(259-261). Similarly, at this time we do not know to what extent the increase in viremia observed following CD8+ depletion in SIV-infected, ART-treated reflects *de novo* virus replication versus reactivation of latent virus versus increased virus production from persistently, non-

latently infected cells. Regardless of the mechanisms responsible for the low-level viremia of ART-treated HIV-infected individuals and SIV-infected RM, which is often non-responsive to ART intensification (164, 262), the current study suggests a direct *in vivo* role for CD8+ lymphocytes in maintaining very low to undetectable viral loads under ART. In this study, the observation that the increased viremia of CD8+ lymphocyte depleted RM is not associated with increased levels of cell-associated SIV-DNA indirectly suggests that *de novo* rounds of virus replication are not a major feature of this experimental system. Future studies in which virus sequencing is conducted longitudinally during the period of detectable viremia after CD8+ lymphocyte depletion may help answer this question. Similarly, the current study does not define whether the antiviral role of CD8+ lymphocytes under ART involves classical cytotoxic T lymphocyte activity, non-cytolytic mechanisms (i.e., chemokine production and inhibition of virus transcription (120, 263)), or both functions of CD8+ T-cells.

Though we cannot definitively show the mechanism of action of CD8+ lymphocytes during SIV-infection and ART, our data revealing the sequence homology of peak viremia and post-depletion viremia is highly suggestive of a non-lytic mechanism of CD8+ lymphocyte mediated control. By definition, a CD8+ T cell that controls viremia by killing a virally infected cell would destroy that cell and the virus resident within. In order to reconcile our data showing the virus that emerges during CD8+ lymphocyte depletion does not resemble the virus that is dominant pre-ART, we must consider a non-lytic mechanism of CD8+ T cell mediated viral control. One theory is non-lytic CD8+ T cells suppress, but don't kill, cells infected with wild-type SIVmac239 during acute infection. Virus that has escaped this immune mediated pressure will be dominant. With the addition of ART, all escaped virus is controlled. However, this would have little impact on the infected cells already suppressed by CD8+ T cells. Depletion of the CD8+

lymphocytes results in the observed increase in plasma viremia from those cells previously suppressed by CD8+ T cells. For future studies, deep sequencing of virus in infected cells pre-ART, pre-depletion, and during depletion will reveal the frequency of cells harboring wild-type virus during chronic infection and after ART.

While the effect of CD8+ lymphocyte depletion on the levels of plasma viremia in ART-treated SIV-infected RM is clear, there are important caveats to this study. The first is that the used MT-807R1 antibody depletes both CD8+ T-cells and CD8α-expressing NK cells. However, our analysis of the correlation between viremia after CD8+ lymphocyte depletion and either the numbers of CD8+ T cells and CD8+ NK cells pre-depletion or their levels during depletion and repopulation strongly suggests that CD8+ T cells, rather than NK cells, are the main contributors of the control of viremia under ART. In addition, we have observed clear reactivation of virus production in lymphoid tissues in which the levels of CD8+ NK cells were extremely low even before administration of MT-807R1. Further studies using a CD8-β-specific antibody that depletes only CD8$\alpha\beta$+ T-cells will formally establish the specific contribution of CD8+ T cells in the control of viremia in ART-treated SIV-infected RMs(264). The second caveat is that the period of ART suppression was relatively short even in the SIV-infected RM defined as persistent suppressors, and therefore the level of virus suppression may not be as complete as in long-term ART-treated HIV-infected humans. To this end, further studies in which animals are treated for longer periods of time (i.e., >1 year) will be needed to determine whether CD8+ lymphocytes contribute to viral suppression during ART only in the relatively early stages of treatment or persists indefinitely. However, we wish to point out that the level of virus suppression was >99.97% in all treated RM (average 99.99%), with five animals showing very low levels of viremia (as determined using the ultra sensitive assay) that are similar to those observed in long-term ART-treated HIV-infected

humans[28]. The third caveat of the current study is that the impact of CD8+ depletion on the reservoir was measured using a relatively insensitive assay, i.e., total cell-associated SIV-DNA, which does not provide a functional analysis of the reservoir of replication competent virus. While interesting, analyses such as the quantitative viral outgrowth assay (QVOA) require a large number of cells and were not feasible in this cohort of animals undergoing an already very heavy schedule of blood and tissue collections. These caveats notwithstanding, the current set of data has important implications for understanding how the host antiviral cellular immune response works in concert with antiretroviral drugs in suppressing SIV replication, and defining the rationale for therapeutic interventions aimed at boosting the virus-specific CD8+ lymphocyte response in ART-treated HIV-infected individuals.

Acknowledgements

The authors declare no conflicts of interest.

We wish to thank D.G. Carnathan, M. Mavigner, C.S. McGary and G. Mylvaganam for helpful discussions; B. Cervasi, K. P. Gill and J.P. Mackel for technical support; S. Ehnert for study organization and scheduling; Yerkes National Primate Center veterinary staff, especially S. Jean for caring for the animals and daily ART administration; Merck (I.MTA.14.232) Gilead Sciences (I.MTA.14.230), and Janssen R&D Ireland (I.MTA.14.231) for generously providing Raltegravir, PMPA and FTC, and Darunavir, respectively. This work was funded in part by R01-AI90797 (to G.S.), RR000165/OD011132 to the Yerkes National Primate Research Center, the Emory Center for AIDS Research, NIH Grant # P30-AI-504 and supported in part with federal funds from the National Cancer Institute, National Institutes of Health, under Contract No. HHSN261200800001E (J.L.).

Online Methods

Animals

Sixteen Indian origin rhesus macaques (RM) were enrolled in this study. They were all infected intravenously with 3,000 tissue culture infectious dose (TCID50) of SIV_{mac239}. Three animals had to be euthanized due to extreme weight loss prior to CD8+ depletion and were thus excluded from analysis. The remaining 13 animals consisted of 6 females and 7 males and ranged in age from 4-13 years at time of infection. Nine were *Mamu-A*01* positive, and all 16 were *Mamu-B*08* and *B*17* negative. All animals were housed at the Yerkes National Primate Research Center of Emory University and maintained in accordance with US National Institutes of Health guidelines. Anesthesia was used for all blood and tissue collections. All studies were approved by the Emory University Institutional Animal Care and Usage Committee (IACUC).

Antiretroviral therapy

All RM were put on a four-drug regimen at 8 weeks post SIV infection. The drug regimen started as 20 mg/kg PMPA (Tenofovir) and 30 mg/kg FTC (Emtricitabine) administered once a day by subcutaneous (s.c) injection along with 100 mg Raltegravir and 400 mg Darunavir orally twice daily. During the first 8 weeks, all RM received this treatment regimen. Three animals remained on this regimen for the duration of the study. In the remaining 10 animals, we increased the dose of Darunavir and Raltegravir as described in Supplemental Table 1**,** until suppression was achieved. Animals were given oral antiretroviral treatment (Raltegravir and Darunavir) via orogastric tube on days where anesthesia was also administered, due to fasting and nausea. Suppression of plasma viremia to below the limit of detection of our assay (60 copies/ml of plasma) was achieved in 12 out of 13 animals, with persistent suppression observed in 7 out of 13

animals. Of note, the original study design planned for CD8+ lymphocyte depletion after two consecutive undetectable viremia measured 2 weeks apart. However, some animals who had not reached this definition of viremic suppression started to manifest significant side effects of ART. As such, we decided to perform CD8+ lymphocyte depletion in all enrolled animals to generate as much data as possible regarding CD8+ depletion in ART-treated macaques, even if the planned ART end-point was not always reached.

CD8+ lymphocyte depletion

We administered one dose of MT-807R1 intravenously at 50 mg/kg (<0.25 EU/ml of endotoxin by LAL gel-clot). MT-807R1 was purchased from the NIH Non-human Primate Reagent Resource Program. The extent of CD8+ lymphocyte depletion was determined (i) in peripheral blood by flow cytometric staining and complete blood counts (CBC) as both number of cells/cmm of blood and percentage of CD3+ T cells, and (ii) in tissues by flow cytometry and measuring the level of CD8+ T-cells as fraction of their pre-depletion frequency.

Sample collection & tissue processing

Blood was collected in EDTA tubes. Plasma was obtained throughout the study by centrifugation. Peripheral blood mononuclear cells (PBMC) were obtained by density-gradient centrifugation using 90% Lymphocyte Separation Media (LSM) from Lonza. Lymph node biopsies were taken at 4 time points throughout the study (Day 56 pre-ART initiation, 1 week pre-CD8+ depletion, weeks 1 and 3 post-CD8+ depletion) as well as at necropsy. Lymph nodes were cut in half and one half was put in 4% paraformaldehyde (PFA) and then embedded in paraffin. Lymph node mononuclear cells (LMNC) were obtained by removing excess fat and connective tissue and grinding over a 70-μm cell-

strainer. Rectal biopsies (RB) were taken throughout the study. To obtain lymphocytes, RB were digested in 0.75 mg/ml Collagenase (Sigma-Aldrich) and 0.15 μ L DNase in 10% fetal calf serum (FCS), 1% penicillin-streptomycin, and 1% L-glutamine at 37° C with gentle shaking. After 2 hours, tissues were mechanically separated using plastic cannula and run over a 70-µm filter.

Immunophenotype by flow cytometry

Multiparametric flow cytometry was performed according to a standard protocol on PBMC, LNMC, and cells isolated from rectal biopsies using fluorescently labeled monoclonal antibodies cross-reactive in rhesus macaques. The following antibodies were used for immunophenotyping of CD4+ and CD8+ memory subsets: CD3-APC/Cy7 (SP34-2), CD4-PE-CF594 (L200) CD8-BV711 (RPA-T8), CCR7-FITC (150503), CD45RA-PE/Cy7 (L48), CD95-PE/Cy5 (DX2), Ki67-AlexaFluor700 (B56), CD14-BV650 (M5E2), CD56-BV605 (NCAM16.2), CD62L-PE (SK11), HLA-DR-PerCPCy5.5 (G46.6), CCR5-APC (3A9) from BD Biosciences; CD28-ECD (CD28-2) from Beckman Coulter; CD16-BV421 (3G8), CD4-BV650 (OK-T4), PD-1-BV421 (EH12.2H7), from Biolegend; CD8-APC (DK25) from DAKO. All flow cytometry specimens were acquired on an LSR II (BD Biosciences) and analysis of the acquired data was performed using FlowJo software (Tree Star).

Cell sorting

After isolation, cells were resuspended in 50 ml PBS containing 2 mM EDTA and spun at 200 x g for 15-20 minutes (depending on cell volume) to remove contaminating platelets. Prior to sorting, CD4+ T cells in PBMC and LNMC were enriched using magnetic beads and column purification (Miltenyi Biotec). Enriched cells were then stained with

previously determined volumes of CD3-APC/Cy7 (SP34-2), CD4-Brilliant Violet 650 (OKT-4) CD8-Brilliant Violet 421 (RPA-T8), Live/Dead-Aqua, CD45RA-APC (5H9), CCR7-PE/Cy7 (3D12), CD95-PE/Cy5 (DX2), CD28-ECD (CD28-2), CD62L-PE (SK11). Populations for sorting were defined as follows: T_{SCM} (CD45RA+CCR7+CD95+CD62L+), T_{CM} (CD45RA-CD95+CD28+CCR7+CD62L+), T_{TM} (CD95+CCR7+CD62L-), and T_{EM} (CD95+CCR7-CD62L-). Sorting was performed on a FACSAria LSR II (BD Biosciences) equipped with FACS Diva software.

Plasma viral load and cell-associated SIVgag DNA

Plasma viral quantification was performed as described previously (211) DNA was extracted from sorted peripheral and lymph node CD4+ T-cells and CD4+ T-cell memory subsets using the Blood DNA Mini Kit (QIAGEN). Quantification of SIV_{mac} *gag* DNA was performed as previously described on the extracted cell-associated DNA by quantitative PCR using the 5′ nuclease (TaqMan) assay with an ABI7500 system (PerkinElmer Life Sciences) (235). The sequence of the forward primer for SIVmac *gag* was 5′-GCAGAGGAGGAAATTACCCAGTAC-3′; the reverse primer sequence was 5′-CAATTTTACCCAGGCATTTAATGTT-3′; and the probe sequence was 5′-6 FAM-TGTCCACCTGCCATTAAGCCCGA-TAMRA-3′. For cell number quantification, quantitative PCR was performed simultaneously for monkey albumin gene copy number.

***In situ* hybridization (ISH)**

We used a next-generation, ultra-sensitive RNA *in situ* hybridization technology, RNAscope (Advanced Cell Diagnostics), as described previously (265).

Ultrasensitive viral load assay

Ultrasensitive plasma viral RNA measurements were performed essentially as described previously (251).

SGA for SIVenv

SGA was performed as described preiviously (266).

Statistical analysis

The data presented in Figure 2D and E were analyzed by fixing a mixed effects linear model that allows estimation of mean viral load by period. Comparisons between frequency of infection pre- and post-depletion were carried out using either Kruskal-Wallis (Figure 4A and B) or Wilcoxon matched-pairs signed rank test (Figure S3A and B). Correlations were determined using the non-Gaussian Spearman correlation. Significance was attributed at $p<0.05$. Analysis was done using GraphPad Prism 6.0, and R 3.1.3.

Figures and Figure Legends

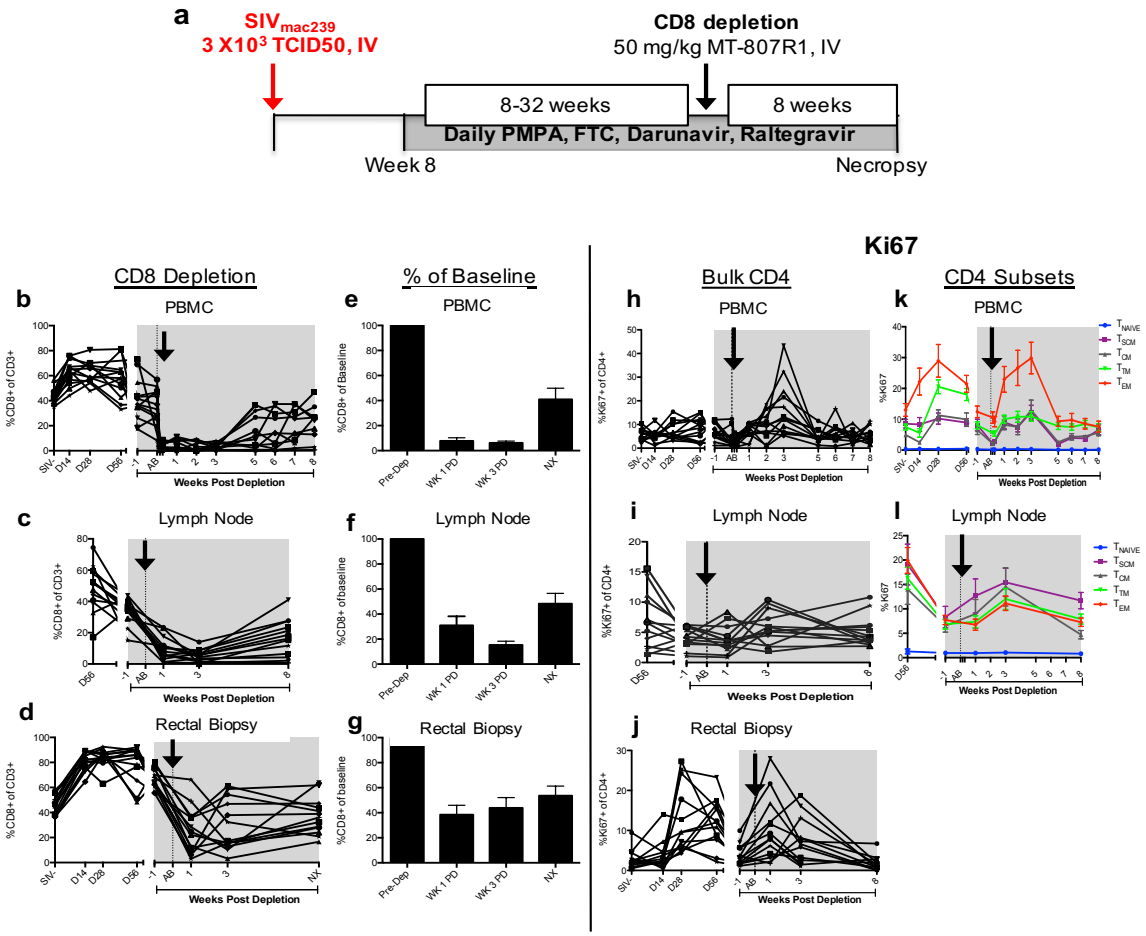


Figure 4.1. Treatment of SIV-infected ART-treated RM with the anti-CD8+ antibody MT-807R1 results in CD8+ lymphocyte depletion from peripheral blood, lymph node and rectal mucosa and a variable increase in CD4+ T-cell proliferation. (a) Study design. CD3+CD8+ T-cells as percentage of CD3+ lymphocytes in (b) PBMC, (c) lymph node, and (d) rectal biopsy. CD8+ T-cells as percentage of pre-depletion frequencies ("baseline") in (e) PBMC, (f) lymph node, and (g) rectal mucosa. Proliferation (Ki67+) of total CD4+ T-cells in (h) PBMC, (i) lymph node, and (j) rectal mucosa. Proliferation (Ki67+) of CD4+ T-cell subsets in (k) PBMC and (l) lymph node. Gap in x-axis represents 8-32 weeks of continuous ART. Shaded area represents ART period. Black arrow and dotted vertical line indicate MT-807R1 administration. NX=necropsy.

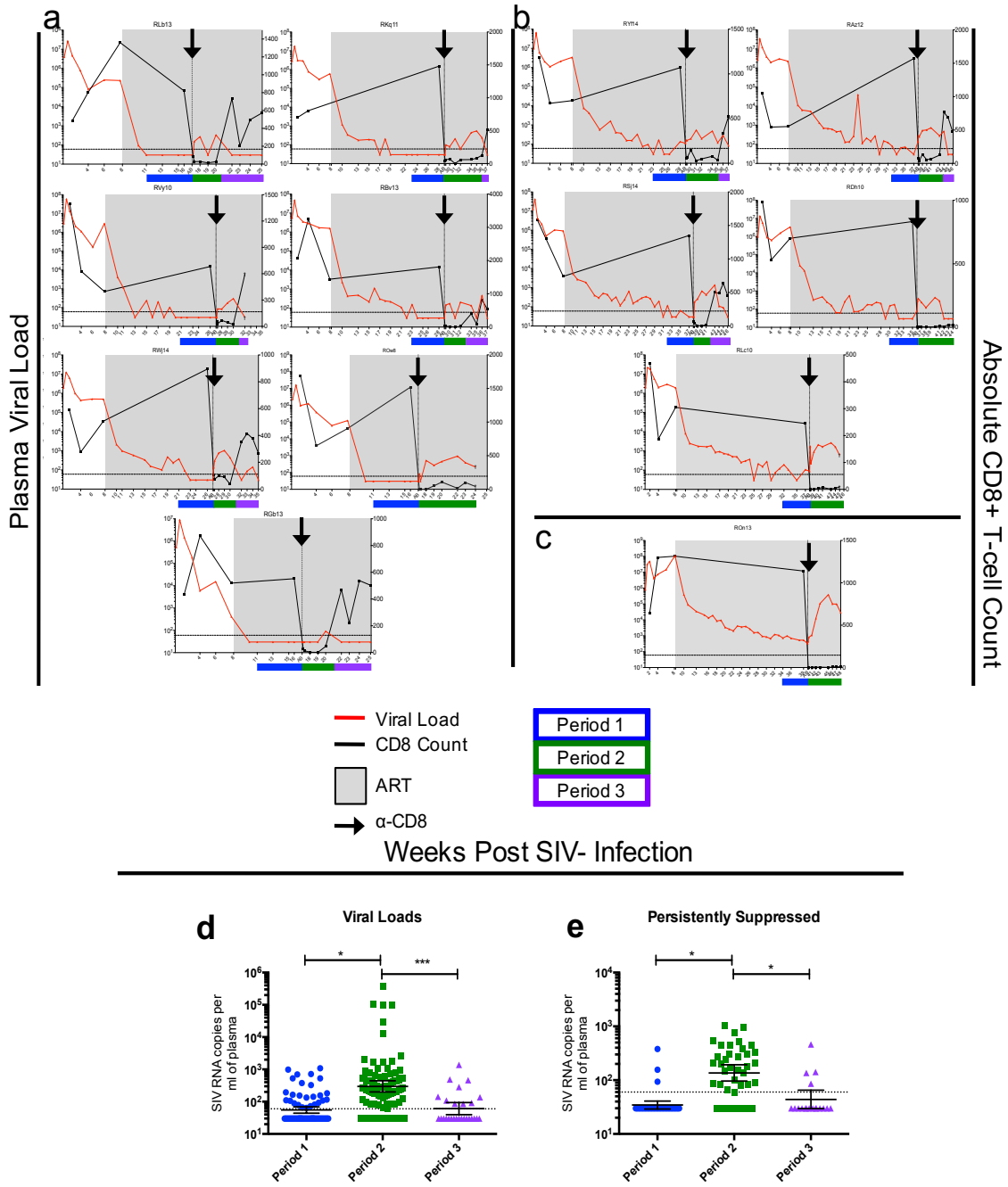


Figure 2. CD8+ lymphocyte depletion results in measureable increase in plasma viremia in 13/13 ART-treated SIV-infected macaques. Viral load (red line) and CD8+ T-cell counts (black line) of SIV-infected RM that were (a) persistently suppressed, (b) intermittently suppressed, and (c) never fully suppressed on ART. Shaded area represents ART period. Black arrow and dotted vertical line indicate anti-CD8+ antibody MT-807R1 administration. (d) Geometric mean viremia for each statistical period for all 13 animals. (Described fully in Table S2). Bars show the geometric mean with 95% CI, *p< 0.05, ***p<0.001. (e) Geometric mean viremia for each statistical period for 7 persistent suppressor animals. Bars show the geometric mean with 95% CI, *p <0.05.

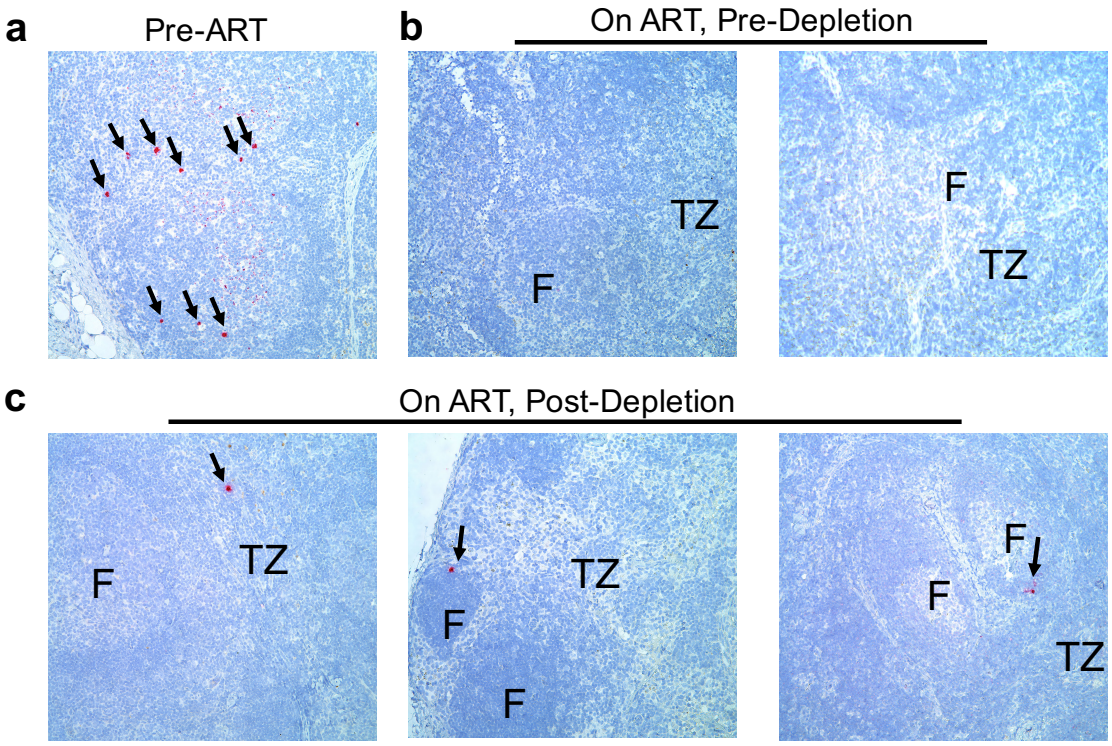


Figure 3. CD8+ depletion results in increased SIV-RNA in lymph nodes of ART-treated SIV-infected macaques. Representative *in situ* hybridization of SIV-RNA in lymph nodes of (a) chronically infected, (b) ART-treated, pre-CD8+ depletion, and (c) ART-treated, post-CD8+ depletion macaques. Data presented in (b) and (c) are from lymph nodes of two and three different animals, respectively. Red indicates SIV-RNA positive, productively infected cell; F=follicle, TZ= T-cell zone. Images are 20X magnification.

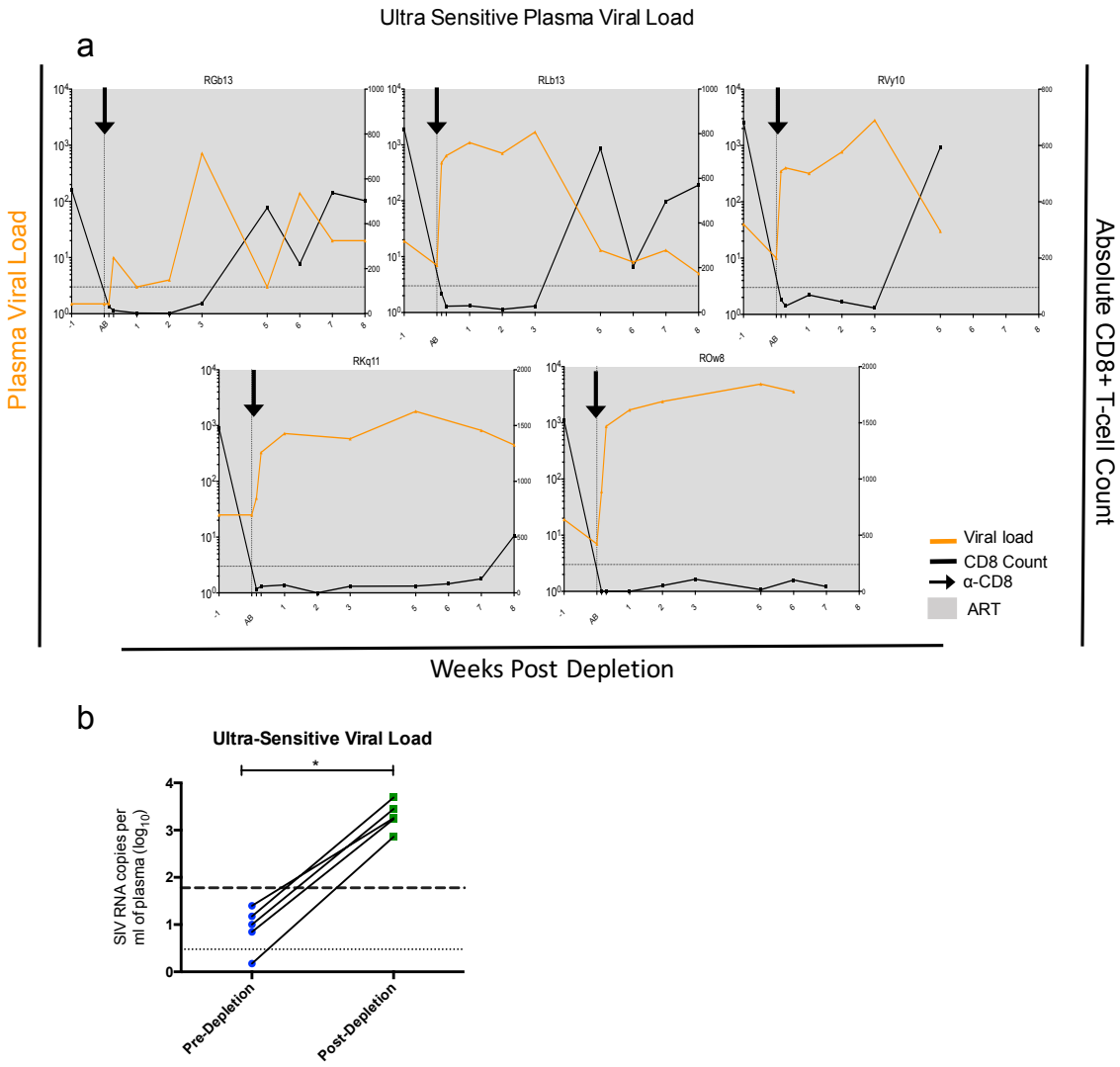


Figure 4. Ultrasensitive viral load assay confirms dramatic increase in viremia post-CD8+ lymphocyte depletion. (a) Viral load (orange line) and CD8+ T-cell counts (black line) of SIV-infected RM that were persistently suppressed. Shaded area represents ART period. Black arrow and dotted vertical line indicate anti-CD8+ antibody MT-807R1 administration. (b) Pre-depletion and peak post-depletion viremia (log_{10}) for 5 RM. Dotted line is limit of detection of ultrasensitive viral load assay (3 copies/ml plasma). Dashed line is limit of detection of standard viral load assay (60 copies/ml plasma). Paired t-test, *p< 0.05.

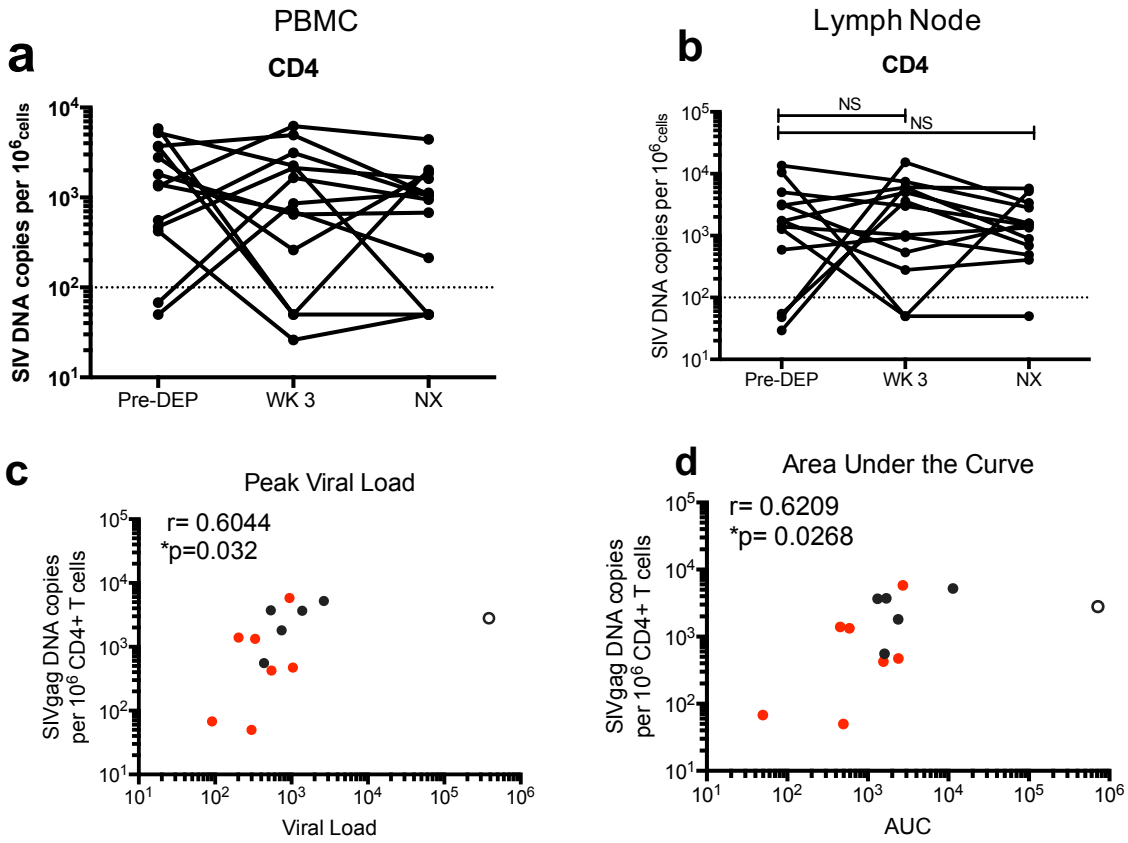


Figure 5. Pre-depletion levels of SIV infection in CD4+ T-cells correlates with post-depletion changes in viral load in ART-treated SIV-infected macaques. Fraction of SIV-infected sorted CD4+ T-cells in (a) PBMC and (b) lymph node. WK 3= week 3 post-depletion, NX= necropsy. Correlation of cell-associated SIV-DNA in peripheral CD4+ T-cells pre-depletion and the peak viral rebound (c) and area-under-the-curve (d) post depletion. Data is shown for all 13 animals. (●) persistent suppressors, (●) intermittent suppressors,(○) never suppressed.

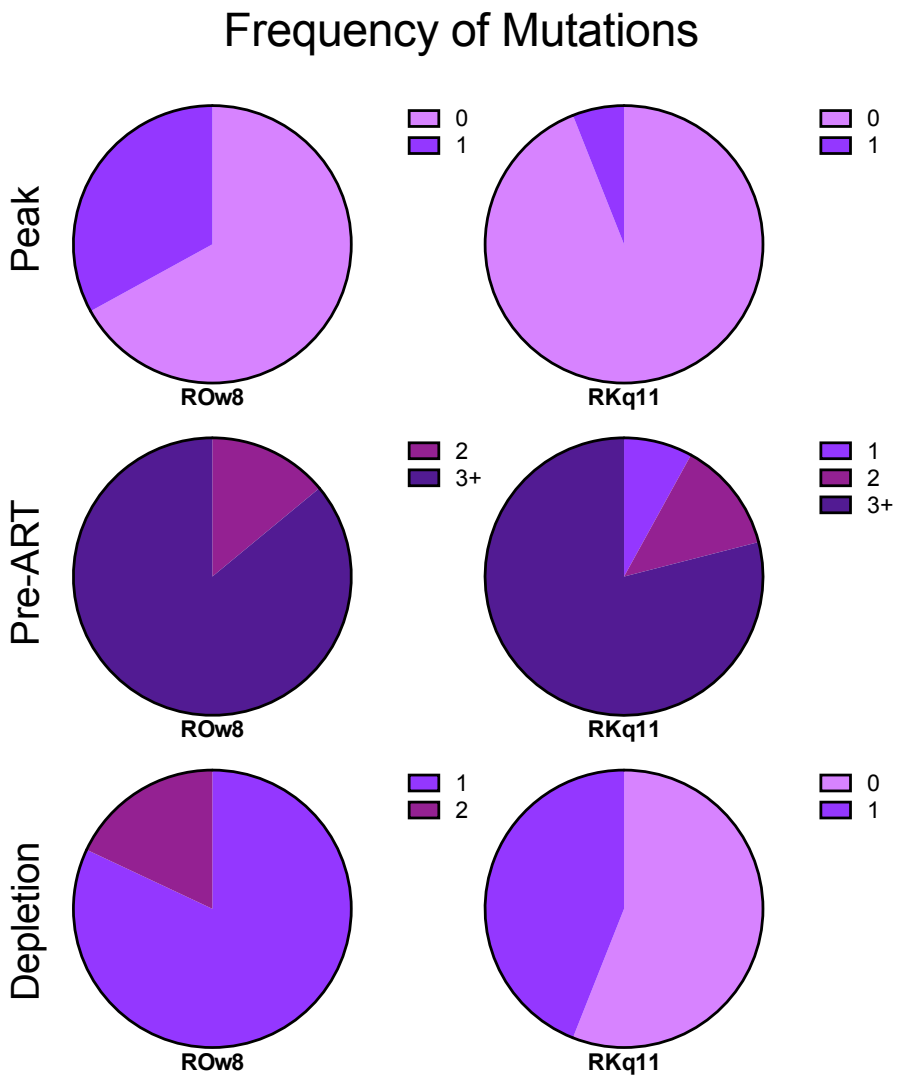


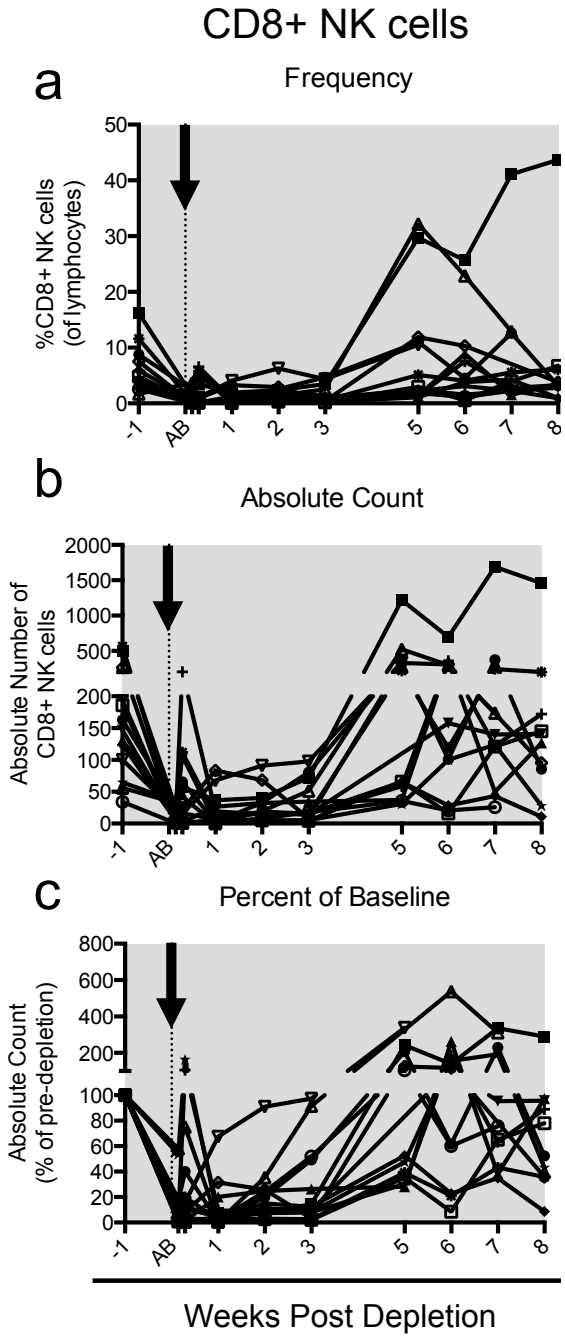
Figure 6. Virus emerging during CD8+ lymphocyte depletion closely resembles virus during the peak of acute infection. Single genome analysis (SGA) of SIV_{mac239} *env* in 2 rhesus macaques at peak (Day 10), pre-ART (Day 56), and during CD8+ T cell depletion. Frequency of mutations was determined by counting the number of sequences with amino acid substitutions (0, 1, 2, and 3+) and dividing by the number of sequences analyzed for that time point.

Frequency of Mutations in SIVmac239 Envelope

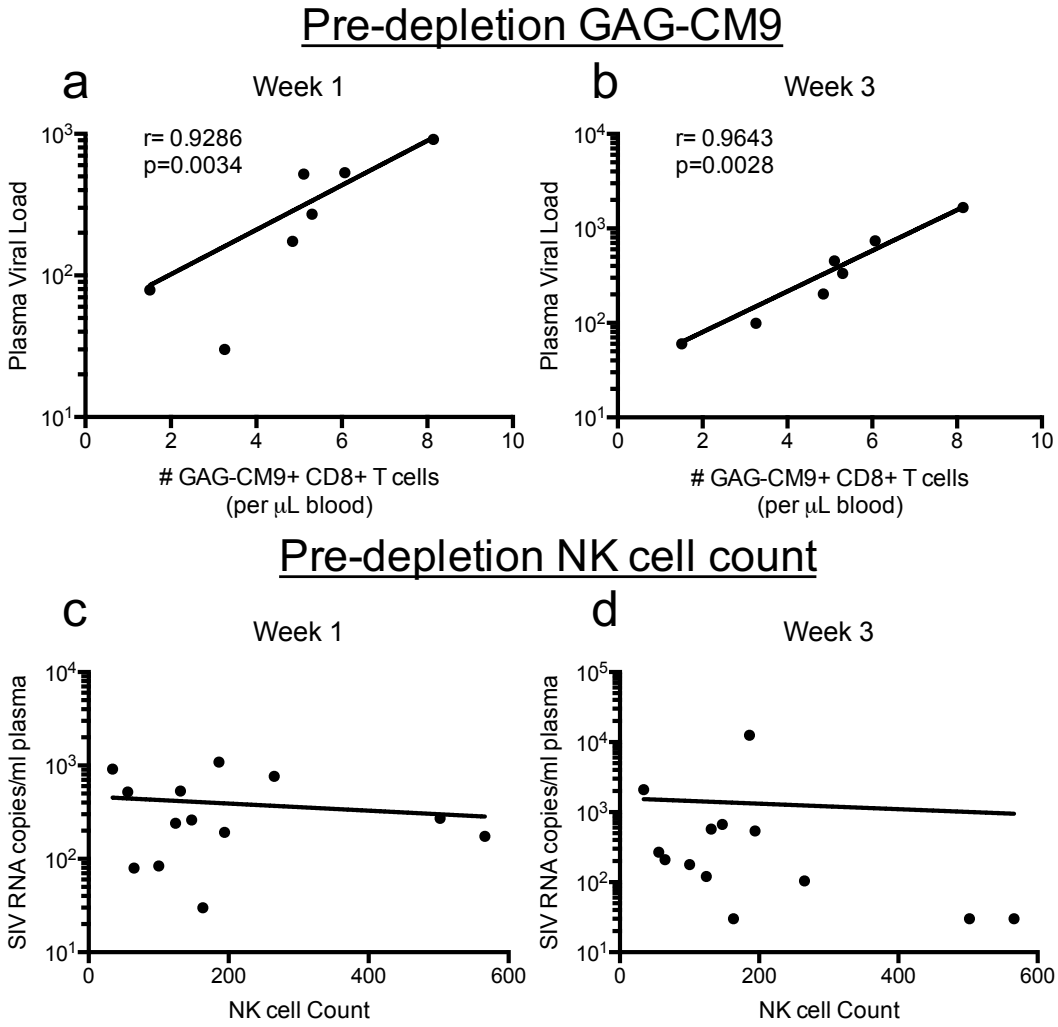
	Mutation	Peak Freq.	Pre-ART Freq.	Depletion Freq.
ROw8				
	Y305S	0/15	0/22	7/11
	Y425H	0/15	9/22	0/11
	I502V	0/15	9/22	0/11
	Q739R	0/15	16/22	1/11
	R751G	0/15	17/22	1/11
RKq11				
	V67M	0/18	19/24	0/9
	R751G	0/18	24/24	0/9

Table 1. Identity of mutations in SIVmac239 envelope identified by single genome analysis .

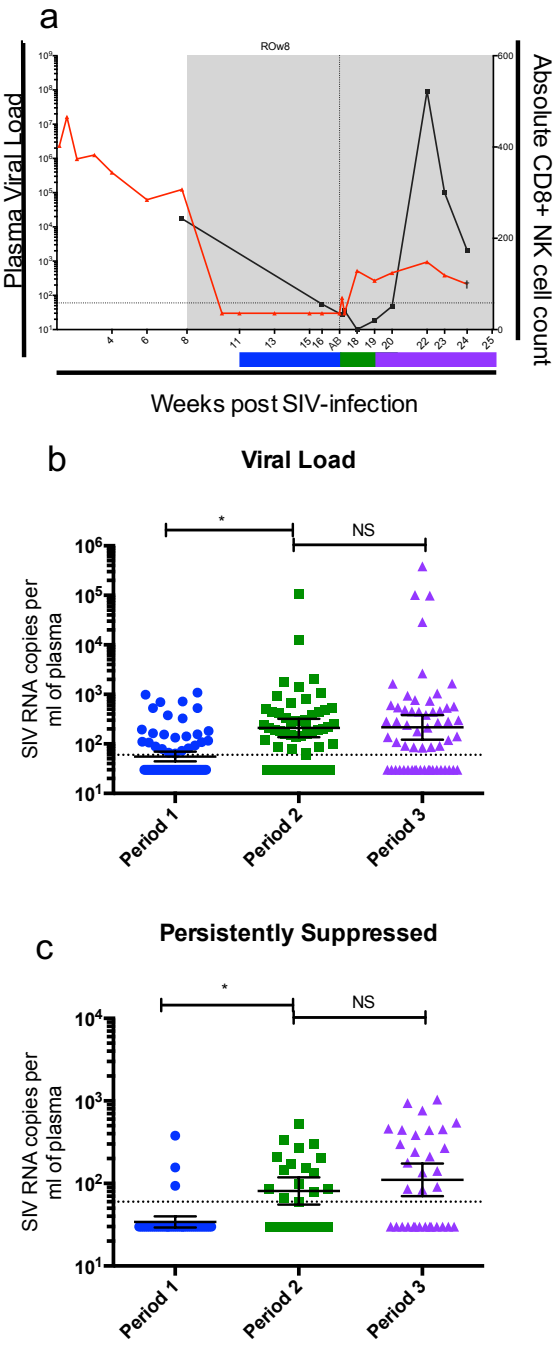
Supplemental Figures and Tables



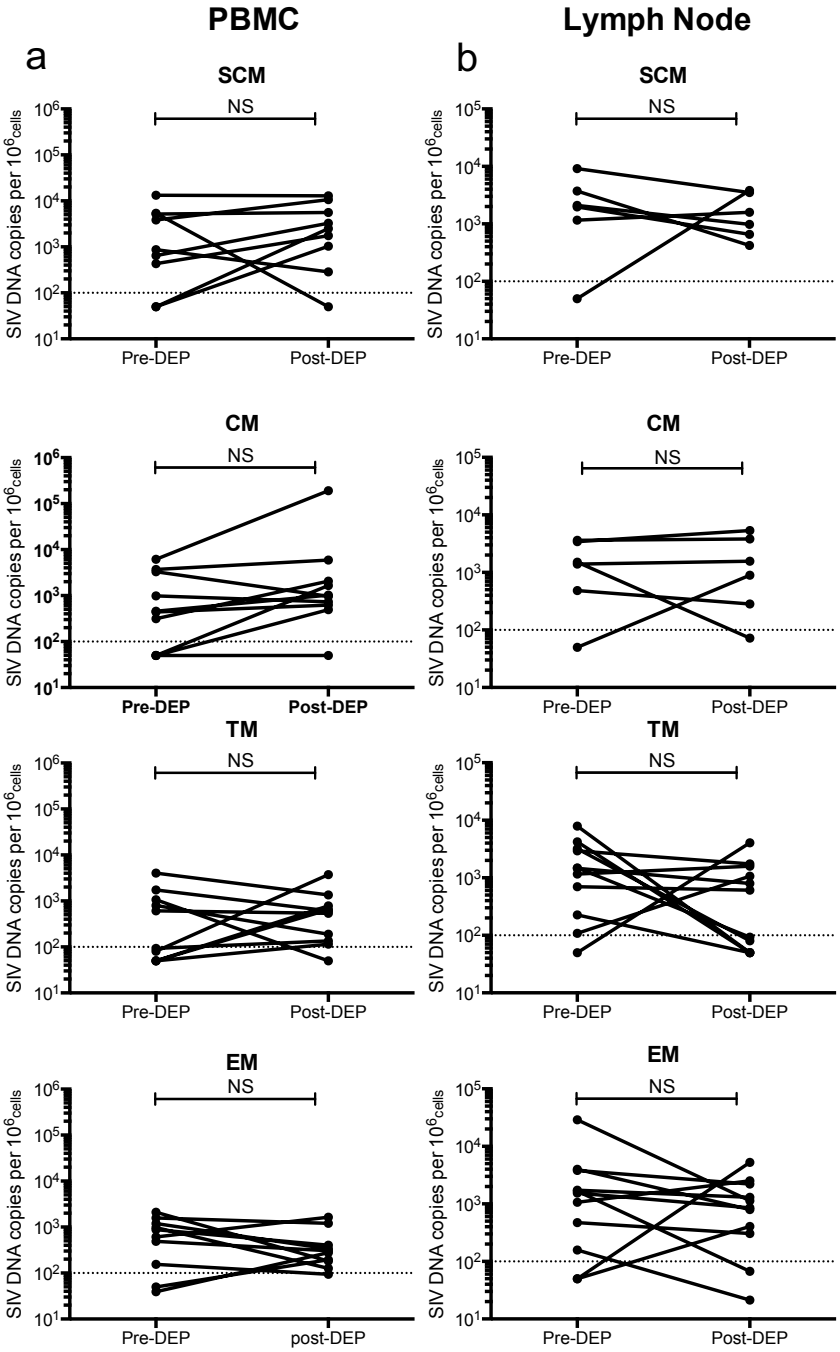
Supplemental Figure 4.1. MT-807R1 administration also depletes CD8α+ NK cells. Cells were gated on lymphocytes, then CD3-CD20-. (A) Frequency of CD8α+ NK cells as a proportion of total lymphocytes and (B) absolute count of CD8α+ NK cells in PBMC pre- and post-MT-807R1 administration. (C) Absolute count of CD8+ NK cells as a proportion of pre-depletion CD8α+ NK cell counts. Data shown is for 13 ART-treated SIV-infected macaques. Vertical dashed line and arrow represent time of anti-CD8α antibody administration.



Supplemental Figure 4.2. Number of pre-depletion SIV-specific CD8+ T cells predicts post depletion viral loads. Panels A-B: Presence of a significant direct correlation of number of GAG-CM9 specific CD8+ T cells in PBMCs pre-depletion with plasma viral load at (a) week 1 and (b) week 3 post-depletion. Panels C-D: Lack of correlation of number of NK cells in PBMCs pre-depletion with plasma viral load at (c) week 1 and (d) week 3 post-depletion. Spearman rank correlation, two tailed.



Supplemental Figure 4.3. NK cell reconstitution does not coincide with viral load decline in ART-treated SIV-infected RM. (a) Viral load (red line) and CD8+ NK cell counts (black line) of a representative SIV-infected RM on ART. Shaded area represents ART treatment, black arrow and dotted line indicates anti-CD8+ MT-807R1 administration. (b) Geometric mean viremia for each statistical period (NK cell) for all 13 animals. Bars show the geometric mean with 95% CI, *p<0.05. (c) Geometric mean viremia for each NK cell statistical period for 7 persistently suppressed animals. Bars shown at the geometric mean with 95% CI, *p<0.05.



Supplemental Figure 4.4. No significant changes in total cell-associated SIV-DNA in sorted memory CD4+ T cell subsets of SIV-infected ART-treated RM before and after CD8+ depletion. Fraction of SIV-infected sorted T_{SCM}, T_{CM}, T_{TM} and T_{EM} (as defined in Methods) in (A) PBMC (data shown for all animals with >10,000 sorted cells (T_{SCM}=6, T_{CM}=6, T_{TM}=11, T_{EM}=11) and (B) lymph node (data shown for all animals with >10,000 sorted cells (T_{SCM}=6, T_{CM}=6, T_{TM}=11, T_{EM}=11). Statistical analysis was conducted using the Wilcoxon matched-pairs signed rank test.

Number of animals	ART Regimen	Duration (post-ART initiation)
3	20 mg/kg PMPA s.c., 30 mg/kg FTC s.c., 100 mg Raltegravir and 400 mg Darunavir orally b.i.d.	From 0 to 16 weeks
5	20 mg/kg PMPA s.c., 30 mg/kg FTC s.c., 100 mg Raltegravir and 400 mg Darunavir orally b.i.d. followed by 20 mg/kg PMPA s.c., 30 mg/kg FTC s.c., 150 mg Raltegravir and 600 mg Darunavir orally b.i.d.	From 0 to 8 weeks From 8 to 24 weeks
5	20 mg/kg PMPA s.c., 30 mg/kg FTC s.c., 100 mg Raltegravir and 400 mg Darunavir orally b.i.d. followed by 20 mg/kg PMPA s.c., 30 mg/kg FTC s.c., 150 mg Raltegravir and 600 mg Darunavir orally b.i.d. followed by 20 mg/kg PMPA s.c., 30 mg/kg FTC s.c., 150 mg Raltegravir and 800 mg Darunavir orally b.i.d.	From 0 to 8 weeks From 8 to 24 weeks From 24 to 40 weeks

Supplemental Table 4.1. Antiretroviral drug regimen and length of treatment for SIV-infected ART-treated RM. All 13 animals were treated for at least 16 weeks. After 8 weeks of continuous treatment, 10 of the animals were given increased doses of Raltegravir and Darunavir. After another 16 weeks of increased Raltegravir and Darunavir, 5 animals received a further increased dose of Darunavir.

Magnitude of SIV suppression during ART

	Pre-ART	Post-ART (Pre CD8+ Depl.)	% Suppression
	(copies/ml of plasma)	(copies/ml of plasma)	
RGb13	433	Below 3	100
RLb13	233,000	7	99.992
RVy10	2,940,000	10	99.998
ROw8	124,000	15	99.985
RKq11	599,000	25	99.993
RBv13	1,580,000	65	99.994
RWj14	496,000	90	99.979
RSj14	921,000	80	99.989
RDh10	3,400,000	90	99.997
RLc10	1,940,000	160	99.985
RAz12	2,270,000	300	99.982
RYf14	5,870,000	330	99.991

Supplemental Table 4.2. Magnitude of virus suppression during ART, prior to CD8+ lymphocyte depletion is >99.9% in all animals where at least 1 undetectable time point was achieved. Animals in black were “persistently suppressed”, animals in red were “intermittently suppressed.”

Code	Period 1	Period 2	Period 3
RGb13	6 weeks	3 weeks	4 weeks
RLb13	6 weeks	3 weeks	4 weeks
RVy10	6 weeks	3 weeks	1 week
RKq11	6 weeks	7 weeks	1 week
RBv13	6 weeks	3 weeks	4 weeks
RWj14	6 weeks	3 weeks	4 weeks
RSj14	6 weeks	3 weeks	4 weeks
RYf14	6 weeks	6 weeks	2 weeks
RAz12	6 weeks	5 weeks	3 weeks
RLc10	6 weeks	7 weeks	n/a
ROw8	6 weeks	7 weeks	n/a
RDh10	6 weeks	8 weeks	n/a
ROn13	6 weeks	8 weeks	n/a

Supplemental Table 4.3. Length of the periods 1-3 used for statistical analysis as described in Figure 2 for each SIV-infected ART-treated RM. Period 1 is pre-CD8+ depletion (blue), Period 2 is post-CD8+ depletion, pre-CD8+ recovery (green), and Period 3 is during CD8+ recovery (purple). CD8+ T-cell reconstitution above 20% of baseline (i.e., pre-depletion) levels was not achieved in four animals (RLc10, ROw8, RDh10, and ROn13).

Code	Period 1	Period 2	Period 3
RGb13	6 weeks	4 weeks	4 weeks
RLb13	6 weeks	3 weeks	5 weeks
RVy10	6 weeks	1 week	4 weeks
RKq11	6 weeks	3 weeks	5 weeks
RBv13	6 weeks	3 weeks	5 weeks
RWj14	6 weeks	1 week	7 weeks
RSj14	6 weeks	5 weeks	3 weeks
RYf14	6 weeks	5 weeks	3 weeks
RAz12	6 weeks	1 weeks	7 weeks
RLc10	6 weeks	2 weeks	5 weeks
ROw8	6 weeks	1 weeks	6 weeks
RDh10	6 weeks	3 weeks	5 weeks
ROn13	6 weeks	3 weeks	5 weeks

Supplemental Table 4.4 . Length of the periods 1-3 used for statistical analysis as described in Supplemental Figure 3 for each SIV-infected ART-treated RM. Period 1 is pre-CD8+ depletion (blue), Period 2 is post-CD8+ depletion, pre- CD8+ NK cell recovery (green), and Period 3 is during CD8+ NK cell recovery (purple).

Subset	Time Point	Tissue	p
Total CD4	WK 1 Post Depletion	PBMC	NS
CD95+ Memory	WK 1 Post Depletion	PBMC	NS
T_{SCM}	WK 1 Post Depletion	PBMC	NS
T_{CM}	WK 1 Post Depletion	PBMC	NS
T_{TM}	WK 1 Post Depletion	PBMC	NS
T_{EM}	WK 1 Post Depletion	PBMC	NS
Total CD4	WK 1 Post Depletion	Lymph node	NS
CD95+ Memory	WK 1 Post Depletion	Lymph Node	NS
T_{SCM}	WK 1 Post Depletion	Lymph Node	NS
T_{CM}	WK 1 Post Depletion	Lymph Node	NS
T_{TM}	WK 1 Post Depletion	Lymph Node	NS
T_{EM}	WK 1 Post Depletion	Lymph Node	NS
Total CD4	WK 1 Post Depletion	Rectal Biopsy	NS
CD95+ Memory	WK 1 Post Depletion	Rectal Biopsy	NS
Total CD4	WK 3 Post Depletion	PBMC	NS
CD95+ Memory	WK 3 Post Depletion	PBMC	NS
T_{SCM}	WK 3 Post Depletion	PBMC	NS
T_{CM}	WK 3 Post Depletion	PBMC	NS
T_{TM}	WK 3 Post Depletion	PBMC	NS
T_{EM}	WK 3 Post Depletion	PBMC	NS
Total CD4	WK 3 Post Depletion	Lymph node	NS
CD95+ Memory	WK 3 Post Depletion	Lymph Node	NS
T_{SCM}	WK 3 Post Depletion	Lymph Node	NS
T_{CM}	WK 3 Post Depletion	Lymph Node	NS
T_{TM}	WK 3 Post Depletion	Lymph Node	NS
T_{EM}	WK 3 Post Depletion	Lymph Node	NS
Total CD4	WK 3 Post Depletion	Rectal Biopsy	NS
CD95+ Memory	WK 3 Post Depletion	Rectal Biopsy	NS

Supplemental Table 4.5. No correlation between the frequency of proliferating (Ki67+) CD4+ T-cells and plasma viral load at weeks 1 and 3 post-CD8+ depletion. Spearman rank correlation.

Chapter 5:

Discussion

HIV Cure: Moving Forward

HIV has proven more resourceful than many other viruses and despite 35 years of intense research, both a preventative vaccine and a cure remain elusive. Multiple factors have prohibited the generation of an effective preventative vaccine, including inability to generate effective broadly neutralizing antibodies in vaccinated individuals as well as the fact that recruitment of activated CD4+ T cells necessary to generate those antibodies increases HIV target cells at sites of transmission. In addition to the caveats and difficulties in generating a preventative vaccine, it will do nothing to relieve the burden of infection in the over 30 million individuals currently infected with HIV worldwide. Though antiretroviral therapy has significantly reduced morbidity and mortality associated with HIV-infection, treatment adherence is life-long due to the presence of a long-lived, latent HIV reservoir inaccessible to current treatment regimens. It is to these patients we direct the current research of “functional cure.” The “functional cure” has many potential definitions but, at its core, it is an intervention that will allow patients to cease antiretroviral therapy and remain disease free with very low transmission rates. A more complete understanding of the cellular localization of the HIV/SIV reservoir, as well as the role of the immune system in cooperating with antiretroviral therapy to maintain virus suppression during ART, as shown in this dissertation, provides important insight into future therapeutic design.

Role of CD4+ memory T cell subsets in HIV/SIV pathogenesis

The relative importance of CD4+ T cell homeostasis to overall immune health is evidenced by non-pathogenic SIV infection of sooty mangabeys (SM). As mentioned previously, SM are a natural host for SIV infection. They are infected with SIV in the wild

and have viral titers similar to SIV-infected RM, yet they will not progress to simian AIDS. Two important hallmarks of non-pathogenic SIV infection of SM are the absence of CD4+ T cell depletion and lack of chronic immune activation. Prior work has shown that low CCR5 expression on CD4+ T cells in SM protects these cells from infection. In particular, low CCR5 expression on CD4+ T_{CM} preferentially preserves these cells during SIV-infection (96). Models predicting the role of CD4+ T_{CM} infection in pathogenesis and disease progression suggest that the preservation of the CD4+ T_{CM} compartment allows SM to maintain their CD4+ T cell homeostasis (223). Building upon this observation, in Chapter 2, we show that disruption of a precursor memory CD4+ T cell subset, T_{SCM}, is also a distinguishing characteristic between pathogenic and non-pathogenic SIV infection. In addition to being the first study to describe CD4+ T_{SCM} in SM, our current work suggests that CD4+ T_{SCM} are also mediators of pathogenesis and T cell homeostasis. Our model for the role of CD4+ T_{SCM} in SIV pathogenesis is illustrated in Discussion Figure 1. Briefly, CD4+ T_{EM} are infected and depleted during pathogenic and non-pathogenic SIV infection. Compensatory homeostatic mechanisms allow for repopulation of this pool by proliferation and differentiation of precursor CD4+ T_{CM}. In pathogenic SIV infection, this step is perturbed due to infection and depletion of CD4+ T_{CM}. This depletion of T_{CM} may put added pressure on the relatively small population of T_{SCM} to try and maintain memory CD4+T cell homeostasis. Based on our comparative data, we hypothesize that eventually this compensatory mechanism is exhausted and CD4+ T_{SCM} can no longer preserve the balance. However, preservation of CD4+ T_{CM} in SIV-infected SM allows for continual repopulation of CD4+ T_{EM} and maintenance of CD4+ T cell homeostasis. This observation has important implications for SIV pathogenesis but also CD4+ T cell homeostasis. If these cells are indeed being "exhausted" by constant proliferation, strategies should be devised to protect

downstream subsets from infection. One such strategy may incorporate editing of the CCR5 gene by zinc finger nucleases (267). To specifically target CD4+ memory T cells,

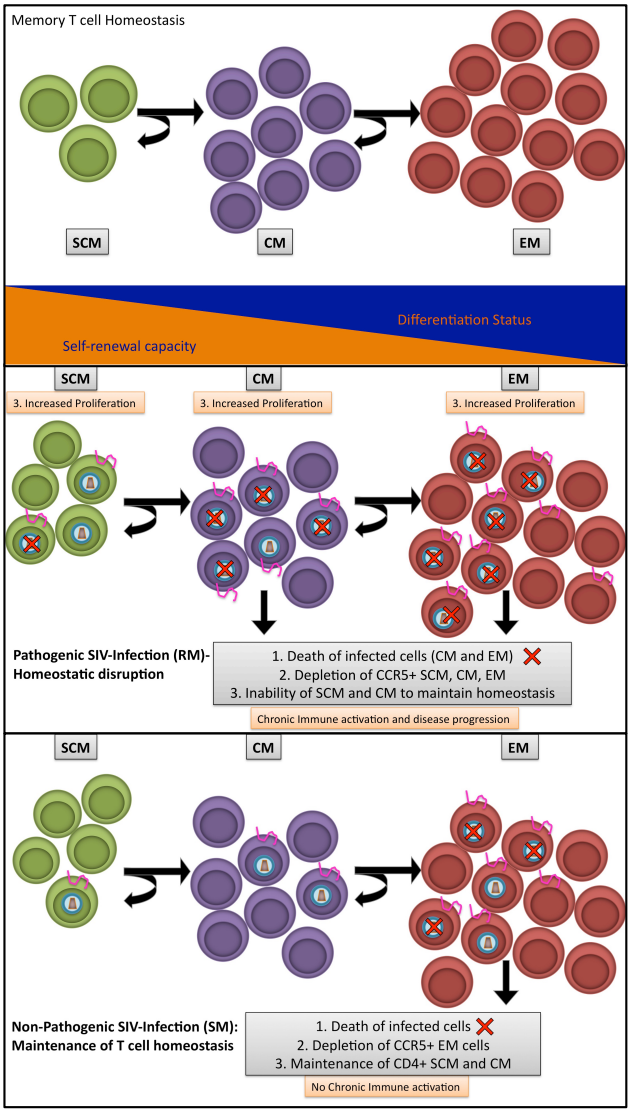


Figure 5.1. Model for differentiation and self-renewal capacity of memory T cells. (Top) Model for CD4+ T cell homeostasis showing T_{SCM} as the precursors for other memory subsets. (Middle) Disruption of CD4+ T cell homeostasis in pathogenic SIV-infection of RM caused by direct viral infection and cell death, depletion of CCR5+ memory T cells, and chronic immune activation preventing T_{SCM} and T_{CM} from maintaining homeostasis. (Bottom) Proposed model for CD4+ T cell homeostasis in SIV-infected SM, showing lack of T_{CM} death and little infection of T_{SCM} compartment, allowing maintenance of CD4+T cell homeostasis.

we can utilize the biology of CD4+ T_{SCM}. These cells can be generated *in vitro* by stimulating naïve CD4+ T cells in the presence of a GSK3β inhibitor to prevent differentiation and promote the generation of T_{SCM} (268). Prior to expansion, the CCR5 locus should be edited out of naïve CD4+ T cells. Second, stimulate the cells in the presence of a β-catenin inhibitor. Next, re-infuse these CCR5-deficient CD4+ T_{SCM} into an ART treated RM. By removing the inhibitor, the cells should differentiate into T_{CM}, T_{TM}, and T_{EM} thus creating a pool of memory CD4+ T cell deficient in CCR5. *In vitro* generation of CD8+ T_{SCM} has already been employed for cancer therapeutics, and editing of CCR5 locus on autologous CD4+ T cells has been tested in HIV-infected humans (268, 269).

Why ART is not enough: Immune activation

We know that disruption of immune homeostasis and subsequent immune activation are critical mediators of pathogenesis, and current ART regimens are insufficient to restore these imbalances to pre-infection levels (270). Even once virus suppression is achieved in patients on ART for >10 years, immune activation remains elevated compared to HIV-uninfected individuals. In concordance with this observation, our results in Chapter 3 show that CD4+ T_{SCM} in the lymph nodes of SIV-infected, ART treated RM remained activated despite viral load suppression. In particular, ART was unable to reduce proliferation to levels seen in SIV-uninfected animals. We and others believe it will be necessary to add additional immunotherapeutics to current ART regimens to help reduce immune activation, particularly in the lymph nodes. Still unknown is the relative contribution of CD4+ T_{SCM}, particularly in the LN to the actively replicating virus present in plasma. It is possible these cells are actively making low levels of viral RNA in the LN, where ART is less penetrant. Recent NHP studies have

shown that administration of IL-21 in RM treated with ART significantly reduces immune activation as well as SIV RNA production (271). Another recent study showed mechanism of action of IL-21 is the induction of microRNA-29 (mir29) in CD4+ T cells (272), which is directly responsible for inhibiting HIV-RNA expression in isolated CD4+ T cells. These studies did not look at the effect of IL-21 administration on CD4+ T_{SCM}, and did not thoroughly investigate CD4+ memory T cell subsets in the lymph nodes. However, if we extrapolate from data collected on T_{CM} in PBMC and rectal biopsies, administration of IL-21 during ART should dramatically reduce immune activation in CD4+ T_{SCM}.

Why ART is not enough: Reservoir

The importance of reducing immune activation while on suppressive ART, while important, is not the ultimate goal of HIV cure research. Though immunomodulation will improve the health of those on ART, the problem of the HIV reservoir remains. We know that current ART, though efficient at suppressing viremia in most patients, does not eradicate HIV/SIV from infected cells. HIV/SIV hides in long-lived CD4+ memory T cells. While we show the disruption of CD4+ T_{SCM} is important to a complete understanding of HIV/SIV pathogenesis, based on their long half-life, CD4+ T_{SCM} are critical to understand in the context of HIV/SIV treatment and cure as a site of HIV/SIV persistence. Our data shown in Chapter 3 provides new insight into this area of research and highlights the importance of the NHP model in accessibility of lymphoid tissues. We show that there is no reduction in the fraction of CD4+ T_{SCM} harboring SIV-DNA after ART initiation. As mentioned previously, T_{SCM} have been shown to persist in humans for as long as 12 years (195). Estimations of the half-life of the HIV reservoir must begin to take into account the lifespan of this extremely stable and long-lived population of infected cells.

Recent calculations estimate it would take 100 years of suppressive ART to eradicate just the CD4+ T_{EM} "sub-reservoir", with T_{EM} having the shortest half-life (203). They also point out that the CD4+ T_{SCM} and T_{CM} are inherently more stable than T_{EM}, and will therefore take longer to turnover. The extremely long half-life of the stable reservoir makes strategies for eradication with ART alone futile. A ready-made system in which we could study the half-life of the HIV/SIV reservoir *in vivo* is SIV-infected SM treated with ART. These animals have low infection of T_{SCM} and T_{CM} and presumably, the SIV reservoir would decay more rapidly in these animals than in SIV-infected RM treated with ART. However, taking into account the calculations above, 100 years to eradicate the T_{EM} sub-reservoir, monitoring the decay using ART alone may take more time than is feasible to demonstrate experimentally. This experiment was attempted in our laboratory with SM on ART for 3 months-1 year. Upon interruption, we saw no reduction in time to viral rebound in the short-term versus long-term treated animals, though some SM tended to have a slight reduction in set-point viremia after ART interruption (273). Again, these data suggest that even when T_{TM} and T_{EM} are the major contributors to the reservoir, eradication with ART alone is improbable at best.

One way to enhance decay of the HIV/SIV reservoir is to disrupt the self-renewal pathways maintaining the long-lived cells harboring latent HIV/SIV. While this strategy may seem counterintuitive, having just described the importance of these cells in T cell homeostasis and long-term health, controlled disruption may ultimately be beneficial. One way to selectively target T_{SCM} is by targeting the Wnt/β-catenin signaling pathway. T_{SCM} and to a lesser extent T_{CM} utilize this signaling pathway to self-renew. There are several small molecule inhibitors to target multiple stages of the Wnt/β-catenin pathway. One in particular, PRI-724, is already in use *in vivo* to target myeloid leukemia cells (274). This drug acts by inhibiting the interaction of β-catenin with its co-factor Creb-

Binding-Protein (CBP). Binding of β-catenin to this co-factor (along with Tcf-1) initiates the transcriptional profile of "stem-cellness", including transcription of the gene *survivin* (275 2005). Inhibition of CBP binding allows β-catenin to more frequently bind a second co-factor, p300. P300 acetylates β-catenin and facilitates its binding to Tcf-4, initiating the "differentiation" pathway (276). Studies are underway to use this drug in SIV-infected ART treated RM. While it is a sound strategy for cancer cells, which rely heavily on Wnt/β-catenin signaling, little work has been done on the requirement of β-catenin signaling to maintain T_{SCM}. Most of the work has focused on generation of these cells by inhibiting the differentiation pathway, not promoting their differentiation (268). Another caveat to this approach is that homeostatic proliferation, in which T_{SCM} appear to stochastically proliferate and generate T_{SCM} and other memory subsets, may not occur frequently enough to drive more differentiation in the presence of low doses of CBP/β-catenin inhibitor. Therefore, in SIV-infected ART treated RM, where little or no antigen is present, it may be difficult to detect differences in T_{SCM} compartment *in vivo*. Future studies will need to be executed to determine the viability of this approach including testing the ability of this drug to act on RM cells, as well as correct dosing and treatment regimen.

Functional Cure: *Early Treatment*

Another approach actively under investigation to help patients achieve a functional cure is to put patients on ART early during infection to reduce the potential size of the viral reservoir, limit immune activation, and prevent immune exhaustion. One such cohort of patients, the VISCONTI cohort is a group of 14 patients who initiated HAART during acute infection and then underwent treatment interruption after a prolonged period of undetectable viremia. Encouragingly, these 14 patients, referred to as post-treatment controllers (PTC), have undetectable viremia in the absence of

HAART (207). These patients are particularly interesting because they display none of the previously reported characteristics associated with spontaneous control, such as protective HLA alleles or highly activated CD8+ T cell responses (207). HLA-B*35 is associated with increased risk to rapid progression and is overrepresented in these PTC (207). In this cohort, the patients who became PTC had higher viral loads and lower CD4 T cell nadirs than those who did not end up controlling post-treatment interruption. Another important characteristic is the pattern of infected cells in these patients. While not significantly different than spontaneous controllers, the contribution of T_{CM} to the total reservoir in PTC is significantly less than T_{TM} (206). This observation, combined with their observation that the amount of HIV DNA decreases in PTC even after ART interruption has important implications for our examination of CD4+ T_{SCM}. It suggests that limiting infection of long-lived subsets in acute infection may allow for a smaller HIV reservoir and more potential for control. Recently, the WHO changed its recommendations on when to initiate ART in HIV-infected individuals. Previously, the recommendation was to wait until a patient's CD4 T cell count reached below 350. However, recent data about improved prognoses with early ART forced them to change the recommendation to treat infected individuals as early as possible.

Additionally, work by Siliciano et al showed the viral reservoir in patients who initiated ART during chronic HIV infection is highly mutated, with much of the virus showing CD8+ T cell escape mutations (109). This is in contrast to patients who initiate ART during the acute phase, whose viral reservoirs harbored fewer mutations (109). The earlier viremia is suppressed, the less time for viral escape and functional exhaustion of CD8+ T cells to occur. A future study could involve ART initiation at various time points during SIV infection. For example, during acute infection (Day 7 or 10), during early chronic (between weeks 8-12 after set point establishment) and very late chronic

infection (around week 24 post-infection). We then keep these animals on treatment for at least 2 months and then deplete the CD8+ T cells. We can hypothesize that the role for CD8+ T cell mediated suppression during ART would decrease as the amount of escape mutations within the reservoir increased.

Functional Cure: Role of CD8+ T cells

Our data showing a critical role for CD8+ T cells in maintaining virus suppression in SIV-infected ART treated RM provides important insights into future therapeutic interventions and allows us to re-interpret a study conducted using CD8+ lymphocyte depletion in sooty mangabeys (SM) (129). Upon administration of CD8+ lymphocyte depleting antibody, the viral load significantly increased in all animals that received the antibody. The original interpretation of the data was, since the increases seen were modest (3 to116-fold), that CD8+ T cells did not play a major role in controlling viremia in chronically infected SM. They arrived at this conclusion based on the data available at the time that depletion of CD8+ T cells in chronically infected RM resulted in an increase in viremia of 1-3 logs (127). However, our data using CD8+ lymphocyte depletion in the presence of ART closely resembles that of the data found in SM. So we offer an alternative hypothesis. CD8+ T cells in SM are essential for a portion of viral control, based on an increase in viremia seen during depletion. However, SM have other mechanisms of controlling viremia and progression to disease such as low CCR5 expression on CD4+ T cells, protection of precursor memory CD4+ T cell subsets T_{SCM} and T_{CM}, and lower chronic immune activation (54). These host control mechanisms in SM may prevent an increase in viremia like that seen in RM, where CD8+ T cells play a larger role in control. Additionally, compared to increases seen in chronically infected RM, the viral load increases during CD8+ lymphocyte depletion in our animals was

relatively modest (though by ultrasensitive techniques the increase was 72-350 fold). We hypothesize that ART is acting as an additional mechanism of control preventing infection of new cells and propagation of virus. In short, the CD8+ T cells in both the SM and our ART treated RM are not responsible for controlling all virus, but they are controlling some portion of infected cells. The magnitude of this contribution has yet to be established, but our data suggests it is at much as 2 logs of virus.

Based on our observations, CD8+ T cells are involved in maintaining suppression of virus production during ART. Our data also suggest an association between pre-depletion SIV-specific CD8+ T cells and viremia post-depletion. This data is somewhat contrary to current dogma in the CD8+ T cell field as it has been previously shown that the frequency of antigen-specific CD8+ T cells decreases while on ART, concomitant with a decrease in antigen load. We also find this is true, though SIV-specific cells are still detectable by tetramer on ART. It is also well established that ART decreases the expression of effector molecules such as perforin and granzyme in CD8+ T cells (277). It is important to note that HIV/SIV specific CD8+ T cells undergo more phenotypic and functional changes than decreased granzyme and perforin expression (149). They express more naïve and T_{SCM}-like properties including CD45RA, CD127, and CD28 (in RM). Interestingly, they also have increased polyfunctionality (secretion of IFN-γ, TNF-α and IL-2), when compared to antigen specific T cells from chronically infected individuals. Though they decrease in frequency and cytotoxic potential, antigen specific CD8+ T cells persist during ART and it is possible they are mediating control via a non-cytolytic mechanism. We propose these are the cells that are actively suppressing HIV/SIV transcription during ART and that boosting of this CD8+ T cell response may lead to better control of virus post-ART interruption.

Importantly, there is precedence of HIV infected individuals controlling viremia in the absence of treatment. Elite controllers, long-term non-progressors, and viremic controllers are all able to control viremia to some degree and delay disease progression without any therapeutic intervention. However, we still do not fully understand the mechanisms of control in any of these cohorts. Particularly attractive are the elite controllers, a small cohort of individuals who control viremia to undetectable levels in the absence of therapy (115, 278). Recent studies have suggested the CD8+ T cell repertoire of an individual will determine whether or not that individual is able to control infection (115). Preservation of polyfunctional CD8+ T cells has been shown to correlate with long-term non-progression, in HIV-infected humans. However, as mentioned previously, mechanisms of control are not consistent throughout cohorts and there is still much to learn from these spontaneous controllers.

If we can understand the mechanism of control, be it CD8+ T cell mediated or not, we can try to induce these responses in other individuals. Critically important to this endeavor is early and longitudinal monitoring of patients. We believe this is crucial as events during acute infection may determine disease outcome. For example, we see a dichotomy in the resolution of immune activation in SM and RM during acute infection, where SM exhibit robust early responses that are eventually controlled, while the RM continue to exhibit elevated levels of immune activation throughout chronic infection (55). Recent work in HIV-infected humans has been able to monitor women longitudinally for the first year of infection. These women are given access to services that train them for careers and education throughout the year, as well as monitoring for HIV infection twice per week. This FRESH cohort allows access to unprecedented events in acute HIV infection in human, so early its termed “hyperacute”. The study of hyperacute HIV infection in these women reveal robust early HIV-specific CD8+ T cell responses are

associated with eventual disease progression (279). Namely, that the greater the hyperacute CD8+ T cell response, the lower the set-point viral load. There will be extremely important insights to come out of this cohort and studies are now underway to put these women on early treatment interventions.

Functional Cure: Kick and Kill

Another approach currently under investigation to relieve the burden of infection in those who were unable to initiate early treatment, is colloquially known as “kick and kill.” This approach combines therapeutic vaccination with administration of ART as well as an agent to reactivate latently infected cells. A drug is administered to patients on ART to induce expression of latent virus. This is the “kick.” Prior to or concurrently with reactivation, individuals may receive a vaccine or other immunomodulatory agent to boost the immune system. Presumably, the primed immune system will be able to “kill” the newly activated cells, or reactivated cells will simply die due to virus-induced cytopathology. Eventually, these individuals will be taken off ART, with the expectation of a reduced pool of infected cells remaining. Additionally, their immune system is better able to response to the virus, and even if there is no reduction in the total reservoir, perhaps the immune system will be able to control infection. Efforts in this direction have so far, proven less than fruitful. Several attempts to achieve this have shown latency reactivation agents (LRAs) are able to induce viremia in ART suppressed patients and NHPs, but no one has shown a decrease in the size of the HIV/SIV reservoir, or a significant ability to control viremia upon interruption (280, 281).

This “kill” strategy relies heavily on the ability of CD8+ T cells to kill virally reactivated cells and the potency of the latency reversal agents to activate the majority of the latent reservoir. As mentioned previously, one recent study showed the virus harbored in CD4+ T cells of ART treated individuals who initiated therapy during chronic

HIV infection is highly mutated and contains many viruses that have escaped the CD8+ T cell response (109). If we reactivate virus that has escaped the CD8+ T cell response, significant efforts must be made to ensure the CD8+ T cell response boosted is adequate to control infection. Efforts are underway to vaccinate animals (and eventually patients) to generate a broader CD8+ T cell response, but so far they have not been effective. Additionally, our data suggests an important mechanism of CD8+ T cell mediated control may be non-lytic.

Evidence of a CD8-specific factor that controls HIV replication can be found in the literature, but its identity has yet to be established. However, as mentioned previously, multiple studies have shown that a non-lytic CD8+ T cell response is at least partially responsible for controlling viremia during SIV infection (130-134). Several mechanisms for non-cytolytic CD8+ T cell antiviral function have been proposed. The results to date are inconclusive as it appears to be independent of TCR:pMHC, but partially dependent on cell-cell contact (122). In addition, the mechanism appears to be dependent on STAT-1 signaling. One interesting study found that exosomes secreted by CD8+ T cells could be responsible for mediating the non-cytolytic antiviral effect (122). They show that membrane and exosomal fractions (i.e. exosomes isolated from membrane fraction of CD8+ T cells) are able to mediate potent suppression of HIV-1 LTR activity, in addition to bulk CD8+ T cells and whole membrane fractions. The CD8+ T cells mediating this effect were preferentially CD28+HLA-DR+. Whether or not it is one factor, or several factors working in combination has yet to be established.

Our data that most strongly suggests a non-lytic mechanism is the observation that plasma virus sequenced during depletion resembles sequences found at peak viremia. We sequenced viremia at three time points: day 10 post-infection, prior to peak expansion of SIV-specific CD8+ T cells, day 56, after contraction of CD8+ T cells but

prior to ART initiation, and during CD8+ T cell depletion in the presence of continuous ART. The virus found at set point prior to ART initiation is highly mutated compared to peak, with 3 or more amino acid changes found in most viral genomes. The cytolytic mechanism of control suggests the cells harboring this virus during peak are killed and the virus that survives has escaped the CD8+ T cell response and infected new cells. If the only mechanism of CD8+ T cell mediated control were cytolytic, it is unlikely the virus emerging during CD8+ T cell depletion would be the same as at peak, since peak sequences were not present in the set point viremia. However, re-emergence of wild-type virus during CD8+ T cell depletion strongly suggests a non-lytic mechanism of CD8-mediated suppression. To investigate this hypothesis, we are conducting deep sequencing of cell-associated virus found prior to ART and prior to depletion to confirm the presence of the wild-type virus within these cells. We also want to understand the frequency of this wild-type virus with respect to the mutant virus within the cells. Additionally, peptides generated based on both wild-type and mutant sequences will be generated and tested to see if this epitope can induce a CD8+ T cell response.

An alternative explanation for our data is the possibility that the virus emerging during CD8+ T cell depletion is coming from a site other than the periphery where ART is less penetrant. To answer this question, in the future, we will sequence virus found in lymph nodes prior to ART initiation. It is possible the virus found in lymph nodes will not be highly mutated and still resemble peak viremia. If we think about CD8+ T cells acting as suppressors and not killers, then actually the CD8+ T cell response becomes another mechanism of immune control co-opted by HIV to ensure survival.

Perhaps our previous attempts at therapeutic vaccinations have failed because we are trying to generate the wrong kind of CD8+ T cell response. Most measure CD8+ T cell functionality and antiviral efficacy by IFN-γ production or degranulation, and this

might not be the CD8+ T cell response we need to induce better control. Since a specific factor has yet to be identified as solely responsible for non-lytic CD8+ T cell function, it is still difficult to measure.

Functional Cure: Checkpoint Blockade

We know that a hallmark of chronic HIV/SIV infection is upregulation of the inhibitory marker PD-1 on antigen specific CD8+ T cells, as shown by the positive association between HIV RNA and PD-1 expression on HIV-specific CD8+ T cells (139). PD-1+ HIV-specific CD8+ T cells are incapable of producing IFN-γ when stimulated. In chronic LCMV infection of mice and HIV infection of humans this has been shown to correlate with decreased killing efficacy, measured again by perforin, granzyme and IFN-γ secretion. Studies in NHP using *in vivo* PD-1 blockade during chronic SIV-infection in RM shows that administration of anti-PD-1 results in increased number of GAG-specific CD8+ T cells (282). Though the trends were not significant, one study saw increased frequency of PD-1+ CD8+ T cells in periphery of HIV-infected, ART treated patients. Perhaps just as interestingly, expression of PDL-1 on CD4+ and CD8+ T cells was also increased in ART treated patients compared to both uninfected and viremic individuals (283). This allows us to posit a potential role for PD-1 on CD8+ T cells playing a part in maintaining virus suppression. Perhaps engagement of PD-1 to PD-L1 on CD8+ T cells causes secretion of a molecule or set of molecules to aid in suppression of viremia during ART, and that is effect can only be seen in low antigen situations. Another observation to support a possible beneficial role of PD-1 during HIV infection is the upregulation of both PD-1 and PDL-1 during HIV-2 infection, in which disease progression is slower and transmission rates lower (284).

When thinking about the role of CD8+ T cells in achieving a functional cure, it is also important to address the question of ongoing replication versus virus production

during ART. One of the arguments against virus replication is the observation that drug-resistant mutations are present in long-term HIV-infected, ART treated individuals (159, 285). This observation is coupled with the fact that there is no sustained increase in plasma viremia containing these mutations during ART. However, our work sheds light on an important aspect of ART treatment to consider, and that is, cooperation from the immune system. Logic allows that residual viremia during ART should and will consist of drug resistant mutations by virtue of the presence of these variants despite the continued presence of drugs. Yet to be explored however, is the role the CD8+ T cells have in controlling drug resistance variants. Further work should be done to examine the sequences of rebounding plasma viremia post-CD8+ lymphocyte depletion to determine if drug resistant mutations are overrepresented. CD8+ T cells may control drug resistant variants by secretion of CCR5 agonists preventing entry of the small number of drug resistant virions into new cells, thus keeping the virus from expanding. This would suggest we only see virus “production”, and not replication due to the efficiency of the CD8+ T cells at mediating entry inhibition and transcriptional suppression. It is also possible, that the effect of CD8+ T cell antiviral factor (CAF) is more global, as suggested by Ashwin et al (122). Studies of CAF have shown it directly acts by suppressing transcription at the HIV-LTR (123). It is possible, therefore, that CD8+ T cells are mediating their effect non-specifically to drug resistant and sensitive variants alike, but the drug resistant variants require it to maintain suppression. This would help explain how virus can be produced in the presence of ART. This hypothesis could also explain the divergent results obtained in the Raltegravir intensification study where only a subset of individuals increased 2 LTR circles upon initiation of Raltegravir (163). Perhaps these individuals have a sub-optimal CD8+ T cell response and are not efficiently preventing virus entry.

Whatever the mechanism of CD8+ mediated suppression of viremia under ART, it is clear from our studies that we do not fully understand the impact of CD8+ T cells in HIV/SIV control. Most likely, a functional cure will be achieved by some combination of these approaches. For example, a foreseeable future treatment regimen for an HIV-infected patient begins with suppressive ART initiated as soon as infection is confirmed, to establish control of viremia. Once suppression is achieved, give patients IL-21 or some other immunomodulatory agent to help restore CD4+ T cell homeostasis, particularly in the rectal mucosa. Once the immune activation and CD4+ T cells have been restored, a patient could choose several different options. The first may consist of a vaccination to boost the CD8+ T cells already cooperating with ART to maintain suppression. Then, these individuals may undergo reactivation by administration of a latency-reversing agent (LRA), such as an HDAC inhibitor or TLR-7 agonist. The combination of these approaches may allow for reduction of the reservoir. During this time, it would be essential for the patients to remain on ART to prevent additional infection. A second approach could be to administer a differentiating agent (like the β-catenin inhibitor) while administering the LRA. This may promote more differentiation due to increased proliferation from antigenic stimulus. ART therapy would be continued for some time after the administration of the new treatment regimen. With careful observation, analytical treatment interruption would then be initiated. The importance of studies designed using multiple interventions is more apparent as single interventions continue to show limited results.

Summary:

Ultimately, this dissertation provides important new insight into the field of HIV cure research. Chapter 2 uncovered a potential novel site of HIV/SIV persistence, CD4+ T_{SCM} and showed disruption of CD4+ T_{SCM} may contribute to pathogenesis. Chapter 3

emphasizes that ART only partially restores immune homeostasis, leaving increased activation of various T cell subsets, particularly CD4+ T_{SCM}. Additionally, no contraction of the frequency of infected CD4+ T_{SCM} and T_{CM} was seen upon initiation of ART, suggesting this reservoir is more stable than other memory T cell subsets. The fourth and final chapter focuses on a previously underappreciated role for CD8+ lymphocytes in controlling SIV viremia during ART. Cooperation of the host immune system with antiretroviral therapy provides important insights into future therapeutic interventions. It also highlights gaps in knowledge of CD8+ T cells and their ability and mechanism to control HIV/SIV infection, particularly in patients on ART.

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