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Functional Cure for HIV: Lymphoid T cell Dynamics and PD-1 Immunotherapy

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Functional Cure for HIV: Lymphoid T cell Dynamics and PD-1 Immunotherapy

By

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B.S. Northeastern University, 2008

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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

Functional Cure for HIV: Lymphoid T cell Dynamics and PD-1 Immunotherapy
By Geetha Mylvaganam

Human immunodeficiency virus (HIV) has emerged as one of the most devastating global health burdens in history with approximately 36 million individuals infected worldwide. The introduction of anti-retroviral therapy (ART) has greatly enhanced viral control and the quality of life for individuals living with HIV, but ART remains a life long therapy due to latently infected CD4 T cells that are seeded early during primary infection. The latent HIV reservoir is directly responsible for viral resurgence post treatment interruption. Due to the limited success in generating a highly effective prophylactic vaccine for HIV and with 15 million and a growing number of HIV infected individuals on ART, researchers have re-directed their focus towards generating a more feasible approach to treating HIV, termed a "functional cure". The key to achieving a functional cure for HIV requires reduction/elimination of viral reservoirs and restoration of functional anti-viral CD8 T cells. The viral reservoirs are concentrated at lymphoid sites and thus there is a need for understanding the dynamics of virus-infected CD4 T cells and anti-viral CD8 T cells in the lymphoid tissue during chronic infection. Using a macaque model system of SIV_{mac251} pathogenesis, our studies revealed critical findings. Firstly, we observed that PD-1^{hi} Tfh cells are aberrantly enriched in the lymph nodes (LN) and rectal mucosa of chronically SIV infected macaques and contribute to ongoing viral replication and production. Secondly, we identified a novel subset of germinal center infiltrating CXCR5+ SIV specific CD8 T cells that rapidly expand in vaccinated SIV controllers, can limit the expansion of virally infected Tfh, and are associated with enhanced viral control. Lastly, immunomodulation of the PD-1 pathway prior to and during the initiation phase of ART in a therapeutic SIV/ART macaque trial demonstrated significantly enhanced proliferation, cytotoxic potential, and polyfunctionality of anti-viral CD8 T cells resulting in markedly faster suppression of virus replication following the initiation of ART. Together, these findings further our understanding of some of the fundamental aspects of basic HIV/SIV biology and provide insight into novel therapeutic interventions that can be administered in combination with ART.

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Dedication

To my grandparents, who love me unconditionally and inspire me to do my very best everyday, no matter how discouraged I get, while always remembering to care and consider those around me.

Acknowledgements

To my parents who have supported me in every aspect of my life and without them I would not be where I am today. Their work ethic, care, and compassion have inspired me everyday to work hard, stay focused, and achieve my very best.

To my siblings who have always motivated me try to be the smartest child (just kidding). We all have motivated each other in many different ways and learned to work hard, always care about what we do, and always care about our family.

To Danny, who made the stress and chaos of lab and science manageable and was always there for me scientifically (hence the second authorship on my second paper), with all the great ideas and knowledge about everything and to be there emotionally (and there was always tons of emotion), always trying to get me to appreciate the more positive outlook on life.

To my friends who were my sounding board the entire way throughout grad school and have supported me academically, listened to me stress out about too many things to count, but have always been there to calm me down, distract me, and make me feel better.

To my lab members, who have made science an ingrained part of my life, and have guided me and mentored me throughout my time in the lab, helping me to be a better scientist day by day.

To Rama, who has been an amazing mentor for the last 5 years. No matter how many questions, how many ideas (good or bad), and how much stress I look like I am under, you have always been there to guide me, mentor me, and help me think about science and life in the most meaningful way. You have helped me develop as a scientist and as an individual and hopefully one day I can write, interpret, and design experiments as intricately as you do while managing to be confident, positive, a leader in science, and a mentor students can look up to.

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Chapter 1: Introduction

History and Global Burden of HIV/AIDS

Scientists first identified human immunodeficiency virus (HIV) in the early 1980's (1) but HIV was likely present as early as the turn of the 20th century. HIV is a single stranded, positive sense RNA virus that emerged after zoonotic transmission of simian immunodeficiency virus (SIV) from African primates to humans (2). It is thought that improper bushmeat practices led to the cross-species transmission event that established HIV. Phylogenetic analysis of viral sequences derived from infected samples revealed that HIV-1 arose from cross-species transmission of a chimpanzee virus (SIVcpz) to humans, particularly from the species *Pan Troglodytes* (3, 4) and HIV-2 was transmitted from a sooty mangabey virus (SIVsm) (5).

HIV is considered a global epidemic with some countries affected far more than others. Reasons for this include economical, social, and biological factors. AIDS was first recognized in the United States (US) and the US remains the most heavily disease burdened industrial country. Other countries and regions affected include Europe, Sub-Saharan Africa (that maintains the highest density of HIV-1 infected individuals), the Caribbean, and South and Southeast Asia. Although prevalence of HIV is still incredibly high in some areas of the globe, there have been significant advances in the field of HIV treatment and prevention that have aided in reducing the infections rates to those seen today. In particular, between 2001 and 2013, new HIV infections have declined more than 38% and AIDS related mortalities have decreased more than 35% between 2005 and 2013 (6, 7). Accessibility to anti-retroviral therapy has significantly increased with more than 13 million people receiving ART as of 2014, 12.1 million individuals of which are from low to middle income families (8). Moreover, in Sub-Saharan Africa, more than 85% of individuals that are aware of their infection status are on ART, with about 76% of those individuals having achieved viral suppression (9). The introduction and administration of ART

has greatly enhanced the quality of life for those living with HIV and is estimated to have provided an additional 40 million life years since the beginning of the epidemic (8).

Transmission and Pathogenesis

Transmission of HIV can occur through multiple routes that include sexual intercourse, injection of blood or blood derived products, and transmission from mother to child during pregnancy. There are many factors that contribute to increased transmission of HIV. A significant amount of viral transmission is thought to occur from sexual exposure of individuals that are acutely infected. A study conducted by Wawer and colleagues of HIV transmission in Uganda, showed that HIV-1 transmission during the first five months of infection was about eight times higher per coital act compared to individuals with chronic HIV-1 infection (10). Moreover, plasma HIV-1 RNA levels have been shown to predict HIV-1 transmission risk (3). A study conducted by Quinn *et al.,* to better understand the influence of viral load in association with other risk factors for heterosexual transmission of HIV, found that a log-fold increase in viral load detected in the plasma was associated with a 2.45-fold increase in seroconversion. Another study found a 0.7 fold reduction in plasma viral load reduced HIV-1 transmission by about 50% (11). Additionally, a study published in 2011 showed that a log-fold increase in genital HIV-1 RNA was associated with an approximately two-fold increase in HIV transmission (12). Several other important factors contribute to increased risk of transmission and those include sexually transmitted diseases, pregnancy, and receptive anal intercourse (13, 14).

Shortly after the identification of HIV-1, the CD4 co-receptor was found to be the primary receptor required for viral entry into a target cell (15). The identification of CD4 T cells as the primary target cell population for HIV infection and recognition of the cytopathic effects of HIV led to the rationale to clinically monitor CD4 T cell counts as a means of following patients with progressive HIV infection. The chemokine receptors CXCR4 and CCR5 were subsequently identified as the co-receptors required for viral entry into T cells and macrophages in the 1990's

(16, 17). HIV binding to both CD4 and the co-receptors either CXCR4 or CCR5 results in a conformational change required for effective viral entry into a target cell. Shortly after the identification of the co-receptors necessary for viral entry, the scientific community identified individuals that have a particular homozygous deletion in the CCR5 gene (referred to as the CCR5delta32 mutation). These individuals are resistant to HIV infection due to the inability of the virus to utilize the co-receptor CCR5 for viral entry into target cells (18-21).

Following HIV infection, the virus preferential targets and infects CD4 T cells generating foci of infection, where the virus replicates, increases exponentially, and then further disseminates to peripheral sites (22). The lymphoid tissue is an active site of viral replication and production (23-25). Many studies investigating simian immunodeficiency virus (SIV-a retrovirus capable of infecting non-human primates and largely studied in rhesus macaques (RM) model system) and HIV infection in humans have shown that gut associated lymphoid tissue (GALT) is a site extremely permissive of infection due to the high density of activated CD4 T cells expressing CCR5 (26, 27). It has been posited that the enrichment of activated CD4 T cells found at mucosal sites is largely due to the interplay between CD4 T cells and the presence of antigens derived from the microbiota. The microbiota within the lumen of the small intestine provides constant exposure of microbial antigens to CD4 T cells within the GALT, reinforcing this activated state. Infection of CD4 T cells in the GALT leads to significant depletion and subsequent mucosal immune dysfunction (28-31). These studies indicated that as many as 60% of GALT CD4+ T cells are possibly infected with SIV and depleted at peak viremia (day 10) (30). Cellular trafficking of CD4 T cells to the GALT versus the skin or cutaneous tissue is attributed to the expression of $\alpha 4\beta 7$ and CCR9, chemokine receptors responding to their cognate ligands MAdCAM and CCL25 expressed by epithelial cells found within the gut (32). Reports have also implicated $\alpha 4\beta 7$ as a receptor for SIV/HIV and thus this receptor may not only promote trafficking of virally infected cells to mucosal sites, but also potentially facilitate cell-to-cell transmission of virus (33). The importance of $\alpha 4\beta 7$+ CD4 T cells was underscored by a study

that demonstrated that the majority of Th17+ CD4 T cells, a mucosal subset critical to mucosal homeostasis, are enriched in the $\alpha4\beta7^{hi}$ population and are preferentially infected during acute SIV infection(34). Ansari *et al*. importantly revealed that blocking $\alpha4\beta7$ with a rhesus monoclonal antibody (mAb) prior to and during acute SIV infection resulted in a delay in viral kinetics, a reduction in plasma viral loads, and a reduction in both viral RNA and DNA in the GALT(35). Moreover, a very recent study by Byrareddy and colleagues determined that anti-$\alpha4\beta7$ administered just before challenge and during acute infection decreased transmission of SIV, in a low-dose intra-vaginal SIVmac251 challenge in RM. Macaques that were treated but did subsequently get infected retained a significantly lower number of SIV infected CD4 T cells in the GALT (36).

Non-pathogenic hosts such as sooty mangabeys (SM) and African green monkeys (AGM) that are infected with SIV but do not progress to AIDS, have greatly improved our understanding of what factors are involved in disease progression. Studies conducted in non-pathogenic hosts, such as sooty mangabeys (SM) have observed reduced frequencies of SIV infected cells in natural hosts of SIV infection. Data has demonstrated that CD4 depletion in the GALT does still occur in non-pathogenic hosts such as SM and African green monkeys (AGM) (37, 38), although in these non-pathogenic hosts, depletion is not predicative of SIV virulence. In recent years, studies have elucidated differences in SIV infection of central memory (T_{CM}) CD4 T cell subsets in SMs versus RM. An important study my Paiardini *et al*. demonstrated decreased expression of CCR5 on T_{CM} CD4 T cells in non-pathogenic SMs versus pathogenic RM(39). Additional studies have found that effector memory (T_{EM}) CD4 T cells compared to T_{CM} CD4 T cells are preferentially infected in RM. These findings suggest that despite high viral loads and memory CD4 T cells being infected in SM, infection of particular memory CD4 memory T cell subsets i.e. T_{CM} may more highly contribute to immunodeficiency and AIDS progression (40-44). The regulatory immune mechanisms that are present in natural hosts that may contribute to limited pathogenesis experience in these macaques include resolution of immune activation and

restricted target cell infection. These findings provide important insights into SIV/HIV pathogenesis and what aspects of the immune system can be strategically manipulated to more effectively limit HIV replication.

Systemic immune activation, a symptom of viral pathogenesis, was determined shortly after HIV-1 was discovered as the etiological agent of AIDS (45, 46). Systemic immune activation is an immune state in HIV infected individuals characterized by higher T cell turnover, B and T cell activation (47, 48), and raised levels of pro-inflammatory cytokines (49) and pro-fibrotic mediators (50-53). Over the years, many studies have helped improve the fields understanding of the effects of the mucosal microenvironment on systemic immune activation, particularly due to the high susceptibility of mucosal CD4 T cells to HIV-1 infection. Many studies have determined that microbial translocation, which is the movement of commensal microbial products from the intestinal lumen into the systemic circulation, strongly influences the state of chronic immune activation (54, 55). Microbial translocation has been shown to occur using assays to detect microbial byproducts such as peptidoglycan, lipotechoic acid, lipopolysaccharide (LPS), and flagellin in the plasma (56). Non-pathogenic hosts display limited immune activation and microbial translocation, and studies have shown that systemic injections of LPS in SIV-infected non-pathogenic hosts such as AGMs results in increased immune activation, supporting the role microbial translocation plays in facilitating SIV/HIV pathogenesis and AIDS progression (38, 54, 57). An important study by Kristoff and colleagues, in an effort to better understand the role of microbial translocation in immune activation and HIV pathogenesis, treated SIV infected pig-tail macaques with rifaximine (a member of the RNA binding ansamycin class of antibiotics) and sulfasalazine (an anti-inflammatory drug used in patients with inflammatory bowel disease). This combination treatment effectively reduced plasma levels of LPS and macaques showed significantly lower levels of immune activation compared to control animals. This study demonstrated that blocking translocation of microbial byproducts into circulation in a macaque model of HIV infection resulted in a dramatic reduction in T cell

activation, pro-inflammatory cytokine production, and plasma viral RNA levels (58). Microbial translocation has been found to occur significantly in conditions that cause direct insult to the gut intestinal barrier, such as Celiac disease and colitis (59). A more recent non-human primate study demonstrated that disruption of the gut epithelial barrier using a chemically induced colitis model in SIV-uninfected RM with dextran sulfate sodium (DSS) resulted in microbial translocation and resultant inflammation and immune activation. These data highlight the detrimental systemic effects that result from damage to the GI tract and support findings that dysfunction of the GALT can facilitate pathogenesis and progression of HIV infection (60).

ART and Viral reservoirs

The emergence of anti-retroviral therapy (ART) in the late 1990's as a clinical treatment for HIV both improved viral control and significantly reduced AIDS related mortalities, greatly enhancing the quality of life for individuals living with HIV/AIDS. (1). Each class of antiretroviral drugs (ARV) targets different parts of the HIV life cycle. The first class of anti-HIV drugs were the nucleoside reverse transcriptase inhibitors (NRTIs), which block reverse transcription of HIV RNA into HIV DNA and include Zidovudine (13) (61), Emtricitabine, Tenofovir, Truvada, and others (62). The second class of ARVs are non-nucleoside reverse transcriptase inhibitor that similarly targets reverse transcription of HIV RNA such as Nevirapine, Delavirdine, and Efavirenz in addition to others (62). Protease inhibitors such as Ritonavir, lopinavir/ritonavir, Tipranavir and others are examples of the third class of ARVs that target the assembly of a functioning virus by interfering with the protease enzyme (62). A fourth class of ARVs are entry inhibitors that prevent HIV from entering a cell, and examples of these drugs include Enfuvirtide and Maraviroc. HIV integrase inhibitors are a fifth class of ARVs that limit integration of HIV DNA into the host genome and these include Raltegravir, Elvitegravir, and Dolutegravir (63). The use of multiple classes of these drugs as combination therapy for the treatment of HIV is commonly referred to as highly active anti-retroviral therapy (HAART).

The co-expression of CD4 and CXCR4/CCR5 by CD4 T cells makes these cells the most suitable target for viral infection and the cellular population most enriched in the latent HIV reservoir. HIV's viral life cycle has pre-dominantly two phases, an early phase that is defined by viral entry into the host cell and integration into the host genome and the late phase, which is characterized by an integrated provirus and viral replication (64). Once infection into the host cell has occurred, two types of viral latency are thought to occur. The first of which is considered pre-integration latency and is the stage in which different forms of viral DNA exist in the cell but the viral genome has yet to fully integrate into the host genome. The second stage is termed post-integration latency and is considered a point at which the virus is no longer actively replicating and has inserted its viral genome into the host genome. Stable post-integration latency is considered an important feature of HIV-1 persistence and the major obstacle to viral eradication (14).

Stable post-integration latency generates a form of HIV known as the latent viral reservoir. It is thought that this reservoir is seeded very early after HIV infection, and likely earlier than day 3 of infection (65), as a study showed that ART administered as early as 30 to 36 hours after infection is still unable to prevent the establishment of viral reservoirs (66). It still remains to be determined when, where, and how the stable and persistent viral reservoir is generated. It is estimated that the half-life of the HIV reservoir is about 44 months and is insensitive to antiretroviral therapy. Once therapy is interrupted, viral resurgence occurs in the vast majority of HIV infected individuals, with set-point viral loads returning to levels that mirror those seen prior to the initiation of ART (67). It is also estimated that about 10^6 to 10^7 cells comprise the entire viral reservoir in ART suppressed HIV infected individuals (60, 68-70). Additionally, it is thought that about 3-4 copies of integrated provirus exist per cell in infected individuals on ART (46). The two sources within the CD4 T cell population that are thought to harbor virus are resting CD4 T cells that contain linear non-integrated viral DNA and latently infected cells that contain integrated proviral DNA and are thought to be derived from activated

memory T cells that have returned to a quiescent less active state (14). It has also been postulated that memory CD4 T cells may become infected during the contraction phase after they have expanded in response to virus allowing viral infection and integration to occur without continued viral replication. Quiescent memory CD4 T cells are thought to actively contribute to the ongoing viral reservoir through homeostatic proliferation via IL-7R/IL-7 responses or low levels of antigen (71). This can drive low-level expansion and maintenance of latently infected CD4 T cells. It is understood that once the viral reservoir is established, it continues to persist in the target cell it resides within.

In patients not undergoing ART therapy, the viremia present in the blood is mainly a result of continuous infection of target cells and ongoing viral replication in these activated CD4 T cell targets, whereas it is thought that latently infected T cells only minimally contribute to the total viral burden. However, during long-term ART, latently infected CD4 T cells are the primary contributors to total viral burden and are thought to have an estimated half-life of a around 39 weeks to a life-time (45). Cell types that comprise this reservoir include macrophages, infected dendritic cells (DCs) or viruses trapped in follicular DCs, and resting CD4 T cells. Recently, a particular subset of memory cells termed stem-cell memory cell (T_{SCM}) has been shown to be an important memory precursor subset for both CD4 and CD8 T cells, and potential target memory CD4 T cell subset that houses the persistent viral reservoir. Stem-cell memory cells are considered to be a multi-potent subset, driven by homeostatic cytokines and can give rise to central memory (T_{CM}) and effector memory (T_{EM}) CD4 and CD8 T cells. In the context of the latent HIV reservoir, a recent study elucidated the dynamics of the latent HIV reservoir during long-term suppressive ART and found that there is a progressive reduction in the blood latent reservoir around a core pool of less differentiation cells that include T_{CM} and T_{SCM}. More specifically Jaafoura *et al* demonstrated significant long-term stability of the T_{SCM} reservoir, which is directly related to the cumulative plasma virus exposure before the initiation of ART (72). The specific origin of residual viremia occurring under suppressive ART is somewhat

controversial and three hypotheses have been proposed to explain the residual viremia. The first hypothesis is that ongoing replication is occurring as a consequence of incomplete ART suppression (ART not completely limiting viral replication). The second hypothesis is that residual viremia is a result of viral reactivation of latently infected T cells and the third hypothesis proposed is that virus is continuously released from a stable unknown reservoir that leads to production of viral particles that do not undergo additional rounds of infection but allow for detectable RNA (73). Residual viremia is thought to be proof of ongoing viral replication but recent studies that access the efficacy of HAART regimens found that ongoing HIV replication can be controlled at accessible systemic sites. However, there are anatomical sites such as the GALT and central nervous system that ART is less accessible to and thus ongoing replication could potentially progress at these anatomical sites. To this end, studies have shown that despite no detectable virus in the blood, HIV-1 RNA can be measured in the lymph nodes and the GALT (74-77). It is thought that the presence of DNA in the peripheral blood and gut, after the initial decay of virus with the administration of ART, may be a source of ongoing HIV replication(69, 78, 79). DNA levels have been found to correlate with viral suppression and DNA has been thought to be a marker of persistent viral replication despite HAART(80). However, DNA levels detected under ART can also be due to the stability of the reservoir in resting CD4 T cells that can take the form as replication competent or replication incompetent integrated DNA.

Immune Drivers of Viral Persistence

Anti-retroviral therapy is highly efficient at reducing viral loads to below the limit of detection (by commercially available tests) in up to 90% of HIV infected individuals (81) and although some patients experience immune reconstitution, there are a significant number of individuals that do not. An impaired immunological response under ART is associated with more rapid disease progression and death. HIV infection causes significant impairments to the innate and adaptive immune system, which include upregulation of interferon stimulated genes that

contribute to on-going systemic immune activation, depletion of pDC's important for induction of anti-viral immune response, depletion of CD4 T cells, and expansion of dysfunctional CD8 T cells. Although ART dramatically improves viral control and may restore to some degree CD4 T cell frequencies in the blood, GALT immune reconstitution remains incomplete. Studies have shown that early initiation of ART can provide better reconstitution of CD4 T cells compared to ART initiated during late chronic infection (82-84). Chronic inflammation also persists despite long-term ART and is driven by factors such as ongoing viral replication, impaired mucosal homeostasis, and outgrowth of other co-infections such as CMV, due to an overall compromised immune system.

Activated memory CD4 T cells express higher levels of the co-receptor CCR5 required for entry into a target cell compared to naïve and resting CD4 T cells and macrophages, but these cellular populations can be infected as well (47, 48, 53) and significantly contribute to ongoing viral persistence. During HIV infection, the majority of infected and activated T cells have been shown to die through pyroptosis, with the infection status mainly being abortive and non-productive (49). A small fraction of cells become productively infected, revert to a resting state, and can contribute to the latent reservoir. ART blocks the majority of all new infections that occur during treatment therefore the reservoir that exists at the initiation of ART is believed to be the viral reservoir that persists in the individual long-term (85). There are multiple factors that are thought to contribute to the stability of the viral reservoir and these include homeostatic proliferation of infected memory CD4 T cells that have a very long half-life, cell to cell transfer of virus (cryptic replication), and recent data has suggested that HIV integrates into host genes known to be involved in cell growth and proliferation, possibly further facilitating persistence and expansion of the latent reservoir (50). The reservoir found in the peripheral blood is mainly restricted to memory CD4 T cells, in particular central memory and stem cell memory CD4 T cells, which are known to possess a heightened regenerative potential. The distribution of the viral reservoir in tissues is thought to be higher than the blood. Secondary lymphoid sites such as

the lymph nodes, ileum, and spleen are highly enriched in viral DNA compared to the peripheral blood (23, 86, 87). The higher frequency of target cell infection in these tissues might suggest more of a cell-to-cell transmission of virus versus direction infection. Recently, a particular subset of memory CD4 T cells, termed T follicular helper cells (Tfh) have been shown to be highly enriched in viral DNA and RNA and contribute to ongoing viral replication and production(88). This cellular subset will be discussed in detail in a subsequent section.

Certain studies have demonstrated a relationship between the frequency of activated/proliferating CD4 T cells and the size of the viral reservoir under suppressive ART. One study found a significant association between the level of HIV DNA during ART and the frequency of PD-1+ CD4 T cells (89). More recently, a study by Cockerham and colleagues studied 30 HIV infected ART treated individuals. This study revealed a significant correlation between the frequency of CD4 and CD8 T cells expressing HLA-DR and the frequency of resting CD4 T cells that contained viral DNA(90). It is not known what mechanisms underlying these associations, but the data suggest an important connection between T cell activation and viral replication and/or production. In a subsequent study, the addition of the more potent integrase inhibitor, raltegravir, to intensify ART did demonstrate a decline in HIV-1 replication and inflammation in some individuals suggesting that some low level of replication does persist under suppressive ART and can contribute to the ongoing inflammatory state and persistent infection (91).

An inflammatory immune microenvironment may also contribute to ongoing viral persistence by a number of different mechanisms. T cell activation in response to inflammation can facilitate increased cell-to-cell transmission, allowing the infected cell to produce more virus and transfer infection (92-94). Additionally, chronic inflammation simulates the expansion of T regulatory cells and increased expression of PD-1 and other negative regulators that impair the function of effector cells limiting the effective clearance of virally infected cells. Furthermore, HIV associated inflammation can lead to collagen deposition in secondary lymphoid organs,

which leads to tissue fibrosis, chronic immunodeficiency, and incomplete clearance of HIV(95).

Immune response to HIV

Innate immune response:

The innate immune response during HIV infection is a critical first line of defense and comprises of a variety of cell types important for sensing HIV infection. Innate immune responses require cells to directly interact with the invading pathogen therefore innate cells during HIV infection will often be localized to sites of infection where virus and the target cell are at high densities. Cell types critical to the innate immune response include plasmacytoid dendritic cells (pDCs), monocyte-derived dendritic cells (MDDCs), and monocyte derived macrophages (MDMs), natural killer (NK) cells, and neutrophils. PDCs, monocyte derived DCs, and monocyte derived macrophages can all serve as targets for HIV. One of the predominant cytokines produced by pDCs in response to TLR7 sensing of viral RNA is IFN-α(96-98). PDCs productively infected with HIV can be found at low levels *in vivo* although *in vitro* studies suggest that limited HIV-1 replication occurs in pDC's isolated from the blood of HIV-1 infected patients. MDDC express HIV receptors but again do not efficiently support viral replication compared to productive targets such as CD4 T cells (99-101). Infection of MDDCs can also be restricted by SAMHD1, a deoxynucleoside triphosphate phosphohydrolase that limits the efficiency of HIV-1 reverse transcriptase. Unlike HIV-1, HIV-2 encodes the accessory protein Vpx, an accessory protein carried by lentiviruses. Vpx is present in viral particles in HIV-2 allowing for the degradation of SAMHD1 in target cells, and continued viral production. Vpx, however, is not present in HIV-1 and thus SAMHD1 cannot afford the same protection against infection of MDDCs (102). Natural killer (NK) cells are important players of the innate immune system and contribute to the immune systems defense against HIV. NK dysfunction in patients with AIDS was first described in the early 1980's (103, 104) and was associated with progression of disease (105-108). NK cells are a subset of lymphoid cells that are found in multiple locations

including the peripheral blood, liver, peritoneum, placenta, and other lymphoid tissue. NK cells are thought to mediate control of HIV infection through multiple mechanisms. One mechanism in which NK cells contribute to control of HIV is targeting of HIV infected cells that have down regulated MHC class I expression. HIV-1 will downregulate MHC class I expression as a means of viral escape and to circumvent anti-HIV specific CD8 T cell responses. Infected cells with low levels of MHC class I can be targeted by NK cells either through direct cell lysis, or through antibody dependent cellular cytotoxicity means (ADCC)(109-111). Neutrophils are another important leukocyte subset and an important part of the innate immune response against HIV. A recent study has demonstrated that neutrophils are capable of communicating and co-localizing with T cells at sites of infection (112). It has also recently been determined that neutrophils in the blood of HIV-1 infected individuals express high levels of surface PD-L1, a ligand expressed on a variety of hematopoietic and non-hematopoietic cells. This ligand interacts with its cognate receptor PD-1, an inhibitory receptor important for regulating/inhibiting T cell, B cell, and monocyte responses. This recent study found that PD-L1 is induced on neutrophils by HIV-1 virions, IFNα, TLR7/8 ligand R848, and LPS and PD-L1 suppresses T cell function through ROS and the PD-L1/PD-1 axis(113). These data support a role neutrophils may have in further contributing to chronic T cell exhaustion and immune suppression during HIV infection.

Adaptive immune response:

Humoral Immunity

The humoral immune response is essential for protection against HIV infection. Moreover, hypergammaglobulinemia, a condition in which abnormally high levels of certain immunoglobulins are present in the serum, is a hallmark clinical feature of chronic HIV infection. Hypergammaglobulinemia is a consequence of polyclonal B cell activation, due to ongoing viral replication that results in terminal differentiation of B cells into plasmablasts or plasma cells that secrete large amounts of antibodies (114-117). Several cytokine such as interferon α

(IFNα)(118), tumor necrosis factor (TNF)(119), interleukin-6 (IL-6), IL-10, CD40 ligand (CD40L)(120, 121), and B-cell activating factor (BAFF)(122) have been shown to contribute to B cell hyperactivation during HIV infection. Persistent viral replication can also contribute to the exhaustion of memory B cells. Similar to CD4 and CD8 T cells, B cells can exhibit decreased proliferation and functionality due to the upregulation of co-inhibitory receptors(123, 124) in response to continued exposure to viral antigens. HIV infection is also associated with changes in the composition of memory B cell repertoire found in the peripheral blood. In particular, resting memory B cells, IgM+ B cells, and PD-1+ B cells are significantly depleted during acute infection (124, 125). IgM positive memory B cells are more profoundly depleted in individuals not placed on ART therapy early after HIV infection (126). During chronic HIV infection, these activated memory B cells transition into exhausted B cells that up regulate co-inhibitory markers such as LAIR-1 and PD-1 (123, 124, 127, 128). The level of homing receptors such as CXCR3, CXCR5, and CCR7, and adhesion molecules like CD62L on tissue-like memory B cells are also altered during HIV infection. In particular the chemokine receptor CXCR3 important for homing to inflamed sites is selectively unregulated on B cells and in contrast CXCR5, a chemokine receptor required for homing to B cell follicles is downregulate on B cells (129, 130). The balance of these two homing receptors may have an important effect on T cell dependent B cell responses, that require trafficking to the germinal centers of lymphoid follicles, for these responses to develop.

The specific antibody response that develops as a consequence of B cell activation during HIV infection is important in contributing to protection from infection and control of viral replication once transmission and the establishment of viral infection as occurred. Particular antibody responses develop over the course of infection and can block HIV-1 replication and exert immune pressure by binding to and/or neutralizing free virus, preventing further infection of target cells (termed NAb) and completing with Face receptors to clear virus and minimize cell to cell HIV-1 spread (nab). Approximately 14 to 28 days after detectable plasma viremia anti-gp41

and anti-gp120 antibodies are detected in circulation but these antibody responses have a limited effect on viremia (131). Additionally, these primary antibodies directed against the viral envelope protein exert little immune pressure on the HIV envelope (132). Neutralizing (nab) and non-neutralizing antibody (nab) responses have different and distinct effects during HIV infection (133, 134). Early after infection the specificity of autologous neutralizing antibodies is very narrow and limited to the infecting virus. Due to the lack of neutralizing antibodies capable of being generated early after infection, viral dissemination and replication occur vary rapidly. Nab on the contrary can develop early after HIV infection, bind more sites on a given virus, and have a much greater breadth for more recently transmitted viruses. Over the course of infection, broadly neutralizing antibodies (ban) may develop in a fraction of HIV infected individuals and are most commonly generated against the viral envelope. Elite controllers represent a particular cohort of highly studied individuals that demonstrate consistent and durable control of viremia in the absence of anti-retroviral therapy. These individuals provide important insight into the immunological mechanisms that come into play during immune control of HIV. Studies of NAb responses in elite controllers as compared to aviremic progressors have found that antibody responses unlike HIV specific CD4 and CD8 T cell responses are less frequently observed in individuals that control infection (135, 136). One large-scale efficacy trial, RV144 of the 'Thai trial' recently highlighted the importance of humoral immunity in providing protection from acquisition (134, 137, 138). This large clinical trial identified an important relationship between the binding of non-neutralizing IgG antibodies to envelope proteins and enhanced protection from acquisition of HIV infection. In contrast, serum antibody titers of IgA correlated directly with infection.

Non-neutralizing antibody responses are also important for control of viral replication during HIV infection and although they are not as well understood in elite controllers, there are actively being investigated to better understand their contrition to antibody-mediated immunity. There are conflicting studies that suggest that antibody dependent cellular cytotoxicity (ADCC)

mediated through nNAb might be enhanced in those individuals that control HIV infection in the absence of treatment(135, 139). These individuals have also shown lower Nab titers. This sheds some hope on pre-clinical animal studies in which vaccines have been capable of generating ADCC responses that are associated with better viral control post challenge(140, 141).

Cell mediated immunity

A strong and effective HIV specific CD4 and CD8 T cell response is essential to control of HIV replication. CD4 T cell responses are also integral in generating a humoral immune response and the interplay between helper T cells and B cells are critical for the generation of both a neutralizing and non-neutralizing antibody response that can effectively limiting HIV infection (142-144). Elite controllers possess qualitative differences in the function of their CD4 and CD8 T cells, such as greater cytokine polyfunctionality, which has been associated with more profound control of viral replication (145-147). Once virus trafficks to the local lymph node, antigen presenting cells such as subcapsular macrophages or follicular dendritic cells bring virus or viral antigen to lymphoid follicles to stimulate an effective T cell response. Here, HIV specific CD8 T cells can be primed and clonally expanded, generating a population of effector and memory CD8 T cells critical for targeting virally infected cells (primarily CD4 T cells and macrophages).

Particular subsets of CD4 T cells are important for both viral control and pathogenesis and progression to AIDS. These CD4 subsets include Th17 cells, T regulatory cells (Treg), and Tfh cells. Th17 cells are a subset of CD4 T cells that produce the cytokine interleukin 17 and are required for the immune surveillance and maintenance of the microbiota during mucosal homeostasis. Studies have shown perturbations in the Th17 cell subset during acute and chronic HIV infection. In particular, Th17 CD4 T cells are both preferentially infected by HIV and depleted early after HIV infection, leading to increased microbial translocation and systemic immune activation, immune correlates of progressive HIV infection(148). Higher Th17

frequencies have been associated with lower levels of HIV-1 associated immune activation (148). Treg cells are also important for limiting the extent of immune activation during chronic HIV infection. Multiple studies have reported an increase in Treg frequencies (8), but a decrease in absolute numbers in all immune compartments of HIV infected individuals (149). The role of Tregs in HIV infection is still not fully understood, but two main hypotheses have been proposed for their function and contribution during HIV infection. One hypothesis states that Tregs play a harmful role in HIV infection by suppressing HIV specific immune responses (12, 16, 150), while a second opposing hypothesis is that these cells could in fact play a more beneficial role by limiting chronic immune activation and deceasing the number of immunological pathologies that result from chronic disease (17, 20, 21, 112). Potential mechanisms that have been suggested for why there is an accumulation of Treg cells during HIV infection are: preferential survival of these cells due to a decreased susceptibility to infection, increased proliferation, tissue redistribution from the blood to lymphoid organs and increased peripheral conversion.

T Follicular helper cells and HIV

CD4 T cell help to B cells was first described as early as the 1960's when it was determined that these cells were integral to the development of germinal centers in secondary lymphoid tissue (151). During the 1980's, a number of *in vitro* studies were conducted using T cell clones and recombinant cytokines to show that the TH2 subset of CD4 T cells, a subset that primarily produces IL-4 and IL-10, was the subset important in engaging B cells and aiding in the generation of antibody responses (151). In the early 90's the chemokine receptor CXCR5 was discovered and shortly thereafter shown to be important in the migration of B cells into follicles of lymphoid organs in mice. It was not until the early 2000's that studies demonstrated the capacity of CXCR5+ CD4 T cells in human tonsils to have an enhanced ability to help B cells produce antibodies *in vitro* (152-154). Due to their localization and function, CXCR5+ CD4 T cells were then defined as Tfh cells (155). The highly dynamic interplay of Tfh cells with

germinal center B cells, follicular dendritic cells, regulatory T follicular helper cells (Tfr), and CD8 T cells have become an area of active research in the field of HIV. In particular, it has been observed that there is a noticeable accumulation of Tfh cells in the lymphoid tissue during chronic SIV and HIV infection and these cells contribute to on-going viral replication and production (156-160). Early work has established the germinal center as a site enriched in virus during HIV infection where follicular dendritic cells (FDCs) are saturated with virus (161, 162). FDCs are a cellular subset situated in most secondary lymphoid tissue and retain the capacity to trap and retain antigen on the surface of their dendrites(162), where these cells during HIV infection, can constantly expose Tfh cells to infectious HIV virions (161, 163, 164)There are many factors that contribute to the accumulation of Tfh cells after HIV infection and these include viral load, length of infection, percentage of Tfr cells, germinal center B cells, and CD8 T cells. Patients on long-term ART therapy have a reduction in the total frequency of Tfh cells suggesting that antigen load may drive the accumulation of these cells (88). Moreover, during chronic HIV infection, IL-6R expression increases on Tfh cells in tandem with the overall increase in IL-6 produced by FDCs that harbor HIV virus during chronic infection (165). IL-6R signaling is known to drive the expression of the inhibitory receptor programmed death receptor 1 (PD-1), a check-point inhibitory receptor implicated in chronic immune exhaustion during HIV infection (76). The expression of PD-1 is regulated through the phosphorylation and activation of the STAT3 signaling pathway (166-168). PD-1 is highly expressed on Tfh cells and the PD-1/ PD-L axis is thought to be important in regulating Tfh responses and B cell responses (169). Thus increased inflammation and high concentrations of IL-6 may contribute to the aberrant enrichment of Tfh cells in the follicles during HIV infection. Increased levels of IL-6 found in chronically HIV infected patients may also increase plasma cell survival further perturbing Tfh/B cell homeostasis and antibody production (169). During HIV infection there are a larger number of Tfh cells that produce IL-21, a cytokine important in the differentiation of B cells and in modulating antibody production. Higher frequencies of IL-21 producing Tfh cells may lead to

increased frequencies of low affinity germinal center B cells with impaired function, consequently leading to hypergammaglobulinemia and memory B cell loss, both immunological abnormalities observed during chronic HIV infection. A study by Cubas et al described an equally consistent pathologic situation in which increased PD-1 expression on Tfh cells leads to a defect in antibody production by B cells during chronic HIV infection. This study demonstrated that *in vitro* blockade of PD-L1 increased IL-21 production and enhanced B cell function, suggesting a more positive role persistent IL-21 may play during HIV infection(143). Kinetics of IL-21 production during HIV infection needs to be assessed to better understand when and how IL-21 modulates the B cell response.

Recently it has been shown that HIV replicates in Tfh cells, and it has been suggested that Tfh cells may harbor more actively replicating virus compared to non-Tfh cells, especially under conditions of complete ART suppression or elite controller status (88). It has been shown that replication may be enhanced by the production of cytokines such as TNF that is produced locally by FDCs in the germinal center (163). Increased levels of the protein TGIF1, which limits TGF-β- responsive gene expression, may also promote the accumulation of Tfh cells in the GC (169). This idea of poor regulation via suppressive cytokines may also work in concert with the Tfr subset, a regulatory T cell subset recently described to be reduced during chronic SIV/HIV infection (170). Tfr are thought to be of thymic origin and localize to the follicles and/or GC of lymphoid tissue to regulate Tfh/B cell responses. More recently, it has been hypothesized that germinal centers, thought to be primarily devoid of cytolytic CD8 T cells, may serve as a 'sanctuary' during SIV/HIV infection, It has been postulated that by residing in the GC, Tfh cells may subvert targeted killing by SIV specific CD8 T cells. Furthermore, a recent study has demonstrated that CD8 T cells may play a crucial role in maintaining the B cell follicle as a 'sanctuary' for on-going viral replication (171). In a study by Fukazawa *et al.,* it was observed that Tfh cells are the predominant population of CD4 T cells infected in elite viral controlling (EC) RM infected with nef-deleted $SIVmac_{239}$ compared to viral progressor RM infected with

wild-type SIVmac$_{239}$ (171). Moreover, depleting CD8 T cells in EC RM results in a significant re-distribution of viral replication to non-Tfh cells localized outside the GC proper. The major findings of this study suggest that CD8 T cells exert immune pressure to control viral infection in non-Tfh CD4 T cells outside of the GC during elite control of SIV infection, but have a limited effect on Tfh infection within the GC. Once CD8 T cells are removed, virus can replicate throughout the lymphoid follicle and contribute to ongoing viral production and persistence. A challenge in the field is to understand when and how Tfh cells are infected, in particular due to the low level expression of the viral co-receptor CCR5 required for entry. It is also not well understood how Tfh cells communicate with their surrounding cell partners and what functions carried out throughout the course of infection, or prior to infection post immunization, will modulate Tfh, Tfr, B cell, and CD8 T cell response in the GC. Although there has been considerable progress into understanding the importance of Tfh cells during chronic HIV infection, little is known about the development of these cells over the course of infection and when infection and dysfunction of these cells occurs. Studies need to be carried out to gain a better understanding of the optimal timing to modulate these responses in an effort to generate effective antibody responses capable of affording protection and/or viral control, and limit the generation of persistent Tfh viral targets during HIV infection.

CD8 T cells and HIV

CD8 T cells are thought to be the most essential T cell subset in controlling HIV replication early after HIV infection. Studies have demonstrated that the depletion of CD8 T cells early after SIV infection in rhesus macaque models of HIV infection is associated with an increase in viral replication that presents as up to log higher than plasma viral loads measured in the presence of CD8 T cells (172-176). The importance of CD8 T cells has been further demonstrated in elite controllers in which the most prominent difference between elite controllers and non-controllers is a dominant HIV specific CD8 T cell response primarily against the

dominant and highly conserved HIV epitope Gag (177-179). Differences in the phenotypic and genotypic level of HIV specific CD8 T cells between elite controllers and non-controllers is also observed in terms of the cytotoxic granules, such as granzyme, perforin, and granulysins produced by the CD8 T cells. T-bet (also known as TBX21), a transcription factor that binds to the promoter of both perforin and granzyme is also seen at higher levels in CD8 T cells from elite controllers compared to non-controllers (180, 181). Other functional responses that differentiate elite controllers from non-controllers are a higher proliferative response and greater polyfunctionality of these HIV specific CD8 T cells. CD8 T cells from elite controllers seem to also have enhanced production of the cytokine interleukin-2, which has been linked to the cytotoxic function of the CD8 T cells. The polyfunctionality, or simultaneous production of multiple cytokines, from CD8 T cells in elite controllers also may suggest a more self-renewing memory phenotype and a less terminally differentiated phenotype such as central memory versus effector memory, possibly allowing for better recall of functional CD8 T cells upon increased antigen exposure (146). In contrast, HIV progressors present with a more monofunctional CD8 T cell phenotype (145). There are also certain HLA class I alleles that have been associated with delayed HIV disease progression, and these include HLA-B*57 and B*27, with Mamu-B*17 as the respective protective MHC class I alleles in rhesus macaques (182). Mamu-B*17 is associated with a 26 fold reduction in plasma viral loads in SIV infected rhesus macaques (183). HIV specific CD8 T cells restricted by the HLA alleles HLA-B*57 and B*27 have shown to be less susceptible to the inhibitory effects of regulatory T cells (Treg) likely due to lower T cell immunoglobulin and mucin domain containing protein 3 (TIM3) (184). Furthermore, it has also been demonstrated that these protective alleles have a better ability to present several of the immunodominant epitopes such as Gag (185, 186). CD8 T cells specific for Gag have been inversely correlated with plasma viral loads (186, 187). Despite evidence suggesting better control of viral replication in animals with Gag specific CD8 T cell responses, progressors also generate strong anti-Gag CD8 T cell responses and some macaque SIV infection studies have

shown that control of viral replication can be established without an SIV Gag specific response (188). The variability in anti-HIV SIV specific CD8 T cell responses suggest that a broad CD8 T cell response is more likely required to generate effective control of HIV. A recent study in Nature by Deng *et al*. highlights the need for a broad CTL response to effectively target and clear the latent reservoir in patients on ART (189). There have also been several studies conducted to understand the complexities of T cell receptor (TCR) clonotypes and their association with viral control and have found that TCR clonotypes isolated from patients that control HIV infection seem to retain more cytolytic effects compared to non-progressors despite all other functional properties of the cells in both groups being the same (190). This observation suggests that the specific interactions and possibly the affinity between the CD8 TCR and the MHC class I complex may determine HIV specific CD8 T cell mediated control of HIV infection. It still remains unknown whether polyfunctional CD8 T cell responses in elite controls arise as a defense against HIV infection or as a result of immune control of infection. Individuals that remain on long-term ART regain some of these polyfunctional CD8 T cell characteristics (191) observed in elite controllers suggesting that these polyfunctional CD8 T cell responses from elite controllers may be a consequence of immune control of HIV replication and infection.

Immune Exhaustion and Check-point Inhibition

The balance between co-stimulatory and co-inhibitory signals received from an antigen-presenting cell (APC) dictates the T cell response. Immune exhaustion was first described in a mouse model of lymphocytic choriomeningitis virus (LCMV) infection in late 1990's (192) and was subsequently investigated in other infection models such as chronic SIV/HIV infection and hepatitis B and C infection. Currently, "immune exhaustion" is defined as a state in which a memory cell has lost both effector functions and regenerative capacity. Multiple studies have suggested that immune exhaustion is a response that contributes to viral persistence during chronic infection. In particular, during the course of HIV/SIV infection, immune exhaustion is

thought to be an inevitable consequence of both long-term infection and persistent and uncontrolled antigen exposure leading to decreased anti-viral cellular responses. A molecular signature has been found to be indicative of immune exhaustion and this signature includes the expression of co-inhibitory receptors such as programmed-death 1 (PD-1), cytotoxic t lymphocyte-associated protein 4 (CTLA-4), T cell immunoglobulin mucin-3 (Tim-3), and lymphocyte-activation gene 3 (LAG-3)(193).

PD-1 was the first inhibitory receptor that was associated with immune exhaustion. PD-1 is expressed on T cells, B cells, activated monocytes, and NK cells. Its ligands are PD-L1 (B7-H1; CD274) expressed on most hematopoietic and non-hematopoietic cells and PD-L1 (B7-DC, CD273), which is restricted mainly to professional antigen presenting cells. Barber et al first described PD-1 as an important mediator for antigen specific CD8 T cell dysfunction during chronic LCMV infection, and it was shortly thereafter demonstrated to be important in contributing to immune exhaustion in both SIV and HIV infection. *In vivo* PD-1 blockade during chronic LCMV infection resulted in both a reduction in viral titers and restoring the function of LCMV specific CD8 T cell responses, highlighting the important role PD-1 plays in regulating the function of anti-viral LCMV specific CD8 T cell responses. Although PD-1 is the highly expressed on both anti-viral CD4 and CD8 T cells during HIV infection, other co-inhibitory molecules such as LAG-3, Tim-3, and CTLA-4 can also contribute to dysfunctional cellular responses in the absence of PD-1 expression. Multiple studies carried out in the late 2000's elucidated the role of PD-1 on anti-viral CD8 T cells, but its effects on anti-viral CD4 T cells were less well understood. Earlier studies by Day and colleagues demonstrated that in addition to being expressed on dysfunctional CD8 T cells during HIV infection, PD-1 was also expressed on CD4 T cells that exhibited diminished proliferative capacity and cytokine production(194). A more recent study by Said *et al*. revealed that PD-1 engagement by PD-L1 on monocytes induced the production of IL-10 and reversed CD4 T cell dysfunction associated with chronic HIV infection. Moreover, they showed that TLR ligands such as lipopolysaccharide (LPS), lipotechoic

acid, and CpG DNA resulted in a dose dependent increase in the level of PD-1 expression on monocytes, whereas HIV ssRNA from a CCR5 or CXCR4 tropic virus failed to cause upregulation of PD-1(195). It has also recently been shown that chronic immune activation is a result of microbial products that have translocated into the lumen due to disruption of the mucosal barrier. Said and colleagues tested whether there was any direct association between translocation of microbial byproducts that can act as TLR ligands and PD-1 expression, but found no direct association. They did find increased expression of PD-1 and production of IL-10 from monocytes incubated with sera isolated HIV viremic individuals, but there was no direct connection between PD-1 and IL-10 or plasma LPS levels (195).

More recently, data has emerged on the importance of PD-1 CD4 T cells during chronic infection is its contribution to the maintenance of viral reservoirs. Chomont and colleagues in 2009 assessed PD-1 expression on CD4 T cells and its association with the total level of latent virus found in long-term ART suppressed patients (196). Interestingly, this study found that not only was PD-1 more highly expressed on central memory CD4 T cells in chronically infected ART suppressed patients, but these cells were also the highest cellular contributors to the total reservoir identified in these patients under ART. Furthermore, these cells were found to undergo homeostatic proliferation proliferative in response to IL-7, suggesting that PD-1 may play an active role in the maintenance of the viral reservoir under ART(196). Recent studies have also shown high expression of PD-1 on Tfh cells (157-160, 197) and these PD-1^{hi} Tfh cells have been shown to accumulate in lymphoid tissue during chronic SIV/HIV infection. Moreover a recent study demonstrated the importance of PD-1 on Tfh cells in regulating B cell responses during chronic HIV infection. In this study, Cubas et al. showed that impaired B cell responses observed during chronic HIV infection may result from inadequate Tfh help due to PD-1 signaling on these cells (143). This study shows that engaging the PD-1 axis on Tfh cells leads to impaired proliferation and IL-21 production by Tfh cells, and blocking this pathway on Tfh cells leads to enhanced HIV specific immunoglobulin production *in vitro* (143).

Tim-3 is another important negative regulator that has been actively studied during HIV infection. Tim-3 is highly expressed on Th1 but not Th2 cells and its ligand galectin-9 was demonstrated in a mouse model in which Tim-3 expressing CD4 T cells that engaged with galectin-9 resulted in cell death via calcium flux (198-200). Interfering with the Tim-3/galectin-9 axis through Tim-3 blockade results in hyperproliferation of Th1 CD4 T cells (201). In humans, Tim-3 is expressed on Th1 cells, cytotoxic T cells and is constitutively on macrophages and dendritic cells and was also found to regulate Th17 cells, contributing to enhanced pro-inflammatory cytokine production and IL-17 production in the presence of a Tim-3 blocking antibody (202-204). Multiple studies have demonstrated an observed increase in the frequency of Tim-3 expressing anti-viral CD8 T cells during chronic SIV/HIV infection, and the Tim-3+ anti-viral CD8 T cells fail to produce cytokine or proliferate in response to antigen(205-207). Blocking the Tim-3 pathway *in vitro* in PBMCs isolated from chronically HIV infected patients resulted in restored proliferation and enhanced polyfunctionality (205). Tim-3 expression on anti-viral CD4 and CD8 T cells has been associated with increased disease progression, higher viral loads, increased immune activation, demonstrated by phenotypic expression of both CD38 and HLA-DR and decreased polyfunctionality. LAG-3 was also found to be upregulated on T cells during HIV infection and correlated with HIV viral load. Furthermore, a more recent study by Jones *et al*. studied the co-expression of PD-1 and Tim-3 isolated from a population of chronically infected HIV progressors and found that a small population of PD-1+Tim3+ cells had greater dysfunction than their single positive cellular counterparts (205). Another LCMV infection study by Jin *et al*. demonstrated that PD-1 and Tim-3 co-expression was only transiently upregulated on a small subset of anti-viral CD8 T cells during acute LCMV infection but persisted during chronic LCMV infection (208). Furthermore, the population of Tim3+PD-1+ cells found during chronic infection lacked proliferative capacity and retained minimal polyfunctionality, suggesting that these double positive cells may represent the most dysfunctional subset of antigen specific CD8 T during chronic infection(209). Tim-3 and PD-1

co-blockade resulted in restoration of T cell function and reduction in viral loads(209) suggesting the importance of studying these co-inhibitory axes during chronic HIV infection.

LAG-3, is a member of the immunoglobulin superfamily, and is found on activated Th1 cells. During chronic HIV infection, LAG-3 is significantly upregulated on CD4 and CD8 T cells in the peripheral blood and lymph nodes. The frequency of LAG-3+ CD4 and CD8 T cells correlates with progression of disease during HIV infection (4). Moreover, LAG-3 expression does decrease during ART treatment suggesting that chronic antigen exposure drives increased expression(67). *Ex vivo* blockade of LAG-3 blockade on CD4 and CD8 T cells isolated from HIV infected individuals resulted in enhanced HIV specific T cell responses (4). PD-1 and LAG-3, both check-point inhibitors highly expressed on anti-viral CD4 and CD8 T cell responses during chronic HIV infection, can serve as important immunological targets for check-point blockade during HIV infection and ART suppression. Dual blockade of both inhibitory receptors may potentially result in a more dramatic reconstitution of T cell immune responses; improving clinical outcomes in chronically HIV infected ART suppressed patients.

CTLA-4 is another negative regulatory of the B7-CD28 family and has been shown to have an impact on T cell function during HIV infection. Studies have shown that CTLA-4 expression was more highly expressed on CD4 T cells isolated from HIV infected individuals with progressive disease, reduced CD4 T cell counts, and higher plasma viral loads (210). CTLA-4 expression was also more highly expressed on HIV specific CD4 T cells during most stages of HIV infection except in individuals that control infection in the absence of anti-retroviral therapy (long-term nonprogressors or viral controllers)(5, 210, 211). CTLA-4 unlike PD-1 is not highly expressed on HIV specific CD8 T cells (210). Expression of CTLA-4 was higher on monofunctional HIV specific CD4 T cells producing mainly IFN-γ compared to those cells that produced both IFN-γ and IL-2 (210). Moreover, despite most HIV specific CD4 T cells co-expressing both CTLA-4 and PD-1, duel blockade of both these inhibitory receptors only provided modest effects on improving CD4 T cell proliferation and function and these results

were not consistent and highly variable (210). CD4 T regulatory cells express higher levels of CTLA-4 compared to effector CD4 T cells (211), but even with depletion of CD25+ CD4 Treg cells from the cell culture prior to testing CTLA-4 blockade *in vitro*, proliferation was not enhanced suggesting that in this study most of the T cell dysfunction of CD4 T cells via CTLA-4 expression is most likely due to negative effects on effector CD4 T cells and not through regulation of responses via CTLA-4 expressing Tregs (211). In contrast, in the context of autoimmunity, a more recent study by Jain and colleagues to better understand the role of CTLA-4 on regulatory T cells versus conventional T cells restricted CTLA-4 expression on regulatory but not conventional T cells. This experiment elegantly demonstrated that lack of CTLA-4 expression on regulatory T cells lead to uncontrolled activation and expansion of conventional T cells, albeit CTLA-4 expression on conventional T cells did restrict activated T cells from infiltrating non-lymphoid tissue. These data demonstrate the dynamic role of CTLA-4 on regulatory and conventional T cells, in that CTLA-4 exerts its role on T regulatory cells by restricting aberrant T cell activation, where as on conventional T cells, CTLA-4 is important for limiting infiltration of damaging T cells to non-lymphoid sites (212, 213).

These data highlight the importance of better understanding the synergy between blocking different check-point inhibitors on T cells during chronic HIV infection and support the need to further understand the benefits of co-inhibitory blockade's effects on not only enhancing anti-viral CD8 T cells and functional germinal center responses but also limiting persistent viral reservoirs.

Summary

In the following chapters I identify an important CD4 T cell population that contributes to viral pathogenesis during chronic SIV infection. In assessing a cohort of SIVmac251 intra-rectally infected rhesus macaques for 24-44 weeks post infection, I was able to study the phenotype, function, localization, and contribution of PD-1+ Tfh cells to chronic SIV infection. Furthermore,

through immunofluorescence microscopy, I visualized the localization of these Tfh cells in the secondary lymphoid organs, such as the LN, spleen, and rectum, important sites known to contribute to ongoing viral replication and persistence, and the interplay of Tfh cells with anti-viral CD8 T cells, which are critical to immune control of SIV. Using *in situ* tetramer staining, I was able to assess the location of SIV specific CD8 T cells at sites enriched in PD-1^{hi} Tfh cells and how this relationship developed kinetically overtime. Delineating the cohort of SIV infected macaques into three separate groups, unvaccinated non-controllers, vaccinated- non-controllers, and vaccinated controllers based on plasma viral loads greater than or less than 10^4 copies/mL respectively, allowed me to better understand the dynamics between Tfh cells, anti-viral CD8 T cells localized to lymphoid sites (SIV specific CD8 T cells that co-expressed CXCR5 a chemokine receptor integral for homing to B cell follicles), and viral control. Fluorescence activated cell sorting (FACS sorting) of different PD-1+ CD4 T cell subsets demonstrated PD-1+ CD4 T cells as an active cellular contributor to on-going viral replication during chronic SIV infection and the frequency of CXCR5+ anti-viral CD8 T cells inversely correlated with plasma viral loads at both peak at set-point and the infection status of PD-1^{hi} Tfh cells.

PD-1 expression is inextricably linked to immune exhaustion on CD8 T cells and the persistence of antigen during chronic infections, such as SIV/HIV. *In vivo* PD-1 blockade during acute and chronic SIV infection augmented anti-viral CD8 T cell responses, SIV specific humoral responses, and provided transient control of plasma viremia(214). To better assess what effects PD-1 blockade would have in tandem with ART therapy, I subsequently carried out an *in vivo* therapeutic trial in which I tested the efficacy of PD-1 blockade as an adjunct therapy to ART. This trial allowed us to test whether interfering with the PD-1 pathway on both anti-viral CD4 and CD8 T cells synergized with ART to provide more rapid control of viremia and whether targeted blockade of PD-1 on CD4 T cells destabilized or reduced the size of the memory CD4 T cell viral reservoir under suppressive ART. Taken together, the data from all three chapters highlights the importance of PD-1+ CD4 T cells during chronic SIV infection and the importance

of intra-follicular CD4 (Tfh) and CD8 (Tfc) T cells during pathogenic SIV infection. Our studies highlight the need for developing effective strategies capable of profoundly enhancing anti-viral immunity during suppressive ART and eliminating viral reservoirs to subsequently reduce the viral burden post treatment interruption. Our studies have important implications for functional cure studies for HIV.

Chapter 2

Diminished Viral Control during SIV Infection is Associated with Aberrant PD-1^{hi} CD4 T cell Enrichment in the Lymphoid Follicles of the Rectal Mucosa

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Published in *Journal of Immunology,* 2014, **(193)** 9

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RRA and VV are co-inventors of PD-1 technology that has been licensed to Genentech Inc., by Emory University. All other authors do not have any conflicting financial interests.

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Abstract

The inhibitory receptor Programmed Death-1 (PD-1) has been shown to regulate CD8 T cell function during chronic SIV infection, however its role on CD4 T cells, specifically in the gut associated lymphoid tissue, is less well understood. Here, we show that a subset of CD4 T cells express high levels of PD-1 (PD-1^{hi}) in the rectal mucosa, a preferential site of virus replication. The majority of these PD-1^{hi} CD4 T cells expressed Bcl-6 and CXCR5, markers characteristic of T follicular helper cells in the lymph nodes. Following a pathogenic SIV infection, the frequency of PD-1^{hi} cells (as a percent of CD4 T cells) dramatically increased in the rectal mucosa, however a significant fraction of them did not express CXCR5. Furthermore, only a small fraction of PD-1^{hi} cells expressed CCR5 and despite this low level of viral co-receptor expression a significant fraction of these cells were productively infected. Interestingly, vaccinated SIV controllers did not present with this aberrant PD-1^{hi} CD4 T cell enrichment and this lack of enrichment was associated with the presence of higher frequencies of SIV-specific granzyme B+ CD8 T cells within the lymphoid tissue, suggesting a role for anti-viral CD8 T cells in limiting aberrant expansion of PD-1^{hi} CD4 T cells. These results highlight the importance of developing vaccines that enhance anti-viral CD8 T cells at sites of preferential viral replication and support the need for developing therapeutic interventions that limit expansion of SIV+ PD-1^{hi} CD4 T cells at mucosal sites as a means to enhance viral control.

Introduction

The humoral and cellular immune responses are critical for the control of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) infections. The CD4 T cells play a key role in regulating the magnitude and function of humoral and cellular immunity (22, 172, 215-217). HIV preferentially infects virus specific CD4 T cells, with memory CD4 T cells being the primary target of HIV infection (22, 215). During acute HIV/SIV infection, massive depletion of memory CD4 T cells occurs predominantly at mucosal sites, with over one-half of all memory CD4 T cells in SIV-infected rhesus macaque (RM) being destroyed directly by viral infection. Virus-specific CD8 T cells are induced during acute infection and are important in the containment of viral replication (172, 217). CD4 T cell help has also been shown to play a vital role in the control of HIV infection, as individuals capable of controlling virus to low or undetectable levels maintain a high frequency of HIV specific CD4 T cells with high functional avidity (218-220). Additionally, depletion of CD4 T cells during acute SIV infection leads to abrogation of initial post-peak viral decline (221).

In the setting of chronic infection, T cells have been shown to upregulate the inhibitory receptor programmed death-1 (PD-1), as well as other inhibitory receptors such as CTLA4, LAG-3, Tim-3 and 2B4 (193, 205, 208, 210, 222, 223). Sustained expression of these inhibitory receptors has been associated with immune dysfunction in murine (19, 20), non-human primate (124, 224-227), and human model systems (124, 208, 222, 228). In the context of chronic HIV and SIV infections, it has been well established that there is an appreciable increase in both the frequency and expression of PD-1 on anti-viral CD8 T cells and a preferential depletion of PD-1+ B cells. PD-1+ antigen specific CD8 T cells exhibit impaired proliferation, decreased antigen specific cytokine production, and compromised survival (214, 224, 225, 229). Alternatively, *in vivo* blockade of PD-1 enhances anti-viral CD8 T cell function and viral control (214, 227, 230). Despite the comprehensive characterization of PD-1 on CD8 T cells during chronic SIV/HIV

infection, the role of PD-1 on CD4 T cells has received far less attention in the context of viral infection, specifically in sites of preferential viral replication.

Preliminary studies of PD-1 on CD4 T cells during chronic HIV infection have shown that the frequency of PD-1+ CD4 T cells in the blood correlates with plasma viral load and decreased CD4 T cell counts, and that subsequent *in vitro* PD-1 blockade of peripheral blood mononuclear cells can augment proliferative capacity of virus-specific CD4 T cells (194, 223). It is known that follicular helper CD4 T cells (Tfh) in the lymphoid tissue express high levels of PD-1 (231-233). Recent studies have demonstrated that the frequency of PD-1^{hi} Tfh cells increase significantly in lymph nodes (LN) of HIV-infected humans and SIV-infected non-human primates (NHP) during the chronic stage (88, 157, 159, 197). The reasons for this increase are not yet fully understood. While human studies suggested a direct relationship between the frequency of PD-1+ or Tfh cells and plasma viremia, this association was not observed in NHP studies. Petrovas et al. demonstrated a direct relationship between higher sCD14 levels in plasma and the frequency of Tfh cells suggesting a role for microbial translocation in the gut in regulating Tfh cells in the lymphoid tissue. However, there is no information available on the status of PD-1^{hi} CD4 T cells in the gut, a preferential site of virus replication in HIV-infected humans or SIV-infected NHP, and a site that is constantly exposed to high levels of pathogenic and non-pathogenic bacteria. In addition, it is not clear if vaccine-elicited CD8 T cells have any effect on PD-1^{hi} or Tfh cells in the LN and rectum following SIV infection.

In this study, to understand the influence of chronic SIV infection on PD-1+ CD4 T cells in the gut of RM, we studied the PD-1 expression on CD4 T cells in the rectal mucosal tissue (rectum) and compared it with LNs in the context of SIV-naïve, chronic uncontrolled SIV infection, and vaccine-mediated controlled SIV infection. Our results showed a preferential increase in the frequency of PD-1^{hi} CD4 T cells in the rectum and LNs of uncontrolled SIV infection and revealed important differences between rectal mucosa and LNs.

Materials and methods

Animals. Young adult rhesus macaques from the Yerkes breeding colony were cared for under the guidelines established by the Animal Welfare Act and the National Institute of Health (NIH) *Guide for the Care and Use of Laboratory Animals* using protocols approved by the Emory University Institutional Animal Care and Use Committee. Non-controllers were either unvaccinated or received a DNA/MVA SIV vaccine, and all vaccine-controllers received the DNA/MVA SIV vaccine (234, 235). All animals were infected with SIVmac251 intra-rectally.

Immunizations and Infections. Indian-origin rhesus macaques (*Macaca mulatta*) were unvaccinated or vaccinated with DNA/MVA SIV vaccine (DM). Vaccination consisted of two DNA primes on weeks 0 and 8, and two MVA boosts on weeks 16 and 24. Both DNA and MVA immunogens expressed SIV239 Gag, Pol and Env as described previously (236) Vaccinated animals either received DM vaccine alone (n=3), the CD40L adjuvant during DNA prime and MVA boosts (DM40L) (n=7), or rapamycin for 28 days during each of the MVA boosts (DMRapa) (n=19). All animals were challenged weekly with SIVmac251 starting 21-24 weeks after the final MVA immunization with a dose of 647 $TCID_{50}$ (1.25×10^7 copies of viral RNA) until they were productively infected. All animals were infected by 7 challenges under these conditions. Please see Table 1 for additional details. Dr. N. Miller (NIH, Bethesda, MD) provided the challenge stock. The criteria for defining controllers and non-controllers were based on a plasma viral load cut-off of 10^4 RNA copies/mL of plasma at 24 weeks post infection (237). SIV RNA levels were determined using a qPCR (238).

Isolation of mononuclear cells. Mononuclear cells were isolated from the blood, axillary lymph nodes, and rectal tissue, and flow cytometry analysis was performed as described previously (224). Briefly, peripheral blood mononuclear cells were isolated from blood collected in CPT tubes. Mononuclear cells from the lymph nodes were isolated from axillary and mesenteric lymph

nodes processed in complete medium and ACK lysed to remove residual red blood cells. Mononuclear cells from the rectum were isolated after tissue was digested for 2 hours in complete medium with 10% fetal bovine serum, 1% Penicillin/streptomycin, 0.05% Gentamycin, 1% Hepes, 200U/mL of Collagenase IV (Worthington, Lake Wood, NJ), and DNase I (Roche, Indianapolis, IN). Digested tissue was then passed through decreasing size needles (16-, 18-, and 20-gauge, five to six times).

Antibodies. The following antibodies were used. FITC conjugated Bcl-2 (clone Bcl-2/100; BD Biosciences), PE-conjugated CXCR5 (clone MU5UBEE; eBioscience), PerCP conjugated CD3 (Clone SP-34-2; BD Biosciences), PeCy7 conjugated CD28 (Clone CD28.2; eBioscience), PE-TR conjugated CD95 (clone; BD Biosciences), Brilliant Violet 421- conjugated CD279 (PD-1; Clone EH12.1; Biolegend), V500 conjugated CD8 (Clone SK1; BD Bioscience), APC conjugated CCR5 (Clone 3A9, BD Biosciences), Live Dead-IR stain (Invitrogen), Alexa700 conjugated Ki-67 (Clone B56; BD Biosciences), FITC conjugated Bcl-6 (Clone K112-91; BD Biosciences), Brilliant Violet 650 conjugated CD4 (Clone OKT4; Biolegend), FITC conjugated IL-17A (Clone eBio64Dec15; eBioscience), PE conjugated IL-21 (Clone 3A3-N2; BD Biosciences), PerCP-conjugated CD4 (Clone L200; BD Biosciences), PeCy7 conjugated anti-CD279 (PD-1; clone EH12.1; Biolegend), PacBlue conjugated CD3 (Clone SP-34-2; BD Biosciences), APC conjugated IL-2 (Clone MQ1-17H12; BD Biosciences), Alexa700 conjugated IFNγ (Clone B27; BD Biosciences).

Intracellular cytokine staining. Fresh blood, lymph node, and rectal samples were suspended in RPMI medium (Gibco, Life Technologies) with 10% FBS (HyClone, Thermo Fisher Scientific), 100 IU/mL of penicillin, and 100 μg/mL of streptomycin (Lonza). Stimulations were conducted in the presence of anti-CD28 antibody and anti-CD49d antibody (1μg/ml; BD Pharmingen). One

million cells were stimulated with either 200ng/ml of PMA and 1ug/mL of Ionomycin, CD3/CD28 beads (at a 1:2 ratio beads to cells; Miltenyi Biotech), or pooled peptides spanning the entire SIV Gag protein (single pool of 125 peptides with each peptide at a concentration of 1.0ug/mL; NIH AIDS Research and Reference Reagent Program catalog number 6204) in the presence of Brefeldin A (5 μg/mL; Sigma) and GolgiStop (0.5 μL/mL; BD Pharmingen) after 2 hours of stimulation for 4 hours at 37°C in the presence of 5% CO_2. At the end of stimulation, cells were washed once with FACS wash (PBS containing 2% FBS and 0.25g of sodium azide) and surface stained with anti-CD3, anti-CD8, anti-CD95, and anti-CD279 (PD-1) at room temperature for 20 min. Cells were then fixed with cytofix/cytoperm (BD Pharmingen) for 20min at 4°C and washed with Perm wash (BD Pharmingen). Cells were then incubated for 30 min at 4°C with antibodies specific to IL-2, IL-17A, IFNγ, IL-21, and CD4, washed once with Perm wash, once with FACS wash, and re-suspended in PBS containing 1% formalin. Cells were acquired on LSR-Fortessa with four lasers (205,288, 532, 633nm) and analyzed using the FlowJo software (Treestar Inc. CA). At least 50,000 events were acquired for each sample.

Phenotyping. Mononuclear cells isolated from the blood, lymph node, and rectum were stained with LIVE/DEAD Near-IR Dead Cell stain (Life Technologies) at room temperature for 15 min in PBS to stain for dead cells. Cells were then washed with FACS wash and stained on the surface using antibodies specific to CD3, CD4, CD8, CD28, PD-1, CD95, CXCR5, CCR5, and then treated with 1x BD FACS Lysing solution for 10min at room temperature, permeabilized with 1x BD Permeabilizing solution for 10 min at room temperature, washed with FACS wash, stained with anti-Ki67, anti-Bcl-6, and anti-Bcl-2 antibodies, washed 2x with FACS wash, and assessed by flow cytometry.

Immunofluorescence staining. Rectal tissues were fixed in SafeFix (Fisher Scientific), and embedded in paraffin. Embedded tissue blocks were cut into five microsections, deparaffinized, and rehydrated for immunohistochemical analysis. Some tissues also embedded unfixed in O.C.T medium (TissueTek, Sakura, Finetek, Torrance, CA) for CD8 staining. Immunofluorescence staining was performed for CD20, CD4, CD8, and PD-1 to examine the distribution of PD-1^{hi} cells in tissues, as previously described (239). In brief, heat-induced epitope retrieval was performed with DIVA Decloaker and then blocked with the SNIPER reagent (Biocare, Walnut Creek, CA) for 15 min and in PBS/0.1% triton-X100/4% donkey serum for 30 min at room temperature. Subsequently, the sections were incubated with rabbit anti-human CD20 (Thermo scientific, Rockford, IL), mouse anti-human CD4 (clone BC/1F6, Abcam, Cambridge, MA), mouse anti-human CD8 (clone LT8, Abcam, Cambridge, MA), and goat anti-human PD-1 (R&D system, Minneapolis, MN) antibodies diluted 1:20 to 1:100 in blocking buffer for 1h at room temperature. Thereafter, the sections were incubated with secondary antibodies (Alexa fluor 488/Cy3/Cy5 conjugated appropriate donkey anti-mouse/rabbit/rat/goat antibodies; Jackson ImmunoResearch, West Grove, PA) diluted 1:1000 in blocking buffer for 30 min at room temperature. For the frozen tissues, five-micron-thick sections were fixed with 4% paraformaldehyde for 10 min, followed by washing in 1x TBS buffer (Biocare Medical, Concord, CA). The same process was then performed without heat-induced epitope retrieval. Finally, the sections were mounted in warm glycerol gelatin (Sigma, St Louis, MO) containing 4-mg/ml n-propyl gallate (Fluka, Switzerland). Between each step, the sections were washed three times. All images were acquired and analyzed with an Axio Imager Z1 microscope (Zeiss) using various objectives.

Cell Sorting. Mononuclear cells isolated from the lymph node and rectal tissue were processed and stained with anti-CD3, anti-CD279 (PD-1), anti-CD95, and anti-CD8 for 25 minutes at 4°C

and the CD95+ PD-1^{neg}, CD95+ PD-1^{int}, and CD95+ PD-1^{hi}, and CD95- (naïve) CD4 T cell populations were sorted using a FACSAriaII (BD). In all sorting experiments, the grade of purity on the sorted cells was >93%. SIV RNA levels were determined using a qPCR (238).

***In vitro* killing Assay.** Mononuclear cells isolated from the LN of SIV infected Mamu A*01+ SIV controller RM were processed, stained with Live/Dead IR, anti-CD3, anti-CD4, anti-CD8, anti-CD95, and anti-CXCR5 antibodies, and sorted for CD95+ CD8 T cells and CD95+ CXCR5^{hi} CD4 T cells (Tfh cells) using a FACSAriaII (BD). Tfh cells were then pulsed with P11c peptide for 1 hour at 37°C at a concentration of 0.1 μg/mL and washed. CD8 T cells were co-cultured with unpulsed or pulsed Tfh cells at a 2:1 ratio of CD8 T cells to Tfh cells with no stimulation or anti-CD3/CD28 stimulation at 1 bead to 2 cells (Miltenyi Biotech) for 5 days. Cells were then harvested and analyzed using flow cytometry.

Statistical analysis. Statistical analyses were performed using Prism (version 5.0d; GraphPad Software Inc.). Statistical significance (p-values) was obtained using non-parametric Mann-Whitney test (for comparisons between groups/subsets) or Spearman rank test (for correlations). Statistical analyses of global cytokine profiles were performed by partial permutation tests using SPICE software (NIAID, NIH) as previously described (240).

Results

PD-1^{hi} CD4 T cells are predominantly found at preferential sites of SIV replication in SIV-naïve RM

To understand the role of PD-1 on CD4 T cells during chronic SIV infection, we characterized PD-1 expression in the rectum compared to the LN and peripheral blood of SIV-naïve RM. We observed three subsets of PD-1 expressing memory (CD95+) CD4 T cells namely PD-1^{neg}, PD-1^{int}, and PD-1^{hi} cells **(Fig. 2.1A)**. Interestingly, the PD-1^{hi} CD4 T cells were present predominantly in the rectum and LN, with 2-4% in the LN and 8-12% in the rectum, and less than 1% in the blood **(Fig. 2.1A)**. Thus, PD-1^{hi} memory CD4 T cells are enriched at sites of preferential SIV replication.

Because Tfh cells in the LN are known to express high levels of PD-1, we phenotyped the PD-1^{hi} CD4 T cells in the rectum for CXCR5 and Bcl-6, markers used to define Tfh cells in the LN **(Fig. 2.1B)**. Interestingly, the majority of PD-1^{hi} but not PD-1^{int} and PD-1^{neg} memory CD4 T cells in the rectum and LN expressed CXCR5 and Bcl-6 suggesting that PD-1^{hi} cells in the rectum phenotypically may predominately be Tfh cells. We also accessed PD-1 subsets for the level of CCR5 co-receptor expression. In contrast to PD-1^{int} cells that expressed high levels of CCR5, the majority of PD-1^{hi} cells in the rectum and LN did not express the viral co-receptor CCR5 suggesting that these may not be ideal targets for the virus **(Fig. 2.1C)**.

PD-1^{hi} CD4 T cells increase during uncontrolled SIV infection in the rectum and LN of RM

We next investigated the influence of SIV infection on PD-1^{hi} CD4 T cells in the rectum and LN of unvaccinated and vaccinated animals. For this purpose, we utilized samples from a cohort of SIV-infected RM that were either vaccine-controllers ($<10^4$ RNA copies/mL of plasma at week 24 post infection) or non-controllers ($>10^4$ RNA copies/mL of plasma at week 24 post infection, that include both unvaccinated and vaccinated animals) **(Fig. 2.7A)**. Please note that all conclusions made below remained true even if we defined controllers based on a set point viral

load of less than 10^3 RNA copies/mL. As expected, the frequency of total CD4 T cells was significantly lower in the rectum of non-controllers compared to vaccine-controllers **(Fig. 2.7B)**. However, the frequency of PD-1^{hi} cells within the memory CD4 T cell compartment was dramatically higher both in the rectum and LN of non-controllers compared to SIV-naive and vaccine-controllers **(Fig. 2.2A)**. The frequency of PD-1^{hi} cells in the vaccine-controllers was comparable to the uninfected RM **(Fig. 2.2A)**. To get an estimate of the cell number, we expressed the frequency of PD-1^{hi} cells as a percent of total lymphocytes and found a significant increase in PD-1^{hi} CD4 T cells in the LN as a percent of lymphocytes **(Fig. 2.7C)**, but this was not observed in the rectum **(Fig. 2.7B)**. This finding suggests that despite a decrease in total memory CD4 T cells in the GALT, PD-1^{hi} CD4 T cells remain enriched at this site. Consistent with the increase in the frequency of PD-1^{hi} cells as a percent of memory, the MFI of PD-1 was higher on memory CD4 T cells in the non-controllers than in uninfected and vaccine-controllers (data not shown). Furthermore, the frequency of PD-1^{hi} cells in the LN and rectum correlated directly with plasma viremia **(Fig. 2.2C)**.

To understand the kinetics of expansion of PD-1^{hi} cells following infection, we followed the frequency of PD-1^{hi} cells longitudinally in the rectum of SIV non-controllers in a separate study and found a similar increase in PD-1^{hi} cells during the course of SIV infection, with the increase being observed as early as 2 weeks post infection **(Fig. 2.2C)**. These data demonstrated that despite the loss of total memory CD4 T cells, the PD-1^{hi} memory CD4 T cells are enriched at preferential sites of virus replication in uncontrolled chronic SIV infection very early after infection while interestingly, such enrichment is not seen in vaccine-controllers. We also characterized PD-1 expression in the jejunum of a small group of chronically SIV-infected animals, but we did not find PD-1^{hi} CD4 T cells (data not shown) at this site. There are two possible explanations for this observation: PD-1^{hi} CD4 T cells *are* depleted from the jejunum during chronic SIV infection or PD-1^{hi} CD4 T cells are not present in the jejunum due to a limited number of GALT structures in this region (241, 242).

Altered CXCR5 expression on PD-1^{hi} CD4 T cells in the rectum following SIV infection

Next, we investigated the expression of CXCR5 on the PD-1^{hi} cells following SIV infection in the rectum and LN to understand their Tfh phenotype and localization. In contrast to SIV-naive animals, a significant fraction of PD-1^{hi} cells in the rectum did not express the Tfh marker CXCR5 following SIV infection **(Fig. 2.3A)**. This was true for both non-controllers and controllers except that it was more pronounced in controllers. However, similar to SIV-naïve animals, the majority of PD-1^{hi} cells in the LN expressed CXCR5 although there was a small decrease in the controllers **(Fig. 2.3A)**. Although we observed a decrease for CXCR5 expression on PD-1^{hi} cells in the rectum of non-controllers, because the majority of memory CD4 T cells were PD-1^{hi} **(Fig. 2.2A)**, the overall frequencies of CXCR5+ and CXCR5- PD-1^{hi} cells within the memory CD4 T cell compartment was also higher in the non-controllers compared to uninfected RM **(Fig. 2.3B)**. Consistent with this increase in CXCR5+ PD-1^{hi} cells in the non-controllers by flow cytometry, immunofluorescence analysis of rectal tissue revealed B cell follicles with significantly higher density of PD-1^{hi} CD4 T cells in non-controllers compared to controllers **(Fig. 2.3C)** (239). We phenotyped CXCR5- and CXCR5+ cells for the expression of Bcl-6 in a limited number of SIV infected RM **(Fig. 2.3D)**. These analyses revealed that in the LN CXCR5- cells express lower levels of Bcl-6 compared to CXCR5+ cells however, interestingly in the rectum both the subsets seems to express Bcl-6 at similar levels. These results argue that the phenotype of Tfh cells in the rectum could be different from that of LN during chronic SIV infection and a thorough characterization of their localization and function is critical before they can be classified as Tfh based on CXCR5 and Bcl-6 expression.

PD-1^{hi} CD4 T cells retain survival potential, show enhanced proliferation and albeit decreased IL-2 production in vivo during chronic SIV infection

The enrichment of PD-1^{hi} cells in non-controllers could be due to increased proliferation of these cells while maintaining their survival potential, so we studied the expression of Bcl-2

(anti-apoptotic protein) and Ki-67 (marker for proliferating cells) *ex vivo*. The PD-1^{hi} memory CD4 T cells in the rectum and LN of non-controllers showed either comparable or higher levels of Bcl-2 expression **(Fig. 2.4A, Fig. 2.8)** and markedly enhanced Ki-67 expression compared to uninfected animals **(Fig. 2.4B)**. However, this was also true for vaccine-controllers suggesting that the observed higher proliferation or Bcl-2 expression of PD-1^{hi} CD4 T cells alone did not markedly contribute to their enrichment in non-controllers. These results demonstrated that the uncontrolled chronic SIV infection is associated with an enrichment of PD-1^{hi} cells at preferential sites of virus replication with preserved survival potential and high proliferation status.

To assess the cytokine production capability of these PD-1^{hi} memory CD4 T cells we stimulated cells isolated from the rectum and LN with either PMA/Ionomycin (non-TCR driven cytokine production) or anti-CD3/CD28 (TCR driven cytokine production) **(Fig. 2.4C)**. In general, a significant fraction of PD-1^{hi} cells failed to produce cytokines following anti-CD3/CD28 stimulation (data not shown). However, following stimulation with PMA/Ionomcyin, a significant fraction of PD-1^{hi} cells in the uninfected animals produced cytokines IFN-γ, IL-2, and IL-21 **(Fig. 2.4C)** and a small fraction produced IL-17 (data not shown). In the LN, they produced predominantly IL-2 followed by IL-21, IFN-γ and IL-17. However, in the rectum they produced predominantly IL-21 and IL-2 followed by IFN-γ. PD-1^{hi} cells in the non-controllers and vaccine-controllers largely maintained IL-21 production but non-controllers showed decreased production of IL-2 and IFN-γ. However, this defect was not observed in the rectum **(Fig. 2.4C)**. These results demonstrated that the PD-1^{hi} cells that accumulate at the preferential sites of virus replication during uncontrolled SIV infection maintain the potential to produce IL-21, which may contribute to hypergammaglobulinemia by aiding in the maintenance and proliferation of memory B cells. PD-1^{hi} cells show decreased production of IL-2 and IFN-γ during chronic uncontrolled SIV infection, possibly limiting the potential for the generation of functional antigen specific humoral responses at these sites. The failure of these cells to express cytokines

following TCR driven stimulation could be due to inhibition by PD-1 signaling and needs further investigation.

PD-1^{hi} CD4 T cells express low levels of CCR5 yet support ongoing viral replication during chronic SIV infection

We then assessed the expression of the viral co-receptor CCR5 on these cells to see if these cells can be preferentially infected and killed by the virus **(Fig. 2.5A)**. In general, only a small fraction (2-5%) of PD-1^{hi} cells expressed CCR5 in the uninfected animals and these levels were even lower in non-controllers. Interestingly, the fraction of PD-1^{hi} cells expressing CCR5 increased dramatically in the rectum of vaccine-controller compared to SIV naive and non-controller RM. As expected, the overall frequency of CCR5+ total memory CD4 T cells declined in the rectum and LN of SIV-infected non-controlling animals however this was not evident in controllers **(Fig. 2.5B).**

In an attempt to better understand the contribution of these PD-1^{hi} cells to viral production and persistence during chronic SIV infection, we further studied the infection status of these cells in the rectum and LN of non-controller animals **(Fig. 2.5C)**. The levels of viral RNA were significantly higher in PD-1+ cells than in PD-1 negative cells. Within PD-1+ CD4 T cells, viral RNA was present in both PD-1^{int} and PD-1^{hi} cells. To approximate the production of virus on a per cell basis, we determined the ratio of viral RNA to viral DNA and observed a significantly higher ratio in PD-1^{hi} cells in the rectum compared to PD-1^{int} and PD-1^{neg} cells **(Fig. 2.5C)**. Cell associated viral RNA and DNA were also found predominately in the PD-1+ CD4 T cell subsets in vaccine controllers compared to non-controllers, albeit at 50 to 100-fold lower levels likely due to lower plasma viremia (data not shown). These data demonstrate that a significant fraction of PD-1^{hi} cells in the rectum are productively infected during uncontrolled SIV infection and actively support viral production in lymphoid sites known to highly contribute to viral persistence.

Higher anti-viral CD8 T cells in the LN are associated with a reduction in CXCR5+ PD-1^{hi} CD4 T cells and better viral control

To understand the relationship between anti-viral CD8 T cells and the frequency of PD-1^{hi} cells we determined the frequency of Gag CM9 tetramer specific CD8 T cells in Mamu A01+ animals of our controllers and non-controllers. Previous studies estimated that a significant fraction of the total SIV-specific CD8 T cell response in Mamu A01 animals is directed to this single epitope during chronic phase (243) and the use of tetramer allows us to determine frequency of CD8 T cells independent of function. These properties make it easier to interpret the data. The frequency of tetramer+ CD8 T cells was significantly higher in the LNs of controllers than in non-controllers and interestingly this difference was not observed in the blood **(Fig.2.6A)** suggesting that LN resident CD8 T cells could have contributed to enhanced control in these animals. Furthermore, the frequency of granzyme B+ tetramer+ CD8 T cells was also higher in controllers than in non-controllers B **(Fig. 2.6A)**. In addition, the frequency of tetramer+ or granzyme B+ tetramer+ CD8 T cells correlated inversely with the frequency of total PD-1^{hi} and CXCR5+ PD-1^{hi} CD4 T cells **(Fig. 2.6B)**. These results strongly suggested that LN resident highly functional anti-viral CD8 T cells might limit the aberrant expansion of PD-1^{hi} as well as CXCR5+ PD-1^{hi} CD4 T cells during chronic SIV infection. The anti-viral CD8 T cells could mediate this effect either directly by killing the infected PD-1^{hi} CD4 T cells or indirectly by controlling the virus replication that in turn may limit their expansion. To gain more insight into the mechanism, we co-cultured anti-viral CD8 T cells and Gag CM9 peptide pulsed CXCR5+ PD-1^{hi} CD4 T cells obtained from three controller RM for 5 days and found that in 2 out of 3 animals anti-viral CD8 T cells limit the expansion of the CXCR5+ PD-1^{hi} CD4 T cells. Interestingly, the ability of CD8 T cells to limit the expansion of CD4 T cells was associated with higher frequency of Gag CM9 specific CD8 T cells at baseline in vitro **(Fig. 2.6C)**. In conclusion, these results strongly suggest that LN resident anti-viral CD8 T cells have the potential to kill the

CXCR5+ PD-1^{hi} CD4 T cells and highlight a role for these cells in limiting the aberrant expansion of PD-1^{hi} CD4 T cells in the vaccinated controllers in vivo.

Discussion

Depletion of CD4 T cells in the gut associated lymphoid tissue (GALT) is an important hallmark of SIV/HIV infection and recent studies have sought to investigate the immune responses and cellular populations affected in the mucosal sites during pathogenic infection. In the early 80's, germinal centers were considered as potential sites for long-lasting viral reservoirs and sanctuaries for viral recrudescence but many questions were left unanswered. Recently germinal centers of lymphoid sites, and in particular CD4+ Tfh cells in LN have emerged as an important population of interest during chronic HIV/SIV infection. In particular, several groups have described an increase in the frequency of CXCR5+ PD-1^{hi} Bcl-6+ Tfh cells in the LN during chronic SIV/HIV infection and these cells have been speculated to significantly contribute to B cell dysfunction and hypergammaglobulinemia observed during chronic infection (88, 143, 157, 197). Despite the existing knowledge of Tfh cells in the LN during chronic SIV/HIV infection, the existence of these cells and their contribution to SIV pathogenesis have not been studied in the mucosal tissue, one of the most important sites of preferential viral replication and persistence.

Our data demonstrate that lymphoid follicles in the rectum of RM with uncontrolled chronic SIV infection are highly enriched in actively proliferating PD-1^{hi} CD4 T cells that retain survival potential and harbor a significant fraction of virus-infected cells. This enrichment is impressive considering the widespread depletion of memory CD4 T cells in the gastrointestinal tract following SIV infection. Furthermore, a significant fraction of these PD-1^{hi} cells reside in B cell follicles of lymphoid aggregates in the rectum. Strategically, this seems to be an important mechanism by which these virus-infected PD-1^{hi} CD4 T cells can contribute to ongoing viral replication and persistence while avoiding anti-viral CD8 T cell responses, as it has been shown that germinal centers of LN may act as viral sanctuaries during SIV infection. In addition, these cells maintained their ability to produce IL-21, which may support the uncontrolled or constant

proliferation of memory B cells leading to B cell dysfunction and hypergammaglobulinemia commonly seen in chronic SIV and HIV infections (143).

With little known about PD-1^{hi} CD4 T cells residing in the mucosal tissue of SIV naïve and SIV infected RM, our study revealed some similarities and differences in immune responses in the rectal mucosa compared to the LN. In the absence of SIV infection, PD-1^{hi} memory CD4 T cells are present at higher frequencies in the rectum compared to the LN. This higher frequency in the rectum is most likely due to constitutive germinal centers present in the GALT, as a result of continued sampling of microbial antigens, which stimulate differentiation and maintenance of CD4 Tfh cells (244-246). Moreover, during chronic SIV infection, PD-1^{hi} cells increased as a frequency of total memory CD4 T cells in both the LN and rectum. This may be due to either preferential depletion of PD-1^{int} and PD-1^{neg} cells or preferential differentiation to PD-1^{hi} cells. Nevertheless, this happens in both compartments (LN and rectum). Although we only directly observed an enrichment of PD-1^{hi} CD4 T cells in the rectum during chronic uncontrolled SIV infection, we speculate that we would find a similar enrichment in other mucosal sites known to have a high density of GALT structures, such as the terminal ileum (242). In contrast to the LN, there was not an increase in the frequency of PD-1^{hi} cells as a percent of total lymphocytes in the rectum. This may likely be due to higher depletion of memory CD4 T cells in the GALT compared to LN during chronic uncontrolled HIV/SIV infection.

The increased frequency of PD-1^{hi} memory CD4 T cells in the LN and rectum both consistently associated with viral control, but interestingly we observed an increase in the frequency of CCR5+ PD-1^{hi} CD4 T cells and decrease in CXCR5+ PD-1^{hi} CD4 T cells in the rectum of vaccine controllers, an observation much less apparent in the LN. We can speculate that altered chemokine receptor expression may allow for differential homing of these PD-1^{hi} CD4 T cells to regions close to the periphery of the GC or outside the B cell follicle. This change in localization may allow for increased immune pressure directed at PD-1^{hi} CD4 T cells. Additionally, due to the enrichment of these PD-1^{hi} CD4 T cells in the rectum of SIV infected

non-controllers, as a result of memory CD4 T cell depletion, these PD-1^{hi} CD4 T cells seemed to express higher levels of Bcl-2, which was not observed in the LN. We can hypothesize that as a result of CD4 depletion, immune activation, and widespread epithelial damage in the GALT, these PD-1^{hi} cells that localized to germinal centers of rectal aggregates, may increase Bcl-2 expression to promote survival and persistence in the face of extensive immune dysregulation. Additionally, in contrast to LN PD-1^{hi} CD4 T cells in SIV non-controllers that experienced decreased cytokine poly-functionality, PD-1^{hi} CD4 T cells in the rectum seemed to retain their ability to produce IFN-γ and IL-21. PD-1^{hi} cells in the rectum, compared to PD-1^{int}, and PD-1^{neg} memory CD4 T cells, also seem to represent the most active cellular subset for ongoing viral production as they contained the highest RNA to DNA ratio per cell. These observations suggest that despite an observed aberrant enrichment of PD-1^{hi} CD4 T cells in both the LN and rectum of SIV non-controllers, site-specific immune response during chronic SIV infection may contribute to localized differences in PD-1^{hi} CD4 T cell subsets.

A critical finding of our study is that the vaccine-controllers do not show an enrichment of PD-1^{hi} CD4 T cells in the B cell follicles of rectum or LN. We speculate that multiple mechanisms could have contributed to this outcome. We observed an increase in CCR5 expression and a decrease in CXCR5 expression on PD-1^{hi} CD4 T cells in the controllers. This shift in chemokine receptor expression could promote T cell migration away from the GC area of B cell follicles towards T cell zones leading to enhanced killing by cytotoxic CD8 T cells. A recent study demonstrated that the Tfh cells move from one GC to another GC within a lymph node (247). Therefore another plausible mechanism that prevents the aberrant enrichment of these PD-1^{hi} cells is that before reaching the GC of lymphoid follicles, these PD-1^{hi} cells are targeted and killed by anti-viral CD8 T cells. In addition, the controllers maintained higher frequency of granzyme B+ anti-viral CD8 T cells that correlated inversely with the frequency of PD-1^{hi} CD4 T cells. It is possible that these CD8 T cells restrict virus replication in PD-1^{hi} CD4 T cells through cytolytic as well as non-cytolytic mechanisms. Furthermore, our ongoing work suggests that

some of the anti-viral CD8 T cells express CXCR5 and thus could migrate to B cell zone (data not shown).

Thus, our results highlight the importance of SIV specific CD8 T cells at sites of ongoing viral replication and persistence. Our results also suggest the role of functional anti-viral CD8 T cells in limiting the aberrant enrichment of SIV+ PD-1^{hi} CD4 T cells at lymphoid sites during chronic uncontrolled SIV infection. Finally, It is conceivable that manipulating the localization of Tfh and anti-viral CD8 T cells to and from the germinal center may enhance immune mediated control of HIV/SIV infected target cells. These data highlight the importance of generating strong and potent anti-viral CD8 T cells at sites of active viral replication and persistence and support the rationale for using targeted therapies that promote Tfh migration out of the GC to allow for increased clearance by anti-viral CD8 T cells and enhanced viral control.

Acknowledgements

We thank the veterinary staff at Yerkes for animal care; CFAR virology core for viral RNA and qPCR analysis; and CFAR immunology core for help with flow cytometry. Also, we thank the NIH AIDS Research and Reference Reagent Program for the provision of peptides.

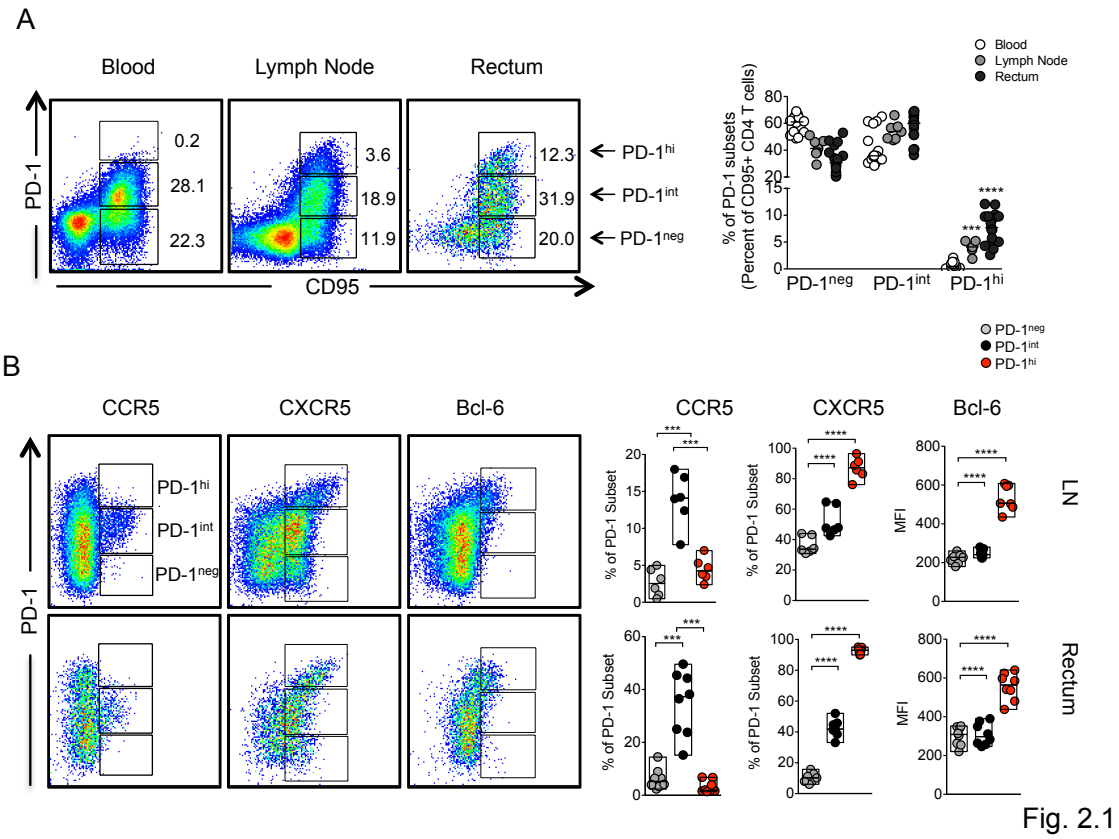

Fig. 2.1

Figure 2.1. Phenotypic characterization of PD-1+ subsets in the blood, lymph node (LN), and rectum of healthy rhesus macaques (RM). (A) Flow cytometric analysis of mononuclear cells isolated from the blood, LN, and rectum of a healthy rhesus macaque. Plots show live CD3+ CD4+ CD95+ PD-1^{neg}, PD-1^{int}, and PD-1^{hi} lymphocytes and scatter plot shows the cumulative data for a group in blood (n=16), LN (n=7), and rectum (n=17). **(B)** Representative histogram plots of CXCR5, BCl-6, and CCR5 expression overlaying PD-1^{neg}, PD-1^{int}, and PD-1^{hi} CD95+ CD4 T cells in the rectum (n=8) and LN (n= 7, 7, and 6, respectively for CXCR5, BCl-6, and CCR5) of healthy SIV naïve RM. Scatter Plots show median. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

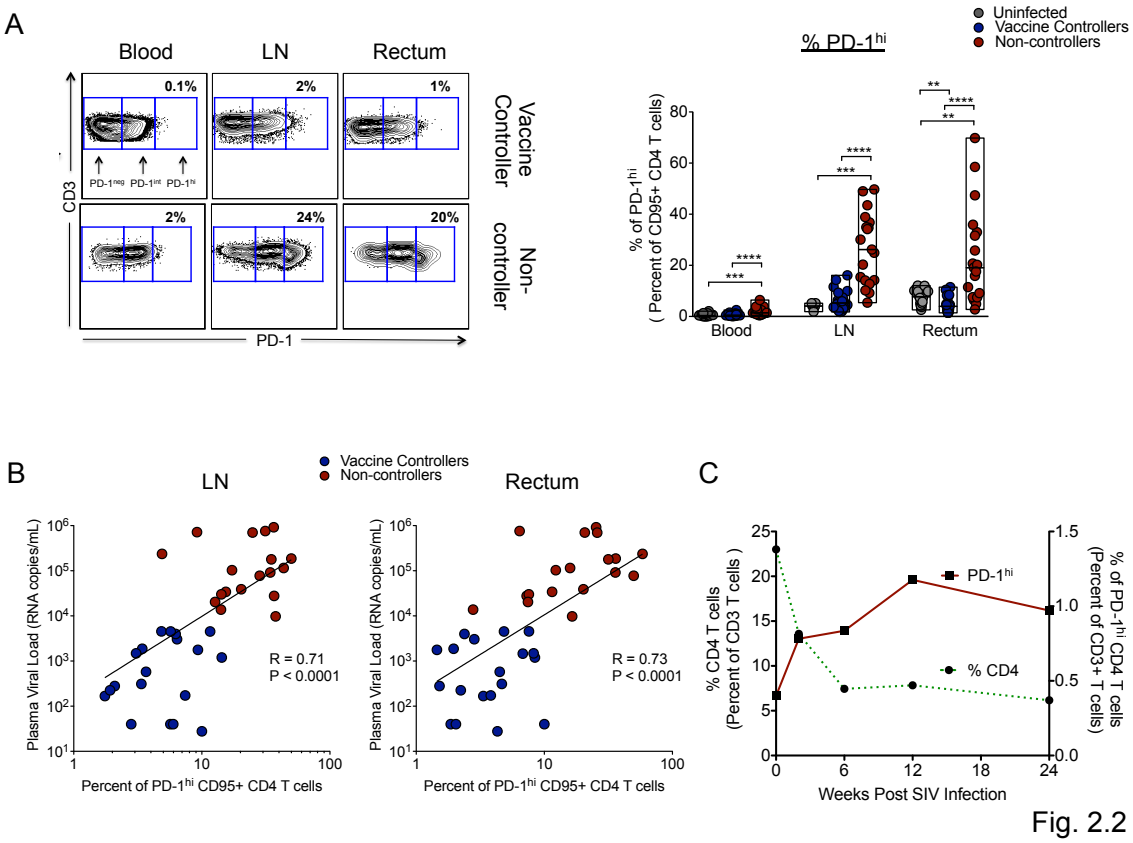

Figure 2.2. Characterization of PD-1^{hi} CD4 T cells in the LN and rectum of chronically SIV infected vaccine-controller and non-controller RM. (A) Flow cytometric analysis of PD-1^{hi} CD95+ CD4 T cells in the blood, LN, and rectum of healthy (n=16, 7, and 23, respectively) SIV+ vaccine-controller (n= 19) and non-controller (n=18) RM. **(B)** Correlation between PD-1^{hi} CD95+ CD4 T cells and viral load at week 24 post SIV infection in the LN and rectum of SIV+ vaccine-controller (n=19) and non-controller (n=18) RM. **(C)** Longitudinal frequencies of PD-1^{hi} CD95+ CD4 T cells and CD4 T cells in the rectum of chronically SIV infected RM as a percent of total CD3 T cells. Scatter Plots show the median. *, P < 0.05; **, P < 0.01; ***, P <0.001; ****, P < 0.0001.

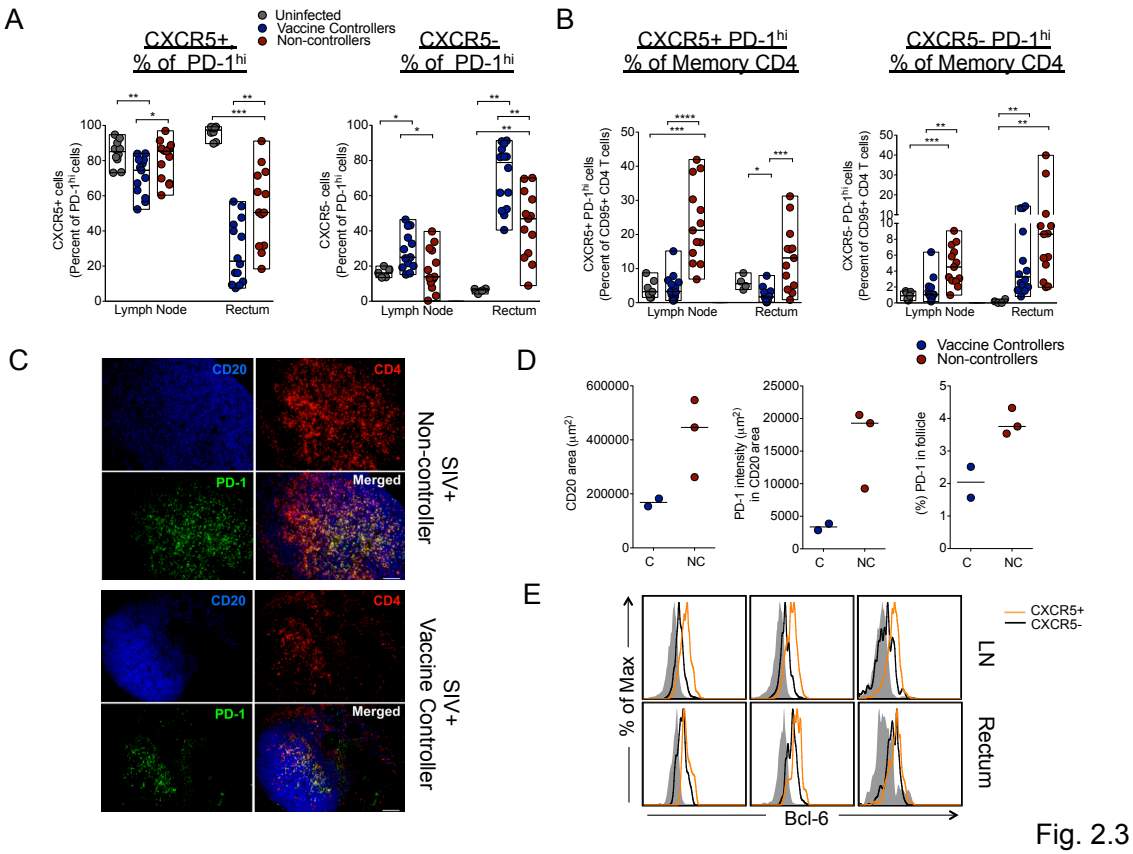

Fig. 2.3

Figure 2.3. CXCR5 expression and localization of PD-1^{hi} CD4 T cells of chronically SIV infected vaccine-controller and non-controller RM. **(A)** Percentage of CXCR5+ and neg PD-1^{hi} CD4 T cells in the LN and rectum of healthy (n=7 and n=4, respectively), SIV+ vaccine-controller (n=13) and SIV+ non-controller (n=13) RM. **(B)** Percentage of CXCR5+ and neg PD-1^{hi} CD4 T cells as a frequency of memory CD95+ CD4 T cells in the LN and rectum of healthy, SIV+ vaccine-controller, and SIV+ non-controller RM. **(C)** A representative immunoflourescent staining for PD-1+, CD4+, and CD20+ cells in a rectal lymphoid aggregate of an SIV+ vaccine-controller and SIV+ non-controller RM and quantitative analysis showing the follicle size and intensity and frequency of PD-1+ CD4 T cells in SIV infected vaccine-controller (n=2) and SIV infected non-controller RM (n=3). **(D)** Expression of Bcl-6 on CXCR5+ and CXCR5- CD95+ CD4 T cells compared to PD-1^{neg} CD95+ CD4 T cells. Scatter Plots show the median. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

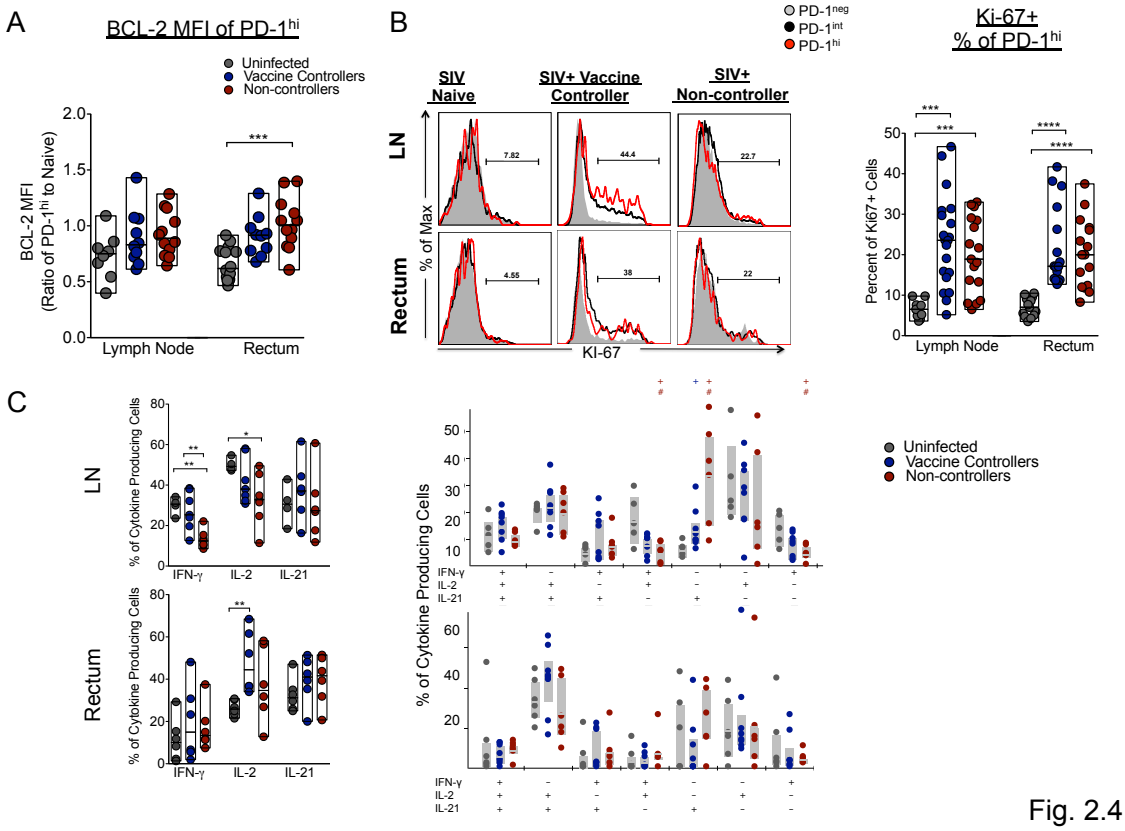

Fig. 2.4

Figure 2.4. Phenotypic characterization of PD-1^{hi} CD4 T cells in the LN and rectum of chronically SIV infected vaccine-controller and non-controller RM. (A) The mean fluorescence intensity (MFI) of Bcl-2 on PD-1^{hi} in the LN and rectum of healthy (n=8 and n=14, respectively), SIV+ vaccine-controller (n=10) and SIV+ non-controller (n=12) RM **(B)** Percentage Ki-67+ PD-1^{hi} cells in the LN and rectum of healthy (n=9 and n=13 respectively), SIV+ vaccine-controller (n=19 and n=16, respectively), and SIV+ non-controller (n=17). RM. **(C)** Flow cytometric analysis of cytokine production by PD-1^{hi} CD95+ CD4 T cell subsets in the LN and rectum. Scatter plot shows the cumulative data of the percentage of IFN-γ, IL-2, and IL-21 producing PD-1^{hi} cells in the LN and rectum of healthy (n=6 and n=5, respectively), SIV+ vaccine-controller (n=6), and SIV+ non-controller (n= 7) RM and SPICE analysis on cytokine co-production is shown on the right. +, $P < 0.05$ and is defined using SPICE software student *t*-test. Since the Bcl-2 analysis was done on multiple days, we expressed the MFI as a ratio of PD-1^{hi} to

naïve to correct for day-to-day variation in cytometer settings. For cytokine production, cells were stimulated with PMA/Ionomycin. Scatter Plots show the median. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

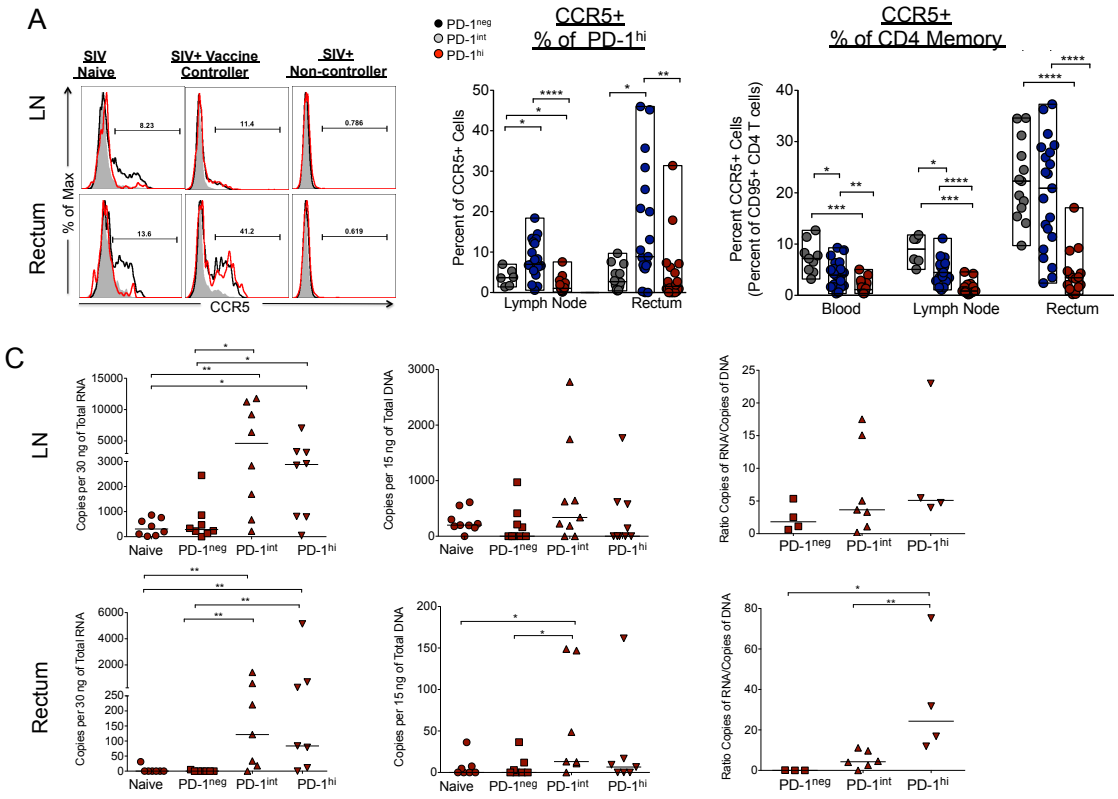

Fig. 2.5

Figure 2.5. **Phenotypic characterization of CCR5 expression and infection status of PD-1^{hi} CD4 T cells in the LN and rectum of chronically SIV infected vaccine-controller and non-controller RM. (A)** Expression of CCR5 on PD-1neg, int, and hi CD95+ CD4 T cells and the percentage of CCR5+ PD-1^{hi} cells in the LN and rectum of healthy SIV naive (n=6 and n=12, respectively), SIV+ vaccine-controller (n=19 and 18, respectively), and SIV+ non-controller (n=17 and 18, respectively) RM and **(B)** Total frequencies of CCR5+ CD95+ CD4 T cells in the blood, LN, and rectum of healthy SIV naive (n= 9, 6, and 23, respectively), SIV+ vaccine-controller (n=19), and SIV+ non-controller (n = 17, 18, and 16, respectively) RM. **(C)** Infection status of PD-1 subsets in the LN and rectum of SIV infected RM. Scatter plots show Gag RNA and DNA copies/ng of input RNA and DNA quantified by RT-PCR from FACS sorted naïve (CD95-) and memory (CD95+) PD-1neg, int, and hi CD4 T cells in the mesenteric LN (n=9) and rectum (n=6) and the ratio of RNA copies/DNA copies in PD-1 neg, int and hi subsets (n=4, 7,

and 4 respectively). Scatter Plots show the median. *, P < 0.05; **, P < 0.01; ***, P <0.001; ****, P < 0.0001.

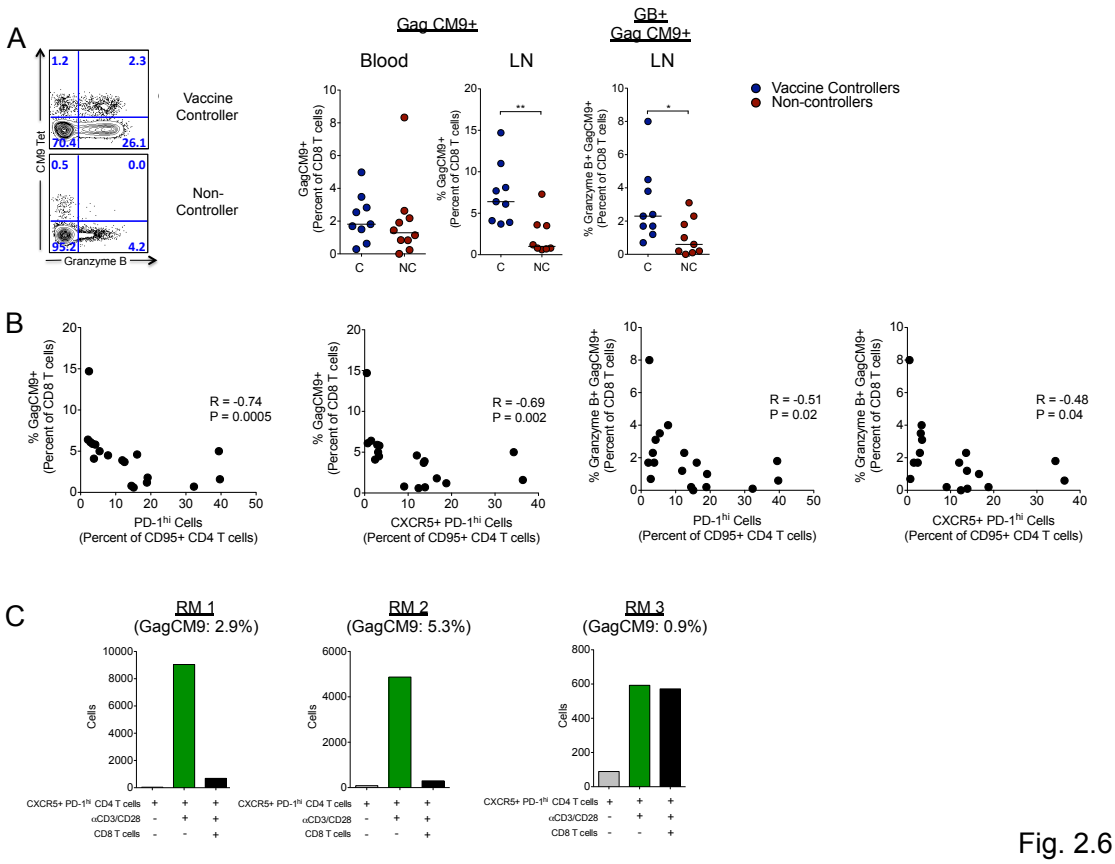

Fig. 2.6

Figure 2.6. Association between anti-viral CD8 T cells and PD-1^{hi} CD4 T cells in the LN following SIV infection. **(A)** Representative plots and percentage of Gag CM9+ and Granzyme B+ GagCM9+ CD8 T cells in the Blood, LN, and Rectum of SIV+ vaccine-controller (n=10, 9, 9) and SIV+ non-controller (n=8, 9, 9) RM. **(B)** Correlations of Gag CM9+ CD8 T cells and Granzyme B+ GagCM9+ CD8 T cells with PD-1^{hi} and CXCR5+ PD-1^{hi} CD4 T cells in the LN of SIV+ vaccine-controller and non-controller RM. **(C)** In vitro killing assay showing the percentage of PD-1^{hi} CXCR5+ CD4 T cells co-cultured with SIV specific CD8 T cells for 5 days from the LN of SIV+ vaccine-controller RM. For each individual animal, the frequencies of Gag CM9 tetramer+ cells ex vivo prior to in vitro stimulation are shown. Scatter Plots show the median. *, $P < 0.05$; **, $P < 0.01$.

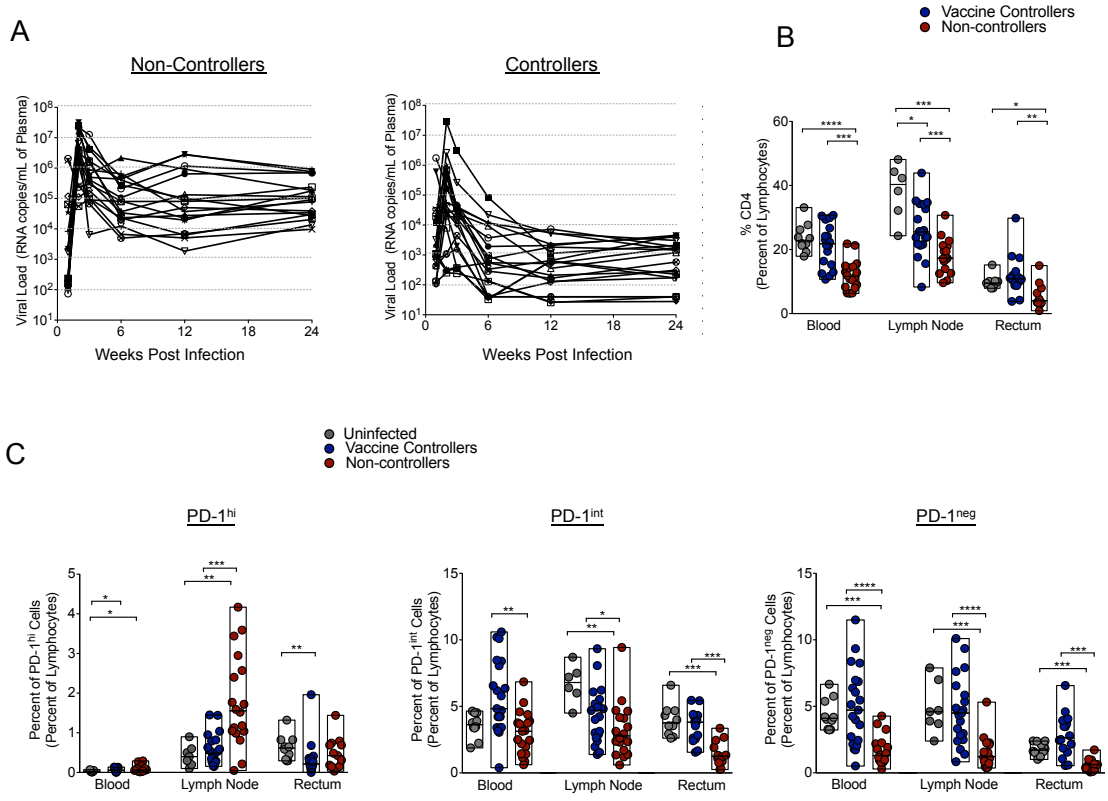

Fig. 2.7

Figure 2.7. Accumulation of PD-1^{hi} CD4 T cells in the LN of SIV infected non-controlling RM. (A) Kinetics of plasma viral load in the two cohorts of SIV infected vaccine controller (n=19) and non-controller (n=18) RM. **(B)** Percent of CD4 T cells as a % of total lymphocytes in the blood, LN and rectum of SIV naïve, SIV vaccine controller, and SIV non-controller RM. **(C)** Percentage of PD-1^{hi}, PD-1^{int}, and PD-1^{neg} CD4 T cells as a percent of lymphocytes in the blood, LN, and rectum of SIV naïve, SIV vaccine controller, and non-controller RM. Scatter Plots show median. *, P < 0.05; **, P < 0.01; ***, P <0.001; ****, P < 0.0001.

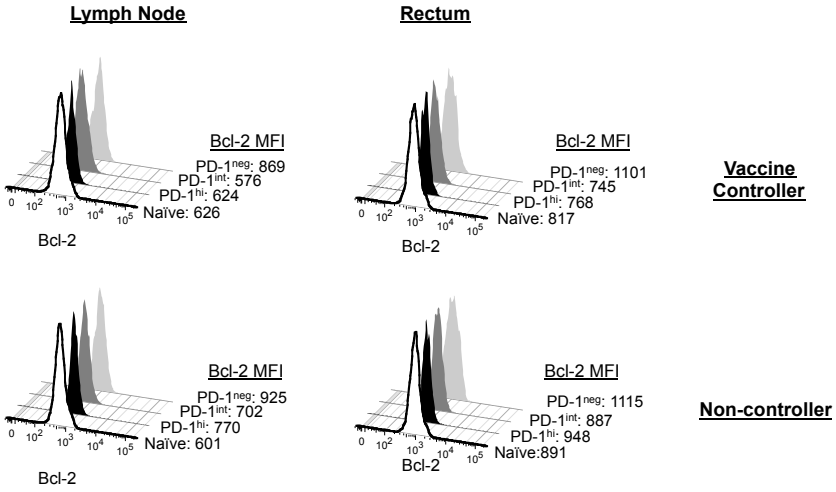

Fig. 2.8

Figure 2.8. **BCL-2 expression on Naïve and Memory CD4 T cells in the LN and Rectum of SIV infected macaques.** Histogram plot showing the expression of Bcl-2 on naïve (CD95-), and memory (CD95+) PD-1^{neg}, PD-1^{int}, and PD-1^{hi} CD4 T cells in the LN and rectum of an SIV+ vaccine-controller and non-controller RM.

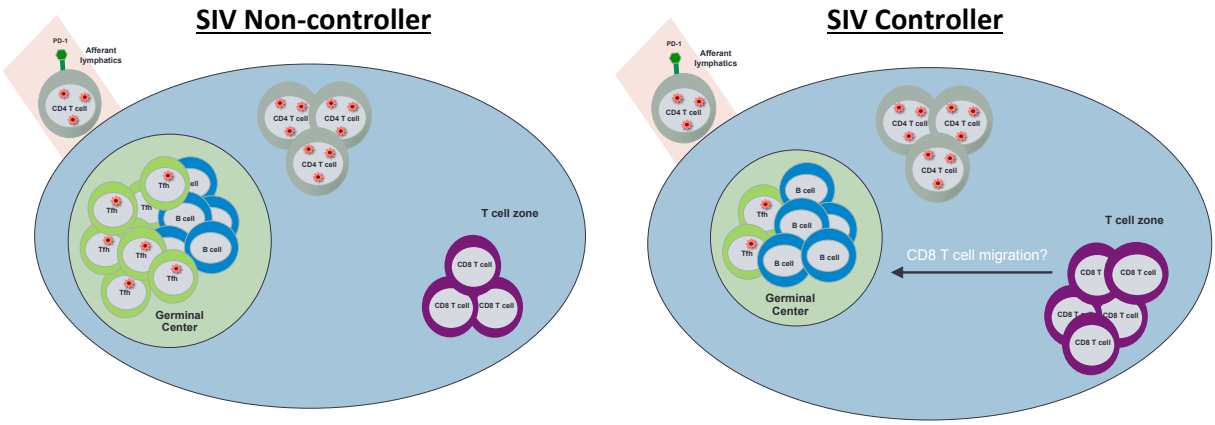

Figure 2.9. Accumulation of SIV infected Tfh within the LN and Rectal mucosa of SIV infected Non-controlling RM. Representative images of 2 lymphoid aggregates (blue) from an SIV infected non-controller (Left) containing a high number of SIV infected T follicular helper cells (Tfh) within a large germinal center/B cell follicle (green) and a vaccinated viral controller (Right) with a lower number of virally infected Tfh cells and a frequency of SIV specific CD8 T cells within the LN that are associated with a limited expansion of Tfh cells within vaccinated viral controllers.

Chapter 3

Follicular Anti-viral CD8 T cells Contribute to Enhanced Control of Pathogenic SIV

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Under consideration (11/20/15)

Abstract

One of the most significant challenges to HIV eradication is the existence of persistent viral reservoirs that are established within days of infection(65) and are seemingly impervious to highly active anti-retroviral therapy (HAART)(69). A major goal for HIV 'cure' studies is to eliminate these viral reservoirs. A significant fraction of HIV/SIV replication and reservoirs are confined to germinal center (GC) resident T follicular helper cells (Tfh) (71, 88, 158, 159, 197). However, GCs are considered to be immune privileged sites for anti-viral CD8 T cells. Here we show that vaccination induces a population of SIV-specific CD8 T cells expressing CXCR5, a chemokine receptor required for homing to B cell follicles, in rhesus macaques. This subset displayed a rapid anamnestic expansion following pathogenic SIV infection that was strongly associated with profound control of viremia and lower viral burden in the Tfh cells. Furthermore, the CXCR5+ SIV-specific CD8 T cells were localized in the GC of SIV controllers and restricted the expansion of SIV antigen-pulsed Tfh cells *in vitro*. Global RNA sequence analysis of purified CXCR5+ SIV-specific CD8 T cells revealed a unique gene expression signature with some genes common to Tfh and Th2 cells. Interestingly, CXCR5+ but not CXCR5- CD8 T cells differentiated into both CXCR5+ and CXCR5- (extra-follicular effector) CD8 T cells following *in vitro* stimulation. Our results identify a unique subset of anti-viral CD8 T cells that migrate to GC, can restrict virus replication in Tfh, and retain the potential to generate extra-follicular effector CD8 T cells, thereby leading to enhanced control of highly pathogenic immunodeficiency virus infection. These findings have important implications for cure strategies to eliminate HIV.

Studies in unvaccinated SIV-infected rhesus macaques (RM) and HIV-infected humans suggested poor migration of anti-viral CD8 T cells to B cell follicles in the LN during chronic infection (248, 249)(250). However, it is not known if anti-viral CD8 T cells elicited by certain vaccines have the potential to migrate to B cell follicles and impact virus replication in the B cell

follicles/GC following HIV/SIV infection. To address this, we characterized the anti-viral CD8 T cell response in a cohort of DNA/MVA vaccinated and unvaccinated RM that exhibited varying degree of viral control following an intra-rectal challenge with a pathogenic SIVmac251 infection (**Table 2**). We segregated vaccinated animals based on their set point viral load into controllers ($<10^4$ copies/ml) and non-controllers ($>10^4$ copies/ml) (**Fig. 3.5a**).

First, we determined the CXCR5 (chemokine receptor required for homing to B cell follicles/GC) expression on anti-viral CD8 T cells in the blood following SIV infection (**Fig. 3.1**). For this purpose, we measured the frequency of Gag-CM9 tetramer+ CD8 T cells (Tet+) and their co-expression of CXCR5 in a group of Mamu A*01+ RM. Prior to infection (~20 weeks after vaccination), the frequency of Tet+ CD8 T cells in the blood of vaccinated animals was small (less than 0.5%) however these cells underwent a rapid anamnestic expansion following SIV infection (**Fig. 3.1a, 3.1b**). At 2 weeks post infection, the frequency of Tet+ cells in the vaccinated animals ranged from 1-30% and was comparable between non-controllers and controllers. At this time, and as expected, the frequency of Tet+ cells in the vaccinated animals was about 2-15 times higher compared to unvaccinated animals. However, by 24 weeks post infection they were comparable between all three groups as they increased in the unvaccinated SIV infected RM and declined in the vaccinated SIV infected RM.

Analysis of CXCR5 expression on Tet+ cells in the blood revealed a different pattern as the controller RM showed a markedly higher CXCR5 expression compared to non-controller RM and unvaccinated RM, and maintained this higher expression even at 24 weeks post infection (**Fig. 3.1a, 3.1b**). At 2 weeks post infection, 2-26% (Median of 5.5%) of the Tet+ cells in the controller RM expressed CXCR5. However, only 0.8-5.3% (Median of 1.2%) of the Tet+ cells in the non-controller RM and 0-1.8% (Median of 0.01%) of the Tet+ cells in the unvaccinated RM expressed CXCR5. Analysis of CXCR5 expression on Tet+ cells in the peripheral LNs showed markedly higher expression in the controller RM (median of 14% at weeks 2-3 and 31% at weeks 12-24) compared to non-controller RM (median of 6.1% at weeks 2-3 and 17% at weeks 12-

24)(**Fig. 3.1c**). In general a higher percentage of the LN resident Tet+ CD8 T cells expressed CXCR5 compared to blood Tet+ CD8 T cells (**Fig. 3.1c**), and the frequency of Tet+ CXCR5+ CD8 T cells in the blood and LN correlated strongly during acute SIV infection (data not shown; $P<0.0001$, $r=0.93$). Moreover, CD8 T cells expressing CXCR5 in the blood or LN at week 2-post infection showed a strong inverse correlation with viremia at week 2 (**Fig. 3.1d**) and week 24 (**Fig. 3.5b**) post infection. A similar strong inverse association between CXCR5 expression on anti-viral CD8 T cells and plasma viremia was also observed at set point (**Fig. 3.5c**). Impressively, this association was also evident in a larger cohort of unvaccinated animals that included non-controllers and spontaneous controllers (**Fig. 3.6a**). Consistent with higher CXCR5 expression on Tet+ CD8 T cells, we observed a significant inverse correlation between the copies of viral DNA in Tfh and the frequency of CXCR5+ Tet+ CD8 in the LN (**Fig. 3.1e**). In addition, higher CXCR5 expression on Tet+ CD8 T cells prior to challenge correlated inversely with set point viral load (**Fig. 3.1f**). Analysis of the expression of CXCR5 on total CD8 T cells in the blood and LN pre and post infection revealed a profound increase in the total CXCR5+ CD8 T cells in the LN during the chronic phase but this increase was evident in all three groups irrespective of the status of viral control (**Fig. 3.6b**). Collectively, these results demonstrated that a rapid expansion of anti-viral CXCR5+ CD8 T cells contributes significantly to early and rapid control of pathogenic SIV infection.

We next characterized the expression of molecules that are associated with cytolytic function, activation status, memory differentiation and proliferation status on CXCR5+ and CXCR5- Tet+ CD8 T cells from the blood of vaccinated controller RMs post-SIV infection (**Fig. 3.2a**). These analyses revealed that a significant fraction of CXCR5+ Tet+ cells express cytolytic molecules granzyme B and perforin, however the fraction of cells expressing these markers was lower compared to CXCR5- Tet+ CD8 T cells. In contrast, a higher percentage of CXCR5+ cells expressed markers associated with proliferation (Ki-67), co-stimulation (CD28) and lymph node homing/memory differentiation (CCR7) compared to CXCR5- Tet+ cells.

To understand the functional quality of CXCR5+ and CXCR5- SIV specific CD8 T cells we characterized their ability to co-produce cytokines IFNγ, TNF-α, and IL-2 in response to stimulation with SIV Gag-CM9 peptide (**Fig. 3.2b, Fig. 3.7**). In controllers, the frequency of CD8 T cells co-producing all three cytokines (TP) was greater (trend, p=0.06) in the CXCR5+ subset compared to CXCR5- subset. Similarly, the frequency of CXCR5+ TP in controllers was greater (p=0.03) than the CXCR5- TP in non-controllers. This was also true when we compared the frequency of TP (expressed as a percent of total cytokine positive cells; qualitative measure) between CXCR5+ and CXCR5- subsets in controllers (p=0.004) and between CXCR5+ subset in controllers and CXCR5- subset in non-controllers (p=0.009)(**Fig. 3.2c**). We could not compare these analyses with CXCR5+ CD8 TP of non-controllers as we failed to see measurable levels of cytokine production from these cells. These results demonstrated that CXCR5+ cells are functionally superior to CXCR5- CD8 T cells for triple cytokine production, a measure that has been shown to be associated with enhanced control of human chronic viral infections (145, 220, 251).

We next performed in situ immunofluorescence staining on LN sections of vaccinated controller RM and non-controller RM to study the localization of total and Gag-CM9 specific CD8 T cells (**Fig. 3.3**). LN sections were stained with CD8, IgD, and PD-1 to visualize the localization of CD8 T cells relative to PD-1 bright Tfh cells in lymphoid follicles. We found that a small, but significant fraction of CD8 T cells were localized to the GC of vaccinated controllers where they were found to be in close proximity to PD-1 high Tfh cells (**Fig. 3.3a, Fig. 3.8a**). Interestingly, we found that in non-controllers CD8 T cells appeared to traffic less to the GC. In addition, we saw this migration as early as 2 weeks post SIV infection in controller RM (**Fig. 3.8b**). We could not determine this for the non-controller RM at this time as we failed to see distinct GC in these animals (data not shown). To confirm that the GC resident CD8 T cells are positive for CXCR5, we stained sections for CD8, CXCR5 and IgD and found that CD8 T cells

that infiltrated the GC in controller RM did indeed co-express CXCR5 (**Fig. 3.3b**). Furthermore, we stained the LN and spleen sections for CD3, Gag-CM9 Tetramer and CD20, and found the presence of CD3+ Tet+ cells within the follicles of controller RM (**Fig. 3.3c**). The density of Tet+ cells in the follicular and extra-follicular regions was either comparable or marginally higher in the latter region (**Fig. 3.3d**). Furthermore, we observed the presence of GagCM9+ CD8 T cells in lymphoid aggregates of rectal sections sampled from two vaccinated controllers (**Fig. 3.9**). Taken together, these data demonstrate that anti-viral CD8 T cells in vaccinated controller RM traffic to GC regions of lymphoid follicles and this may provide an immunological advantage to target and eliminate virus-infected Tfh cells.

We next determined if CXCR5+ CD8 T cells have the potential to limit the *in vitro* expansion of Tfh cells following anti-CD3/CD28 stimulation. For this purpose, we sorted Tfh cells from the LNs of four Mamu A*01+ SIV controller RM and stimulated them with anti-CD3 and anti-CD28 antibodies for 5 days in the absence and presence of autologous CXCR5+ or CXCR5- CD8 T cells. Tfh cells were additionally pulsed with P11C peptide so they could serve as ideal target cells for the responding CXCR5+ and CXCR5- Tet+ cells present in the culture. At the end of 5 days, we determined the number of Tfh cells that remained after stimulation with or without CXCR5+ and CXCR5- CD8 T cells. As can be seen in (**Fig. 3e)**, the number of Tfh cells increased significantly following anti-CD3/CD28 stimulation in the absence of CD8 T cells. However, inclusion of CXCR5+ or CXCR5- CD8 T cells reduced the number of these cells significantly in all 4 animals demonstrating that both CXCR5+ and CXCR5- cells could limit the expansion of SIV antigen-pulsed Tfh *in vitro*.

To understand the mechanism involved in limiting the expansion of TCR stimulated Tfh cells, we studied the expression of the cytolytic molecules granzyme B and perforin on responding Tet+ CD8 T cells. We observed a significant increase in the expression of these effector molecules on both CXCR5+ and CXCR5- CD8 T cells when stimulated with anti-CD3/CD28 antibodies (**Fig. 3.2f, Fig. 3.10a**). This increase was higher in the presence of peptide

pulsed target Tfh cells, particularly on CXCR5+ Tet+ cells (**Fig. 3.2f**). In addition, we investigated the transfer of granzyme B to the target Tfh cells from the CXCR5+ and CXCR5– CD8 T cells co-cultured with these target Tfh cells and observed a higher frequency of granzyme B+ Tfh in some animals when CXCR5+ or CXCR5- CD8 were included in the culture (**Fig. 3.10b**) suggesting cytolytic function of CXCR5+ and CXCR5- CD8 T cells in the culture. Collectively these results demonstrate that both CXCR5+ and CXCR5- CD8 T cells can limit the expansion of antigen-pulsed Tfh *in vitro* potentially through their cytolytic effector function. However, it is important to note that *in vivo* only CXCR5+ CD8 T cells but not CXCR5- CD8 will likely access Tfh because of CXCR5 expression.

Limited information exists on the characteristics of CXCR5+ CD8 T cells during chronic SIV/HIV infection so we performed global gene expression analysis on sorted SIV specific CXCR5+ and CXCR5- CD8 T cells from six vaccinated controller SIV infected RM (**Fig. 3.4**). Interestingly, the CXCR5+ CD8 T cells revealed a distinct gene signature pattern when compared to CXCR5- CD8 T cells. Unlike the CXCR5- CD8 T cells, the CXCR5+ CD8 T cells expressed higher levels of genes associated with Tfh CD4 T cells such as the master transcription factor Bcl6, CD200, and CTLA4 as well as markers associated with Th2 CD4 T cells such as IL-4R (CD124), CCR4, STAT6, NFATC, and IL-10 (**Fig. 3.4a**). Effector molecules typically observed in cytotoxic CD8 T cells such as granzyme A, B, and K were expressed at lower levels on CXCR5+ CD8 T cells compared to their CXCR5- counterparts. However, it is important to note that CXCR5+ CD8 T cells upregulate expression of some of these genes following stimulation (**Fig. 3.3f**). CXCR5+ CD8 T cells also expressed higher levels of molecules associated with co-stimulation/antigen presentation such as CD40, CD83, 41BBL and MAMU-DRA (**Fig. 3.4a**). The CXCR5+ CD8 also expressed higher levels of inhibitory receptors such as CD200 and SPRY2 but lower levels of other inhibitory receptors CD160 and CD244 (**Fig. 3.4a**). The functional consequence of the expression of these molecules is yet to be determined. Additionally, CXCR5+ CD8 T cells expressed higher levels of the anti-apoptotic gene Bcl-2 and lower levels

of the pro-apoptotic gene annexin, suggestive of their better survival potential during chronic SIV infection (**Fig. 3.4a**). We further confirmed the expression of some of these genes on a protein level using flow cytometry (**Fig. 3.11**). Collectively, these results demonstrate that SIV specific CXCR5+ CD8 T cells possess a unique gene expression signature compared to SIV-specific CXCR5- CD8 T cells.

In an effort to understand the modulation of CXCR5 expression following TCR driven CD8 T cell proliferation, we sorted CXCR5+ and CXCR5- CD8 T cells from the LN of chronically infected RM and stimulated them *in vitro* with anti-CD3/CD28 (**Fig. 3.4b**). Both CXCR5+ and CXCR5- CD8 T cells proliferated equally, but interestingly the CXCR5+ CD8 T cells differentiated into both CXCR5+ and CXCR5- CD8 T cell subsets. In contrast, CXCR5- CD8 T cells largely remained CXCR5-. This was also true for Gag-CM9 Tet+ CD8 T cells in the culture. These data suggest that CXCR5+ CD8 T cells have the potential to generate both CXCR5+ and CXCR5- CD8 T cells *in vivo*. If this is true, they not only contribute to control of viral replication in the GC but also in extra-follicular regions of the lymphoid tissue. To further understand if CXCR5 expression on CD8 T cells could be modulated in vitro using certain cytokines, we cultured anti-CD3/CD28 stimulated PBMC in the presence of cytokines IL-12, IL-23, TGF-β and combinations of these and found that TGF-β can significantly increase the expression of CXCR5 on proliferating CD8 T cells (**Fig. 3.4c**). Similar analysis on sorted CXCR5+ and CXCR5- CD8 T cells confirmed that TGF-β enhances CXCR5 expression on CXCR5- CD8 T cells (**Fig. 3.4d**). Previous studies reported that TGFβ levels increase following SIV infection(252, 253) and thus may explain the increase we observed for CXCR5 on total CD8 T cells (**Fig. 3.6b**)**.** However, the increase on SIV-specific CD8 T cells could be due to enhanced priming of these cells following vaccination as we observed a direct correlation for CXCR5 expression on SIV-specific CD8 T cells following vaccination and SIV infection (**Fig. 3.6e**).

In conclusion, our data reveal a strong association between the induction and rapid expansion of a novel subset of highly functional anti-viral CXCR5+ CD8 T cells that migrate to B cell follicles/GC and enhanced control of pathogenic SIV infection in vaccinated macaques. Importantly, the potential of these cells to give rise to both CXCR5+ and CXCR5- CD8 T cells highlights their critical role in the control of virus replication in both follicular and extra-follicular compartments. These results underscore the importance of generating these cells through immune-based therapies to achieve a functional cure for HIV/AIDS.

Material and Methods

Animals. Young adult Indian rhesus macaques (*Macaca mulatta)* from the Yerkes breeding colony were cared for under the guidelines established by the Animal Welfare Act and the National Institute of Health (NIH) *Guide for the Care and Use of Laboratory Animals* using protocols approved by the Emory University Institutional Animal Care and Use Committee. Rhesus macaques (RM) were infected intra-rectally with SIVmac251 at a dose of 647 $TCID_{50}$ studied. All animals studied were MamuA01 positive.

Immunizations and Infections. All RM sampled were unvaccinated or vaccinated with DNA/MVA SIV vaccine (DM). See **Table 2** for additional details. Vaccination consisted of two DNA primes on weeks 0 and 8, and two MVA boosts on weeks 16 and 24. Both DNA and MVA immunogens expressed SIV239 Gag, Pol and Env as described previously(254). Vaccinated animals either received unadjuvanted DNA/MVA vaccine(254), the CD40L adjuvant during DNA prime and MVA boosts(254), or rapamycin for 28 days during each of the MVA boosts. All animals were challenged weekly with SIVmac251 starting 21-24 weeks after the final MVA immunization with a dose of 647 $TCID_{50}$ (1.25×10^7 copies of viral RNA) until they were infected. All animals were infected by 7 challenges under these conditions. Dr. N. Miller (NIH, Bethesda, MD) provided the challenge stock. The criteria for defining controllers and non-controllers was based on a plasma viral load cut-off of $\leq 10^4$ RNA copies/mL of plasma for ≥ 16 weeks prior to sample collection (237). SIV RNA levels were determined using a qPCR (238).

Isolation of mononuclear cells. Mononuclear cells were isolated from the blood, axillary lymph nodes, and rectal tissue, and flow cytometry analysis was performed as described previously (214). Briefly, peripheral blood mononuclear cells were isolated from blood collected in CPT tubes. Mononuclear cells from the lymph nodes were isolated from axillary and mesenteric lymph

nodes processed in complete medium and ACK lysed to remove residual red blood cells. Mononuclear cells from the rectum were isolated (158) after tissue was digested for 2 hours in complete medium with 10% fetal bovine serum, 1% Penicillin/streptomycin, 0.05% Gentamycin, 1% Hepes, 200U/mL of Collagenase IV (Worthington, Lake Wood, NJ), and DNase I (Roche, Indianapolis, IN). Digested tissue was then passed through decreasing size needles (16-, 18-, and 20-gauge, five to six times).

Antibodies. The following antibodies were used. FITC conjugated Perforin (clone Pf-344; Mabtech), PE-conjugated CXCR5 (clone MU5UBEE; eBioscience), PerCP conjugated CD3 (Clone SP-34-2; BD Biosciences), BV605 conjugated CD20 (Clone 2H7, Biolegend), PeCy5 conjugated CD28 (Clone CD28.2; Biolegend), PE-TR conjugated CD95 (clone DX2; BD Biosciences), Brilliant Violet 421- conjugated CD279 (PD-1; Clone EH12.2H7; Biolegend), V500 conjugated CD8 (Clone SK1; BD Biosciences), Streptavidin-APC conjugated P11c tetramer (kindly provided by Dr. Rafi Ahmed's lab at Emory University), PeCy7 and Alexa700 conjugated Ki-67 (Clone B56; BD Biosciences), Brilliant Violet 650 conjugated CD4 (Clone OKT4; Biolegend), BV421 conjugated CD107a (Clone H4A3; Biolegend, Alexa700 conjugated Granzyme B (Clone GB11), FITC conjugated Perforin (Clones Pf-80/164; BD Biosciences). Pe-Cy7 conjugated CD45RA (Clone 5H9; BD Biosciences), FITC and Pe-CF594 conjugated CCR7 (Clone 3D1; BD Biosciences) BV605 conjugated IL-2 (Clone MQ1-17H12; Biolegend), Alexa700 conjugated IFNγ (Clone B27; BD Biosciences), and TNF-α (Clone MAb11; BD Biosciences), PE-CF594 conjugated CD152 (CTLA4; Clone BNI3, BD Biosciences), PeCy5 conjugated CD83 (Clone HB15e; BD Biosciences), PeCy7 conjugated CD200 (Clone MRC OX-104; BD Biosciences), and PE-CF594 conjugated Bcl-6 (Clone K112-91; BD Biosciences).

Intracellular cytokine staining. PBMC were suspended in RPMI medium (Gibco, Life Technologies) with 10% FBS (HyClone, Thermo Fisher Scientific), 100 IU/mL of penicillin, and 100 μg/mL of streptomycin (Lonza). Stimulations were conducted in the presence of anti-CD28 antibody and anti-CD49d antibody (1μg/ml; BD Pharmingen). One million cells were stimulated with either 1μg/mL of pooled peptides spanning the entire SIV Gag protein (single pool of 125 peptides with each peptide at a concentration of 1.0ug/mL; NIH AIDS Research and Reference Reagent Program catalog number 6204), 0.1 μg/mL of P11c peptide (gag sequence CTPYDINQM;), or 80ng/ml of PMA and 1ug/mL of Ionomycin as a positive control. Brefeldin A (5 μg/mL; Sigma) and GolgiStop (0.5 μL/mL; BD Pharmingen) were then added to the cells after 2 hours of stimulation and incubation was continued for 4 hours at 37°C in the presence of 5% CO_2. At the end of stimulation, cells were washed once with FACS wash (PBS containing 2% FBS and 0.25% of sodium azide) and surface stained with anti-CD8, anti-CD4, LIVE/DEAD Near-IR Dead Cell stain (Life Technologies) at room temperature for 20 min. Cells were then fixed with cytofix/cytoperm (BD Pharmingen) for 20min at 4°C and washed with Perm wash (BD Pharmingen). Cells were then incubated for 30 min at 4°C with antibodies specific to IL-2, IFN-γ, TNF-α, CD107a and CD3, washed once with Perm wash, once with FACS wash, and re-suspended in PBS containing 1% formalin. Cells were acquired on LSR-Fortessa with four lasers (205,288, 532, 633nm) and analyzed using the FlowJo software (Treestar Inc. CA). At least 50,000 events were acquired for each sample.

Phenotyping. Mononuclear cells isolated from the blood and lymph node were stained with LIVE/DEAD Near-IR Dead Cell stain at room temperature for 15 min in PBS to stain for dead cells. Cells were then washed with FACS wash and stained on the surface using antibodies specific to CD3, CD4, CD8, CD95, CXCR5, CD28, CCR7, PD-1; and then treated with 1x BD FACS Lysing solution for 10min at room temperature, permeabilized with 1x BD Permeabilizing

solution for 8 min at room temperature, washed with FACS wash, stained intracellularly using antibodies specific to Ki-67, perforin and granzyme B; washed 2x with FACS wash, and assessed by flow cytometry.

Immunofluorescence staining. Freshly isolated lymph node and splenic tissues were snap frozen in Tissue-Tek OCT. 0.5μm sections were fixed for 20 minutes in 100% acetone at -20 degrees Celsius. Sections were then stained overnight at 4°C with the following primary antibodies: anti-IgD at 25ug/ml (Southern Biotech), anti-CD8 5ug/ml (Abcam, ab4055), anti-CXCR5 at 12.5 ug/ml (eBioscience, MU5BEE) and anti-PD-1 at 2.5ug/ml (BioLegend, EH12.2H7). After incubation with primary antibody, sections were washed in PBS for 3 minutes. Sections were then incubated with the following secondary antibodies: donkey anti-goat IgG Alexa488 at 2ug/ml (Life Technologies) and donkey anti-rabbit Alexa 546 at 2ug/ml (Life Technologies) for 2 hours at room temperature. Sections were then counterstained with DAPI and mounted in Prolong Anti-Fade Gold reagent and imaged. For detection of CXCR5, staining was carried out on unfixed tissue overnight at 4°C. After staining with primary antibody, sections were fixed for 20 minutes a room temperature in 4% paraformaldehyde. Secondary antibody staining was then carried out as stated above. All images were acquired and analyzed with a Nikon Eclipse 80i microscope using a 20X objective.

In situ tetramer staining combined with immunohistochemistry. Fresh tissues (LN, spleen and rectum) were sectioned with a compresstome as described(255), and *In situ* tetramer staining combined with immunohistochemistry, confocal image acquisition, and quantitative image analysis was performed as described previously[1,2]. We used FITC-labeled Mamu-A*001:01 tetramers loaded with SIV Gag CM9 (181– 189) (CTPYDINQM) peptide, or with an irrelevant negative control peptide FLP (FLPSDYFPSV) from the hepatitis B viral core protein, and

antibodies including rabbit anti-FITC (AbDSerotec, Raleigh, NC), rat-anti-human CD3 (clone CD3- 12; Thermofisher, Waltham, MA), mouse-anti-human CD20 (Novacastra clone L26; Leica Microsystems, Buffalo Grove, IL), Cy3-conjugated goat-anti-rabbit (Jackson ImmunoResearch, West Grove, PA), Alexa 488–conjugated goat-anti-mouse (Molecular probes), Cy5-conjugated goat-anti-rat (Jackson ImmunoResearch) and Dylight 649-conjugated goat anti-human IgM (Jackson ImmunoResearch). For each lymph node and spleen, we analyzed an average of 8 follicles (range of 6-12). An average of 1.22 mm^2 (range, 0.53–1.91 mm^2) was evaluated for lymph node and 1.54 mm^2 of tissue (range, 1.49–1.58 mm2) for spleen.

Cell Sorting. Mononuclear cells isolated from the lymph node were processed and stained with anti-CD3, anti-CD4, anti-CD8, anti-CD279 (PD-1), anti-CXCR5, anti-CD95, and or P11 tetramer for 25 minutes at 25°C and then either CD95+ PD-1^{neg}CXCR5+, PD-1^{pos}CXCR5+, PD-1^{neg}CXCR5+, and PD-1^{hi}CXCR5^{hi} CD4 T cell populations or CD8+, GagCM9+ CXCR5+ and – CD8 T cells were sorted using a FACSAriaII (BD). In all sorting experiments, the grade of purity on the sorted cells was >93%. SIV RNA levels were determined using a qPCR(238).

***In vitro* Tfh limiting expansion assay.** Mononuclear cells isolated from the LN of SIV infected Mamu A*01+ SIV controller RM were processed, stained with Live/Dead IR, anti-CD3, anti-CD4, anti-CD8, anti-CD95, and anti-CXCR5 antibodies, and sorted for CD95+ CD8 T cells and CD95+ CXCR5^{hi} CD4 T cells (Tfh cells) using a FACSAriaII (BD). Tfh cells were then pulsed with P11c peptide for 1 hour at 37°C at a concentration of 0.1 μg/mL and washed. CXCR5+ and CXCR5- CD8 T cells were co-cultured with unpulsed or pulsed Tfh cells at a 2:1 ratio of CD8 T cells to Tfh cells with no stimulation or anti-CD3/CD28 stimulation at 1 bead to 2 cells (Miltenyi Biotech) for 5 days. Cells were then harvested and analyzed using flow cytometry.

Induction of CXCR5 using cytokines in vitro. PBMCs were stained with Cell Trace Violet (5 μM; Life Technologies) and stimulated with anti-CD3/CD28 at a 1 bead to 2 cells ratio (Miltenyi Biotech) for 5 days in the presence of IL-12 (1 ng/mL; eBioscience), IL-23 (25 ng/mL; eBioscience), or TGF-β (5 ng/mL; eBioscience,). Cells were harvested, stained with Live/Dead IR, anti-CD3, anti-CD4, anti-CD8, anti-CXCR5 and analyzed using flow cytometry. LN cells were stained with anti-CD3, anti-CD4, anti-CD8, Live/Dead Fixable Aqua (Life technologies), anti-CD20, and anti-CXCR5. CXCR5+ and CXCR5- CD8 T cells (CD3+, CD4-, CD20-) were sorted using a FACSAriaII (BD) and stimulated with anti-CD3/CD28 at a 1 bead to 2 cells (Miltenyi Biotech) and cultured for 4 days in the presence of TGF-β (5 ng/mL). Cells were harvested on day 4 and stained with anti-CD3, anti-CD4, anti-CD8, Live/Dead Aqua, anti-CXCR5. Cells were then harvested and analyzed using flow cytometry.

Microarray analysis. Briefly, sorted cells from LNs were lysed in 350 μl of Qiagen RLT buffer. Total RNA extraction was then performed using the RNeasy micro kit (Qiagen, Hilden, Germany) according to manufacturer's specifications with on-column DNAse I digestion to remove Genomic DNA. RNA integrity was assessed by Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) capillary electrophoresis on a RNA Pico chip. all samples had an RNA Integrity Number (RIN) score of 8.5 or higher. For each individual sample, cDNA synthesis and amplification was performed using the NuGEN Ovation Pico WTA V2 system (NuGEN, San Carlos, CA, USA). Briefly, 500 pg of total RNA was used for cDNA synthesis followed by whole transcriptome amplification by NuGEN's Ribo-SPIA® technology. The Ribo-SPIA® technology uses DNA/RNA chimeric primers to amplify cDNA isothermally maintaining the stoichiometry of the input RNA. The amplified single stranded DNA was purified using the AMpure XP beads (Beckman, Indianapolis, IN, USA). Qualitative and Quantitative analyses were performed on the Bioanalyzer and NanoDrop respectively to assess the size distribution of the amplified DNA and quantity. 4.5 μg of the amplified DNA was used for biotinylation and

fragmentation using the NuGEN Ovation Encore Biotin Module (Nugen, San Carlos, CA, USA). All samples were hybridized to Affymetrix GeneChip® Rhesus Macaque Genome Arrays (Affymetrix, Santa Clara, CA, USA), which contains over 52,000 individual probe sets that assay over 47,000 transcripts. The probe arrays were washed, stained and scanned as described in the Affymetrix GeneChip® Expression Analysis Technical Manual. CEL files were extracted from the raw scanned images using the Affymetrix GeneChip® command console Software. Quality Control metrics were monitored on the Affymetrix Expression console software; discordant arrays were excluded from further downstream analyses. Microarray results have been deposited in the Gene Expression Omnibus database (accession number GSE74751). The data can be accessed using the following link: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=epmdikuulfmbhmj&acc=GSE74751

Statistical analysis. Statistical analyses were performed using Prism (version 5.0d; GraphPad Software Inc.). Statistical significance (p-values) was obtained using non-parametric Mann-Whitney test (for comparisons between groups/subsets), paired t-tests (for comparisons between subsets within the same animal), or Spearman rank test (for correlations). Statistical analyses of cytokine co-expression profiles were performed by partial permutation tests using SPICE software (NIAID, NIH) as previously described(199). For microarray analysis, background adjustment, normalization, and median polish summarization of .CEL files were performed with the robust multichip average (RMA) algorithm using the *affy* Bioconductor package. The quality of hybridized chips was assessed after normalization by examining their NUSE and RLE plots. Downstream analyses were performed using Partek Genomics Suite software version 6.5 (Partek Inc., St. Louis, MO). P-values for the contrasts between CXCR5+ and CXCR5- CD8 T cells in the controllers were determined by paired t-test.

Acknowledgements

We thank the Yerkes Division of Research Resources and veterinary staff for animal care and procedures. We thank the Emory Flow Cytometry core for cell sorting, Emory CFAR Virology Core for viral load assays and the NIH AIDS Research and Reference Reagent Program for the provision of peptides. We thank Sakeenah Hicks for technical help. This work was supported by National Institutes of Health Grants R36 AI112787, P01 AI88575 and U19 AI109633 to R.R.A. Yerkes National Primate Research Center Base Grant P51 RR00165, and Emory Center for AIDS Research Grant P30 AI050409.

Author Contributions

G.H.M contributed to the concept and design of experiments, carried out most of the experiments and performed analyses; D.R. and H.M. contributed to the design of IHC experiments and performed IHC experiments and analyses. S.I. provided tissue samples for some of the IHC and flow analyses. G.T performed microarray experiments and analyses. S.B. supervised microarray analyses. I.R.W and P.J.S contributed to the design of IHC experiments and supervised them. V. V contributed to the concept and design of experiments, and performed analyses. R.R.A. contributed to the concept and design of experiments, supervised the project and coordinated the experiments. G.H.M V.V and R.R.A wrote the manuscript. All authors reviewed the manuscript and discussed the work.

R.R.A is a co-inventor of DNA/MVA vaccine technology and Emory University licensed this technology to Geovax Inc.

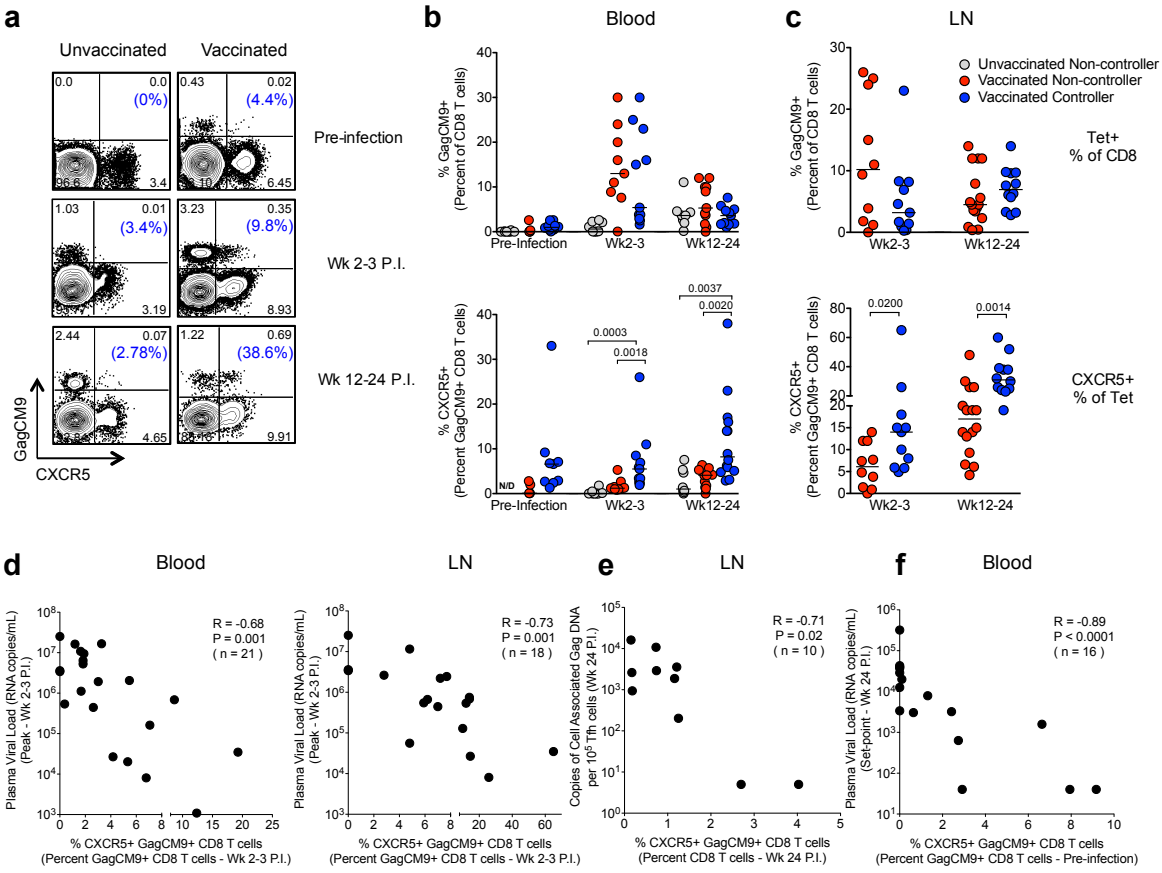

Fig. 3.1

Figure 3.1. Rapid expansion of CXCR5+ SIV specific CD8 T cells is associated with enhanced control of chronic SIV infection. (a-c) Frequency of GagCM9 tetramer+ cells (Tet+) and CXCR5 expression in blood and lymph nodes (LN) following SIVmac251 infection. **(a)** Representative FACS plots. Numbers in parenthesis represent CXCR5 expression on Tet+ cells as a percent of total Tet+ cells. **(b, c)** Summary for three groups of macaques in blood and LN. Data is not available for unvaccinated non-controllers in LN. Scatter plots show the median. (**d**) Correlation between the percentage of CXCR5+ GagCM9+ CD8 T cells and viremia during the acute phase of infection. (**e**) Correlation between the frequency of CXCR5+ GagCM9+ CD8 T cells and the copies of SIV DNA in Tfh cells (CXCR5+ PD-1++). (**f**) Correlation between the frequency of CXCR5+ GagCM9+ CD8 T cells in the blood post vaccination and set point viral load.

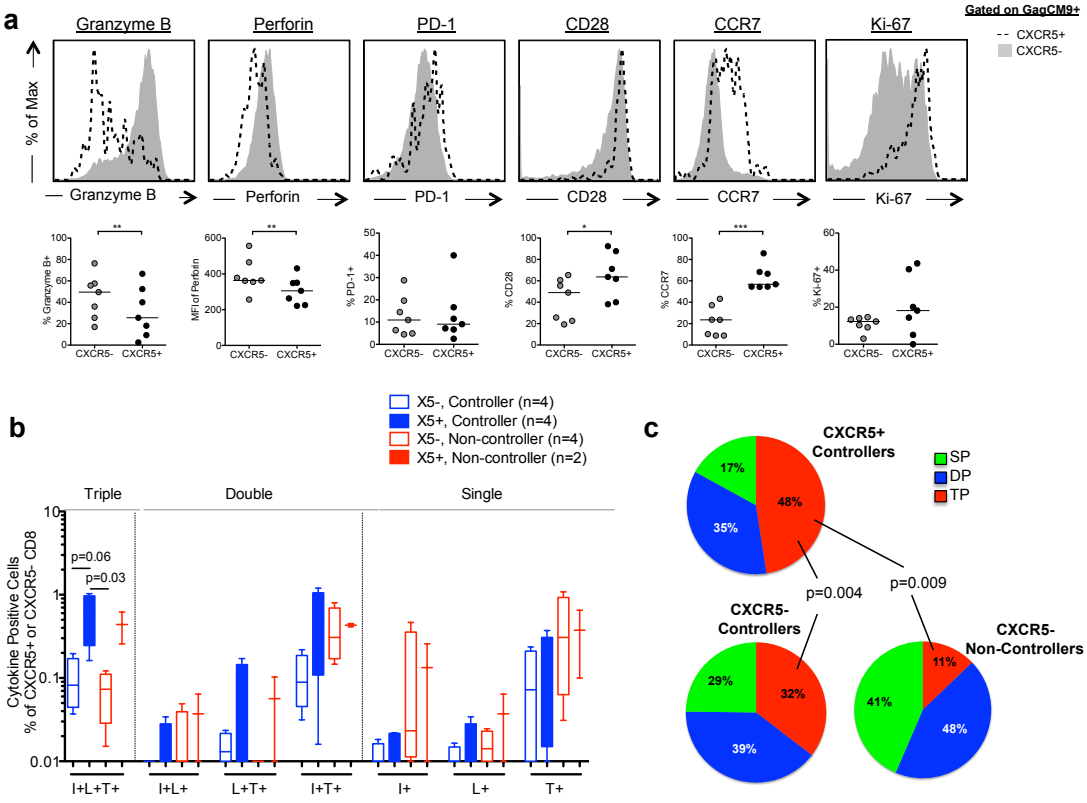

Fig. 3.2

Figure 3.2. CXCR5+ SIV specific CD8 T cells are more polyfunctional than CXCR5- SIV specific CD8 T cells in the blood. (**a**) Representative histograms (top) and frequencies (bottom) showing the expression of the indicated markers on CXCR5+ and CXCR5- GagCM9 tetramer+ CD8 T cells at week 2 post-infection. (**b**) Frequency of cytokine co-expressing cells in response to P11c (GagCM9) peptide stimulation at week 24 post SIV infection. T, TNF-α; I, IFN-γ; L, IL-2. Scatter plots show the median. (**c**) Pie charts showing mean cytokine co-expression represented as a percentage of total cytokine+ cells.

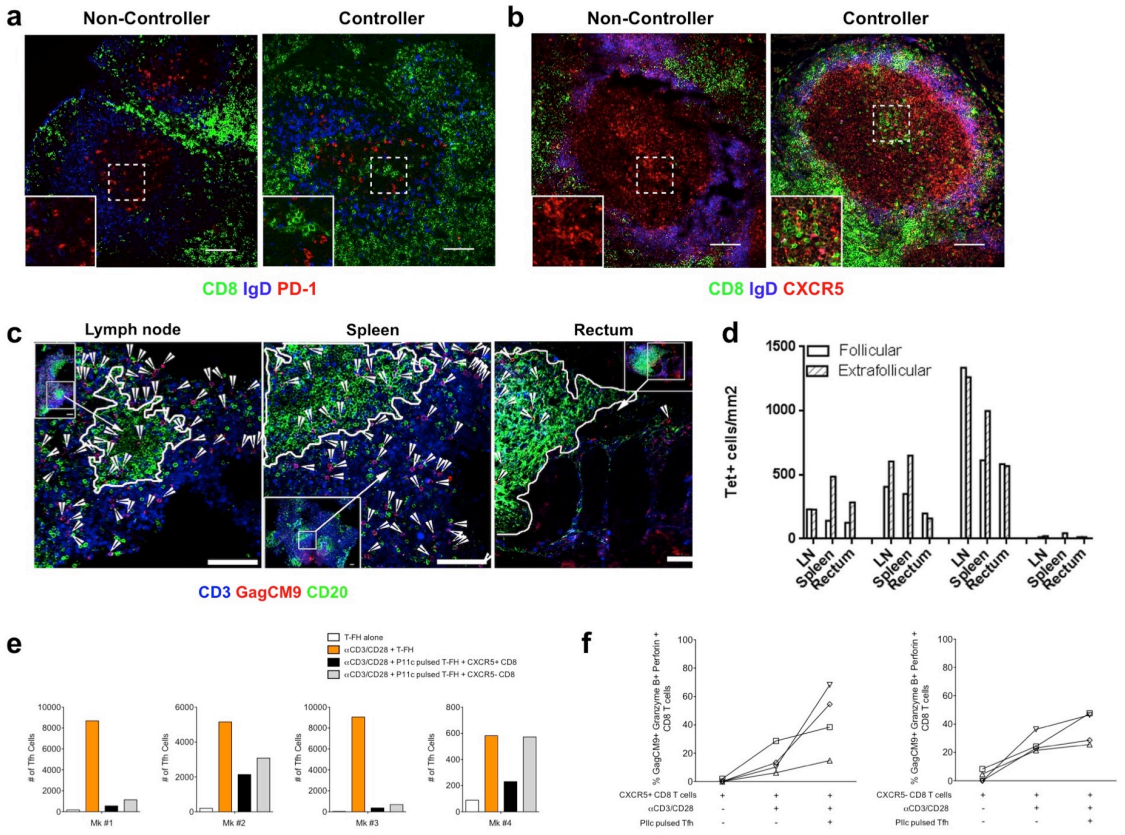

Fig. 3.3

Figure 3.3. CXCR5+ SIV-specific CD8 T cells are localized in the germinal centers (GC) of vaccinated controllers and limit Tfh expansion in vitro. Representative immunofluorescence staining images of the LNs showing the presence of (**a**) CD8 T cells and (**b**) CXCR5+ CD8 in the GC of vaccinated SIV controllers. (**c**) Representative *in situ* tetramer staining images of LN, spleen and rectal tissue sections from a SIV controller RM showing the presence of Gag CM9 tetramer+ cells in the GC. Arrowheads indicate tetramer+ cells. (**d**) Density of follicular and extra-follicular tetramer+ cells. (**e**) CXCR5+ and CXCR5- CD8 T cells from four Mamu A01+ SIV controller RM limit expansion of P11C peptide pulsed Tfh in vitro. Each graph represents data for an individual monkey. (**e**) Expression of granzyme B and perforin by CXCR5+ and CXCR5- CD8 T cells following in vitro culture with P11C peptide pulsed Tfh for 5 days. Scale bars indicate 100μm for all immunofluorescence stained sections.

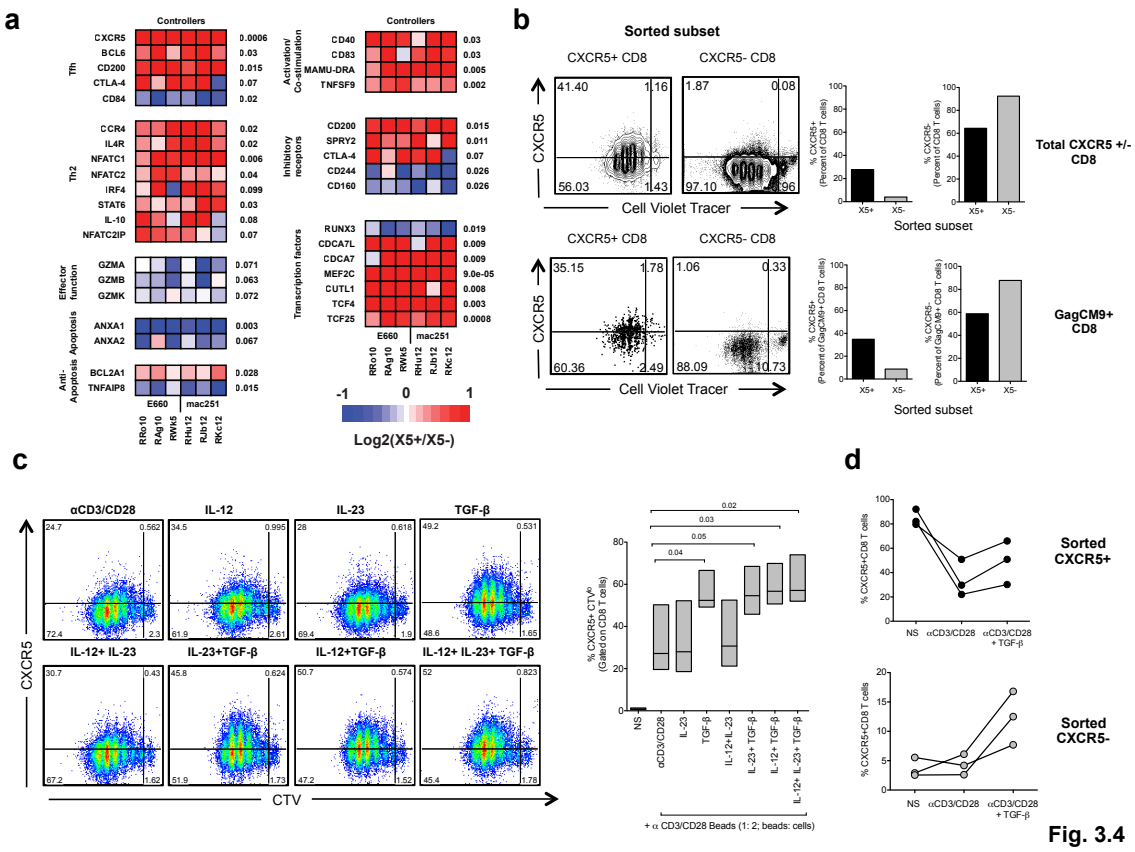

Fig. 3.4

Figure 3.4. Global gene expression analysis revealed distinct gene expression profile for CXCR5+ SIV specific CD8 T cells. (**a**) Microarray analysis of sorted CXCR5+ and CXCR5- GagCM9 tetramer+ CD8 T cells from the LN of six vaccinated SIV controllers. (**b**) CXCR5 expression on purified CXCR5+ or CXCR5- total and GagCM9 tetramer+ CD8 T cells on day 5 following stimulation with anti-CD3 and anti-CD28 antibodies. (**c**) CXCR5 expression on total CD8 T cells on day 4 following stimulation with anti-CD3 and anti-CD28 antibodies in the presence of indicated cytokines. (**d**) CXCR5 expression on sorted CXCR5+ and CXCR5- CD8 T cells on day 4 following stimulation with anti-CD3 and anti-CD28 antibodies in the presence of TGF-β.

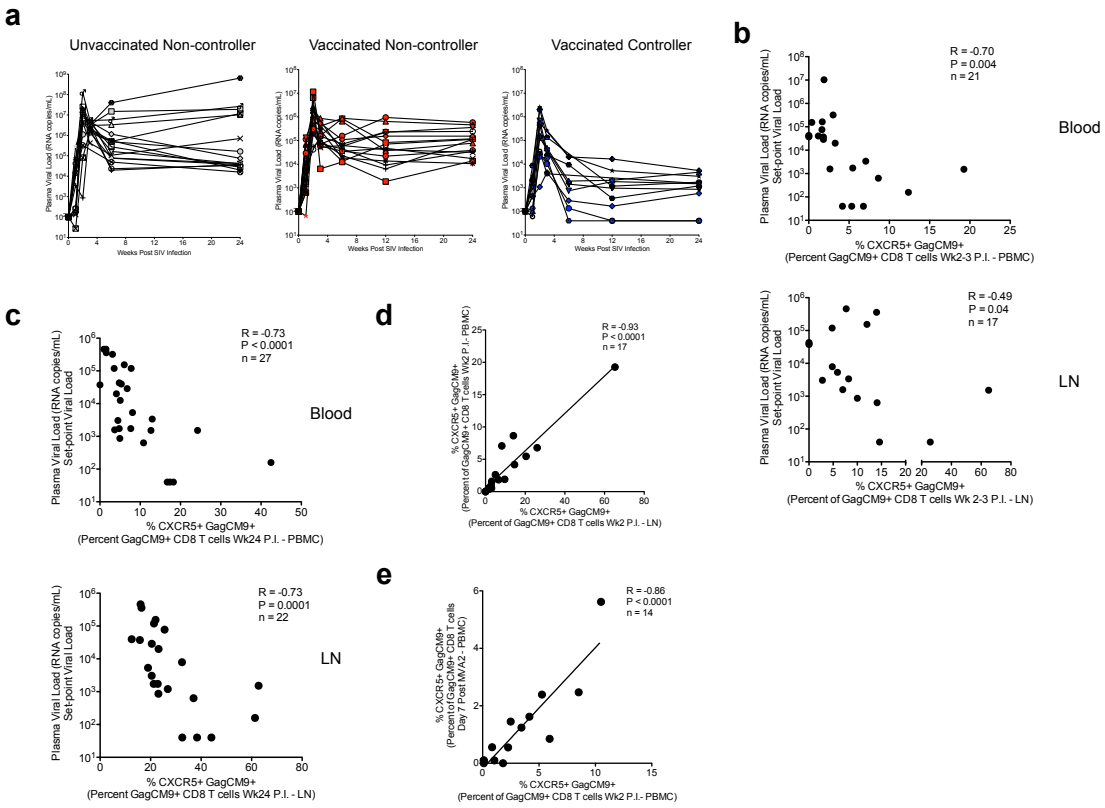

Fig. 3.5

Figure 3.5. Correlations between the percentage of CXCR5+ GagCM9+ CD8 T cells at week 2-3 and week 12-24 post SIV infection and set-point VL. (a) Kinetics of plasma viral loads in unvaccinated (n=19), vaccinated non-controlling (n=11), and vaccinated controlling RM (n=12). (b) Correlations between set-point viral load and CXCR5+ GagCM9+ CD8 T cells in the blood (n=21) and LN (n=17) at week 2-3 post infection and (c) week 12-24 post infection blood (n=27) and LN (n=23). (d) Correlation of CXCR5 expression on Gag CM9 Tetramer+ cells in the blood and (n=17) at week 2-3 post infection (e). Correlation of CXCR5 expression on Gag CM9 Tetramer+ cells following vaccination (week 1 post MVA2) and SIV infection (2 weeks post SIV infection) in blood (n= 14).

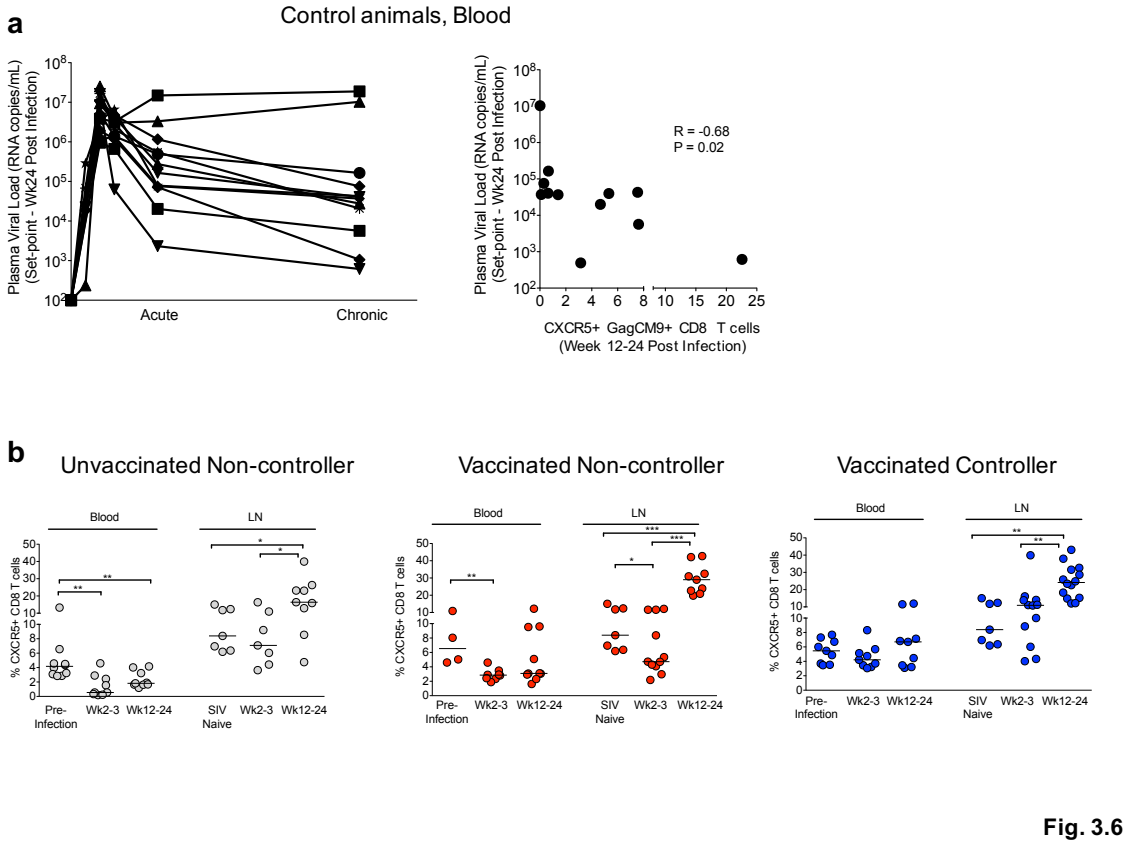

Fig. 3.6

Figure 3.6. Percentage of CXCR5+ GagCM9 CD8 T cells in unvaccinated animals at week 12-24 post SIV infection inversely correlates with set-point viral load. (a) Kinetics of plasma viral load of unvaccinated controller and unvaccinated non-controller RM (n=12) (left) and a correlation between the frequency of CXCR5+ GagCM9+ CD8 T cells in the blood at week 12 post SIV infection and set-point viral loads (right). (b) Frequencies of total CXCR5+ CD8 T cells in the blood (n=10, 9, and 9 respectively) and LN (n=7, 7 and 9 respectively) of unvaccinated non-controller, vaccinated non-controller blood (4, 8 and 10 respectively) and LN (n= 7, 11 and 9 respectively), and vaccinated controller RM blood (n=9,9,and 9 respectively) and LN (=7, 12, and 14 respectively) at a pre-infection time-point, at week 2-3 post SIV infection and week 12-24 post SIV infection.

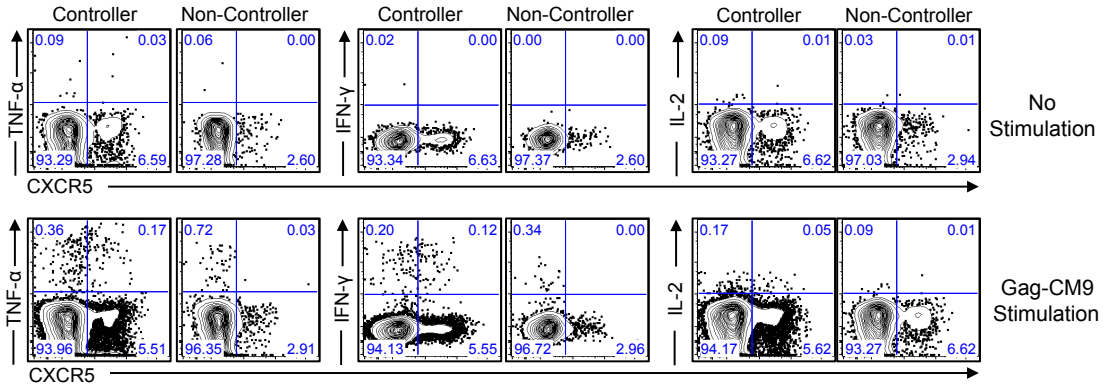

Fig. 3.7

Figure 3.7. Representative FACS plots from a controller and non-controller RM at week 24 post SIV infection, showing TNF-α, IFN-γ, and IL-2 cytokine production by CXCR5+ and CXCR5- CD8 T cells after 6 hours of P11c (GagCM9) peptide stimulation.

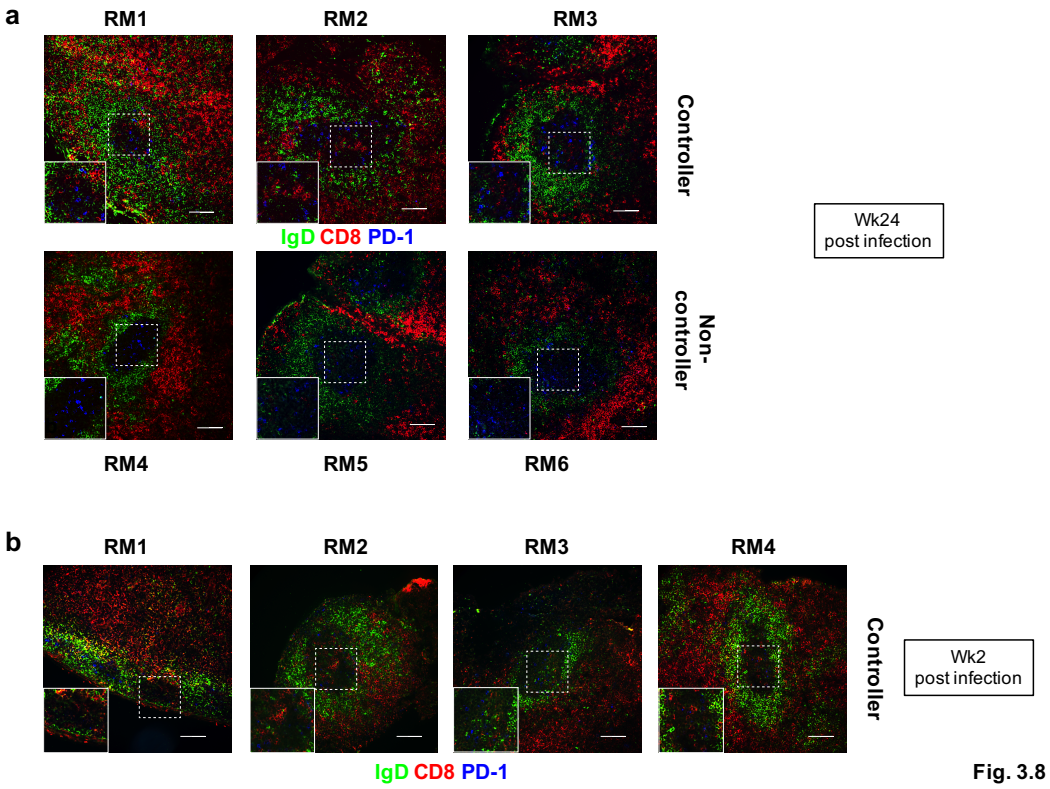

Fig. 3.8

Figure 3.8. (a) Representative immunofluorescence staining for CD8 (red), IgD (green), and PD-1 (blue) of LN sections from SIV+ non-controller (n=3) and SIV+ vaccine controller (n=3) RM at week 24 post SIV infection. (b) Representative immunofluorescence staining for CD8 (red), IgD (green), and PD-1 (blue) of LN sections from vaccinated controller RM (n=4) at week 2-3 post SIV infection.

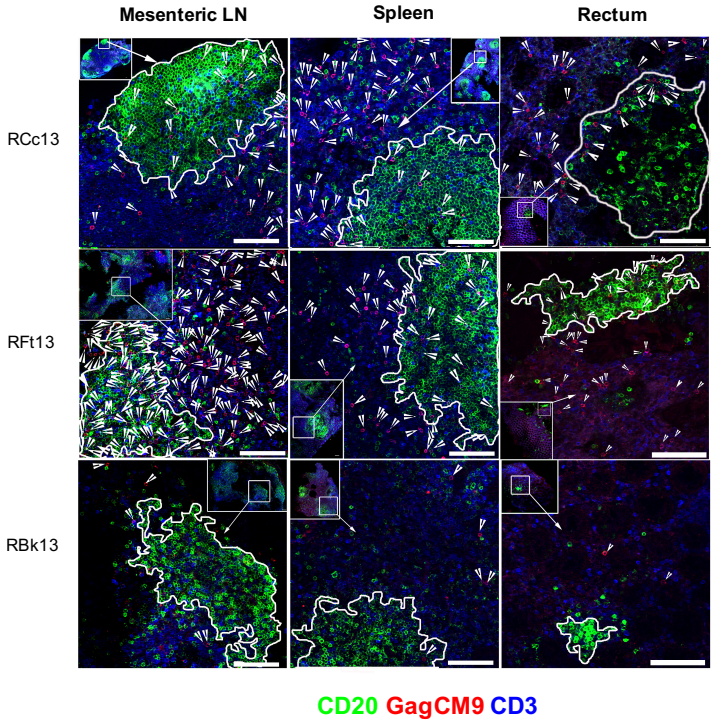

Fig. 3.9

Figure 3.9. Representative *in situ* tetramer staining images of MLN, spleen and rectal tissue sections from SIV+ vaccinated controller RM (n=3) showing the presence of Gag CM9 tetramer+ cells in lymphoid aggregates. Arrowheads indicate follicular tetramer+ cells.

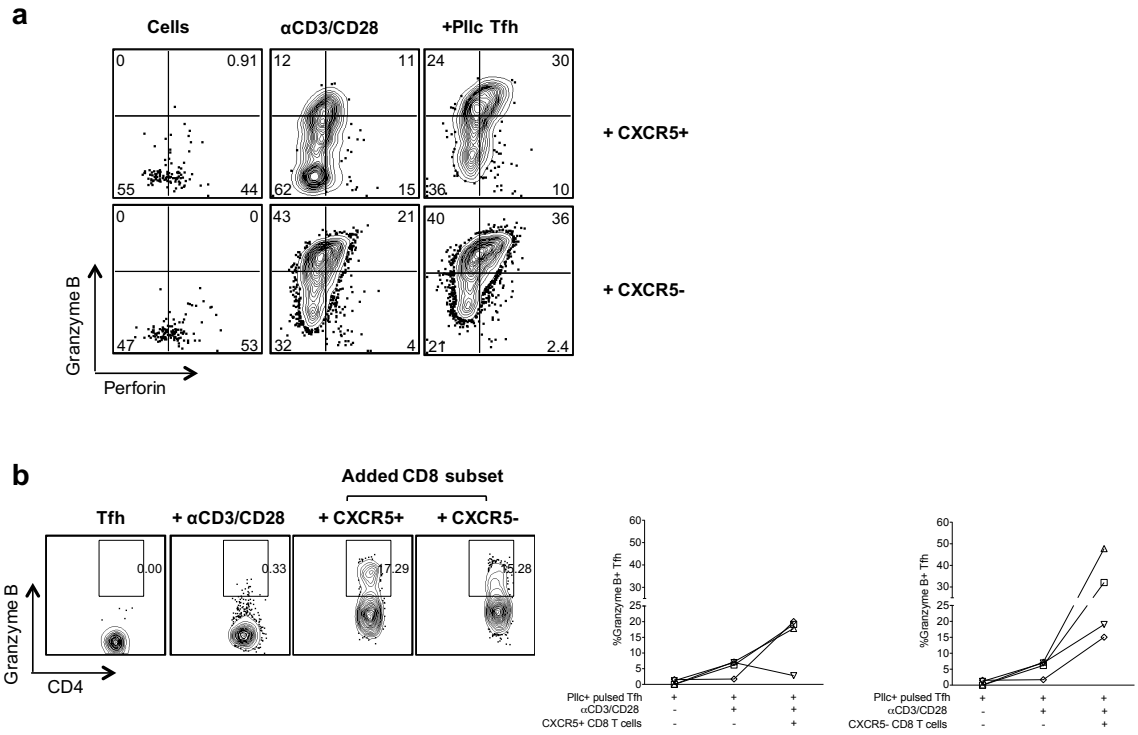

Fig. 3.10

Figure 3.10. Induction of perforin and granzyme co-expression on CXCR5+ and – CD8 T cells co-cultured with P11c antigen pulsed Tfh. (a) Representative FACS plots showing co-expression of granzyme B and perforin on sorted CXCR5+ and CXCR5- CD8 T cells after a 5 day *in vitro* Tfh expansion assay with no stimulation, αCD3/CD28 stimulation, and αCD3/CD28 stimulation in addition to P11c pulsed autologous Tfh cells. (b) Representative FACS plots (left) from the *in vitro* Tfh expansion assay showing granzyme B expression on Tfh cultured alone, with αCD3/CD28 stimulation, and αCD3/CD28 stimulation with CXCR5+ or CXCR5- CD8 T cells and (right) graphical representations of the percentage of granzyme B+ Tfh cells cultured with CXCR5+ or CXCR5- CD8 T cells (n=4).

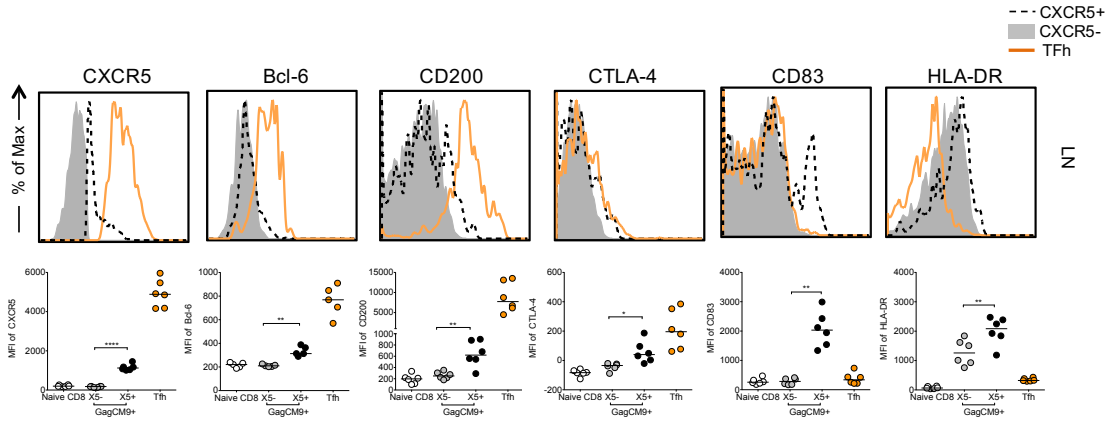

Fig. 3.11

Figure 3.11. Representative histogram plots (above) and scatter plots (below) showing the expression of the indicated markers on naïve CD8 T cells (CD95-, CD45RA+), CXCR5- (X5-) and CXCR5+ (X5+) GagCM9 tetramer+ CD8 T cells, and Tfh cells (n=6).

Table 2. Cohort of SIV infected rhesus macaques used for sample collection

Animal Name	Vaccination status	Set-Point Viral Load*	Challenge Virus	Route of Infection
Controllers				
RKc12	DM	579	$SIVmac_{251}$	Intra-rectal
RAq12	DM	1,200	$SIVmac_{251}$	Intra-rectal
RJp12	D_{40L} M_{40L}	< 40	$SIVmac_{251}$	Intra-rectal
RHb12	D_{40L} M_{40L}	225	$SIVmac_{251}$	Intra-rectal
RJb12	D_{40L} M_{40L}	1,480	$SIVmac_{251}$	Intra-rectal
RHu12	D_{40L} M_{40L}	4,540	$SIVmac_{251}$	Intra-rectal
RAs12	DM_R	< 40	$SIVmac_{251}$	Intra-rectal
RMz11	D_RM_R	3,050	$SIVmac_{251}$	Intra-rectal
RUp12	DM_R	3,980	$SIVmac_{251}$	Intra-rectal
RFd13	DM_R	6000	$SIVmac_{251}$	Intra-rectal
RMo13	DM_R	517	$SIVmac_{251}$	Intra-rectal
RBk13	$DM_{Vehicle}$	1010	$SIVmac_{251}$	Intra-rectal
RFt13	$DM_{Vehicle}$	608	$SIVmac_{251}$	Intra-rectal
RRo10	DDMM	2130	SIVE660	Intra-vaginal
RAg10	D_gD_gMM	< 40	SIVE660	Intra-vaginal
RWk5	D_gD_gMM	86.6	SIVE660	Intra-vaginal
RSi13	$D_{40L}D_{40L}MM$	1120	$SIVmac_{251}$	Intra-rectal
RCc13	$D_{40L}D_{40L}MM$	2020	$SIVmac_{251}$	Intra-rectal
RKt14	Unvaccinated	616	$SIVmac_{251}$	Intra-rectal
RKk14	Unvaccinated	494	$SIVmac_{251}$	Intra-rectal
RLz10	Unvaccinated	5660	$SIVmac_{251}$	Intra-rectal
Non-Controllers				
RRl12	DM_R	10,000	$SIVmac_{251}$	Intra-rectal
RCv12	D_RM_R	13,800	$SIVmac_{251}$	Intra-rectal
RJz12	DM_R	20,400	$SIVmac_{251}$	Intra-rectal
RPn12	D_RM_R	30,100	$SIVmac_{251}$	Intra-rectal
RHy11	D_RM_R	77,500	$SIVmac_{251}$	Intra-rectal
RDm13	DM	37,400	$SIVmac_{251}$	Intra-rectal
RQi14*	DM_R	118,587	$SIVmac_{251}$	Intra-rectal
RUk13*	DM_R	460,414	$SIVmac_{251}$	Intra-rectal
RAz13*	$DM_{Vehicle}$	154,000	$SIVmac_{251}$	Intra-rectal
RJi13*	$DM_{Vehicle}$	358,000	$SIVmac_{251}$	Intra-rectal
RMr13	Unvaccinated	27,700	$SIVmac_{251}$	Intra-rectal
RQi13	Unvaccinated	42,600	$SIVmac_{251}$	Intra-rectal
RYk13	Unvaccinated	20,000	$SIVmac_{251}$	Intra-rectal
RJd11	Unvaccinated	1,030,000	$SIVmac_{251}$	Intra-venous
RNg11	Unvaccinated	40600	$SIVmac_{251}$	Intra-venous
RZh11	Unvaccinated	75400	$SIVmac_{251}$	Intra-venous
RIc11	Unvaccinated	36900	$SIVmac_{251}$	Intra-venous
RKb11	Unvaccinated	164000	$SIVmac_{251}$	Intra-venous

DM, DNA/MVA SIV vaccine; DM_{40L}, CD40L adjuvanted DNA/MVA vaccine; DM_R, DNA/MVA vaccine with rapamycin treatment; D_gM, DNA/MVA vaccine adjuvanted with GM-CSF; Vehicle = Polyethylene glycol; * RNA copies/mL of plasma at week 24 post SIV infect

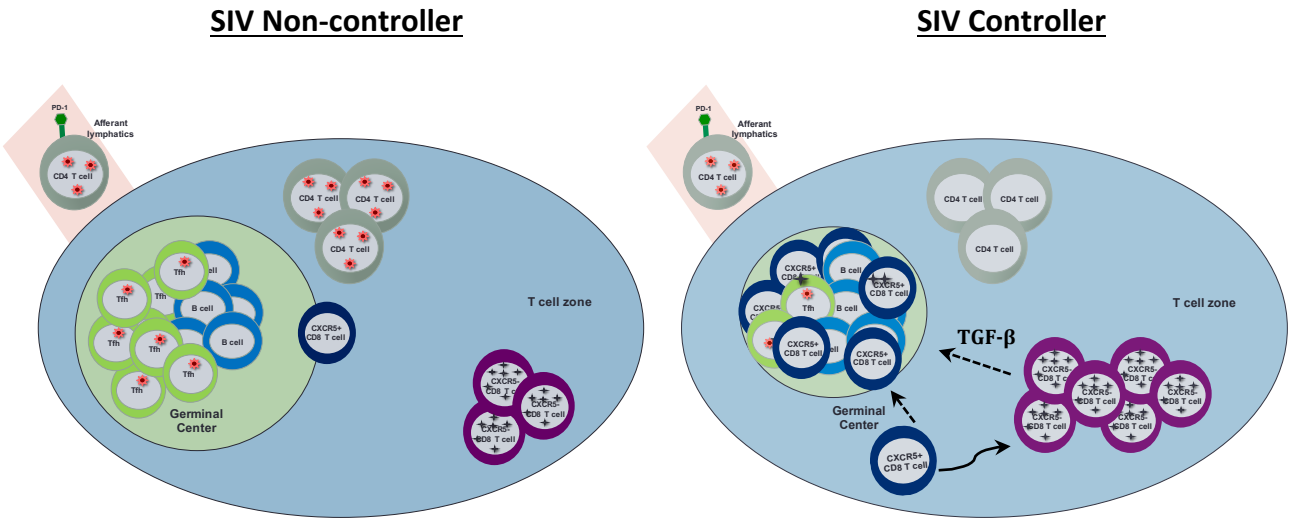

Figure 3.12. Greater Frequency and Function of Germinal Center (GC) Infiltrating CXCR5+ CD8 T Cells During Controlled SIV Infection. Lymph node (LN) sections of an SIV infected non-controller (left) and vaccinated viral controller (right). The LN section of the vaccinated viral controller (right) demonstrates a measurable frequency of intra-follicular (CXCR5+) and extra-follicular (CXCR5-) SIV specific (GagCM9+) CD8 T cells that retain cytotoxic potential, polyfunctionality, and the capacity to localize to the GC during chronic SIV infection. The LN of a SIV infected non-controller (left) demonstrates a reduced frequency of GC infiltrating CXCR5+ SIV specific CD8 T cells and a high frequency of virally infected Tfh cells within the GC. TGF-β may play a potential role in inducing CXCR5 expression on CD8 T cells (shown in the LN from the vaccinated viral controller to the right) and CXCR5+ CD8 T cells, exhibiting a memory phenotype (CCR7+ CD28+), may serve as an important cellular population that can give rise to the effector CXCR5- CD8 T cells localized to extra-follicular sites.

Chapter 4

In vivo PD-1 blockade as a Therapeutic Adjuvant to Antiretroviral Treatment

Abstract

Chronic lentiviral infections such as human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) are characteristically known to induce widespread immune dysfunction. Elevated and persistent expression of the inhibitory receptor programmed death receptor 1 (PD-1) is a well-established immunological signature of dysfunctional anti-viral CD8 T cells and impaired humoral responses during chronic HIV/SIV infection. More recent data has suggested that PD-1 expression identifies a significant population of latently infected CD4 T cells that highly contribute to the persistent viral reservoir that is established under long-term suppressive anti-retroviral therapy (ART). Our lab has previously demonstrated that *in vivo* immunomodulation of PD-1, using a chimerized mouse-anti-human PD-1 blocking antibody, can induce rapid expansion of virus specific CD8 T cells with enhanced functionality both in the blood and rectal mucosa of chronically SIV infected RM. Despite the efficacy of ART in providing control of viral replication and facilitating immune reconstitution in HIV infected individuals, the viral reservoir remains impervious to ART and a major obstacle to complete viral eradication. Therefore, we investigated the safety, tolerability, and immunological benefits of PD-1 blockade as an adjunct therapy to ART. In this study we administered a primatized PD-1 blocking antibody to chronically SIV infected rhesus macaques (RM) prior to and during the first four days of ART. We demonstrated that PD-1 blockade significantly enhanced proliferation of CD4 and total and SIV specific CD8 T cells during the initiation of ART and increased cytotoxic potential and polyfunctionality of SIV specific CD8 T cells. We also observed partial immune reconstitution of CD4 T cell in the blood and rectal mucosa of ART suppressed RM, with enhanced Th17 frequencies in the peripheral blood and rectal mucosa of PD-1 treated animals. More importantly, we observed a significant increase in the kinetics of viral suppression in RM treated with PD-1 blockade compared to control saline treated animals. Taken together, these results establish for the first time the immunological and virological effects of PD-1 blockade

when used as a therapeutic adjuvant to ART. These data highlight the importance of defining combination therapeutic approaches that can be administered with PD-1 blockade to better target and effectively diminish the viral reservoir seeded under suppressive ART as a means to establish a functional cure.

Introduction

The introduction of anti-retroviral therapy (ART) in the mid 1990's transformed clinical management of HIV/AIDS. Prior to the development of ART, limited treatment was available for HIV infected patients, with most treatments involving prophylaxis against opportunistic infections and managing AIDS-related illnesses once they presented. The advent of ART, and in particular the administration of a combination of anti-retroviral drugs known as highly-active anti-retroviral therapy (HAART) also referred to as ART, was essential to providing significant control of viral replication, a reduction in co-morbidities and mortalities associated with HIV infection, and an overall enhancement in the quality of life individuals living with HIV (256-258). Despite the effectiveness of ART in controlling HIV viral replication, patients must receive ART for the duration of their lives because once treatment is halted, plasma viral loads rebounds to pre-treatment levels. The source of this viral rebound is latently infected CD4 T cells that are not eliminated during long-term suppressive ART. Collectively these cells are referred to as the viral reservoir and represent the major obstacle to viral eradication. There has been limited success in generating a prophylactic vaccine capable of affording sterilizing immunity to HIV, and thus the field has redirected its efforts towards generating a more feasible approach to controlling HIV, termed a functional cure, in which treatment can be arrested in HIV infected individuals with limited to negligible viral reemergence. There have been a number of recent studies that have highlighted the significance of HIV reservoirs in limiting viral eradication. One group in particular studied a cohort of intra-rectally SIVmac251 challenged rhesus macaques (RM) to investigate what immunological effects timing of ART, in particular initiation of hyper-acute ART, may have on the generation of viral reservoirs and control of viremia post treatment interruption (65). Although hyper-acute administration (day 3 post SIV infection) of ART during primary infection delayed viral rebound post ART interruption and slightly reduced the total size of the viral reservoir, viral reservoirs were established post infection and contributed to complete

viral resurgence post treatment cessation. Moreover, macaques treated at day 3-post infection did not develop an SIV specific immune response, suggesting that even with a reduction in the size of the viral reservoir that occurs with hyper-acute ART treatment, the absence of a developed anti-viral immune response may limit any significant control of viral reemergence post treatment interruption.

Despite partial immune reconstitution occurring in a small number of HIV infected individuals on long-term ART, including restoration of CD4 T cell counts and a slight increase in the polyfunctionality of T cells (74, 191, 259, 260), residual immune dysfunction still persists and is associated with elevated levels of programmed death receptor 1 (PD-1). A study by Chomont and colleagues sought to better understand the importance of PD-1 on CD4 T cells and its contribution to the viral reservoir under long-term ART (71). This study identified a subset of PD-1^{hi} central memory CD4 T cells that were highly enriched in integrated viral DNA, were the greatest contributors to the total viral reservoir that persisted under suppressive ART, and was continuously maintained throughout the course of long-term ART through homeostatic proliferation. Recent studies by our lab and others have also shown that T follicular helper (Tfh) cells in addition to expressing high levels of PD-1 and are also highly infected both during chronic SIV/HIV infection and under suppressive ART (88, 158, 159). These cells actively participate in ongoing viral production and persistence, particularly in lymphoid sites enriched in the latent viral reservoir. Findings from our study and others highlight the importance of better understanding the mechanisms underlying PD-1 as a marker of latent viral reservoirs and underscores the need to understand how check-point inhibition of PD-1 may modulate and potential destabilize the latent PD-1+ cellular reservoirs during suppressive ART. Our lab previously determined the effects of *in vivo* immunomodulation of PD-1 during acute and chronic SIV infection and observed enhanced SIV specific immunity and a transient decline in plasma viremia. PD-1 blockade also resulted in greater survival of chronically SIV infected rhesus macaques (RM) and decreased mucosal hyper-immune activation (124, 214, 230).

Based on our previous observations, we hypothesized that PD-1 blockade used as an adjunct therapy to ART could result in enhanced anti-viral immune responses and accelerated viral control, which would have important clinical implications for attaining a functional cure for HIV. We conducted an *in vivo* PD-1 blockade trial, in which 22 chronically SIV infected RM were treated with a primatized anti-PD-1 blocking antibody or saline that overlapped for the first four days of ART. Macaques continued on ART for an additional 24 weeks. Our findings from this trial indicate synergy between PD-1 blockade and ART, resulting in an enhanced quality of SIV specific CD8 T cell responses and a more rapid decline in plasma viremia with the initiation of ART. CD4 T cell reconstitution in the blood and rectal mucosa occurred in both treatment groups with long-term ART, with a significant increase in Th17 cells found in the blood and rectal mucosa of PD-1 treated macaques. These findings have important implications for achieving a functional cure for HIV.

Results

PD-1 blockade was administered to 10 RM at 24-30 weeks post SIV infection, with 12 RM receiving saline as a control group **(Fig. 4.1A)**. PD-1 blockade was performed using a primatized antibody specific to PD-1 that blocks the interaction between macaque PD-1 and its ligands (PD-L1/PD-L2). The primatized PD-1 antibody is a humanized EH12 heavy chain variable domain with rhesus macaque IgG4 and has a humanized EH12 kappa variable domain with a rhesus macaque kappa constant region. Blockade was administered intravenously at a 3 milligram per kilogram (mg/kg) dose in 25 milliliters of saline 5 times over the course of two weeks and given at day 0, 3, 7, 10 and 14 **(Fig. 4.1A-B)**. Anti-retroviral therapy (ART) was initiated at day 10 of blockade. A cohort of 22 RM was infected intra-rectally with SIVmac251. The range of set-point viral loads prior to the initiation of PD-1 blockade was between 1 x 10^4 and 5 x 10^5 RNA copies per milliliter of plasma **(Fig. 4.1B)**. The plasma concentration of infused antibody ranged from 15-80 μg/mL and was confirmed in all 10 RM infused with anti-PD-1 treatment **(Fig 4.2A)**. Careful sampling of the peripheral blood and rectal mucosa at baseline and relatively close sequential time-points for the first 2 weeks post antibody treatment allowed us to monitor phenotypic and functional changes in the lymphocyte populations, as well as changes in plasma viremia induced by treatment. This sampling schedule allowed us to 1) determine the effects of PD-1 blockade on plasma viremia prior to and with the initiation of ART; 2) determine the biological effects of PD-1 blockade treatment on both non-specific and SIV specific T cell responses. The inclusion of 12 macaques treated with saline alone allowed us to access any non-specific effects that occur as a result of a saline infusion on viral replication, frequencies of lymphocyte populations, and phenotypic changes in lymphocyte populations.

In vivo PD-1 blockade was confirmed using the fluorescently conjugated version of the infused EH12 anti-PD-1 antibody. We were able to detect PD-1 expression on < 1% of circulating CD28+ CD95+ central memory (T_{CM}) CD4 and CD8 T cells within 3 days of

treatment in all 10 anti-PD-1 antibody treated macaques demonstrating effective blockade of the PD-1 receptor *in vivo* **(Fig. 4.2B)**. PD-1 expression remained undetectable in the blood for approximately 6 weeks, despite the half-life of the PD-1 antibody approximating 7 days. We found a less profound but significant reduction in the expression of PD-1 on CD4 and CD8 T cells in the rectal mucosa of treated macaques suggesting less penetrance of the infused antibody into peripheral tissues such as the rectum **(Fig. 4.2C)**. Previous macaque studies utilizing blocking and/or depleting antibodies have observed less pronounced effects in secondary lymphoid sites (81) compared to peripheral sites. We studied the CD8 T cell response to the immunodominant epitope GagCM9 using major histocompatibility complex (MHC) I tetrameric complexes in macaques that expressed the Mamu A*01 histocompatibility molecule. GagCM9 CD8 T cell responses were studied in 5 A*01 RM treated with anti-PD-1 antibody and 6 A*01 animals treated with saline. Consistent with previously shown data from multiple studies, most (> 98%) of GagCM9+ CD8 T cells were PD-1+ before blockade and < 1% of GagCM9+ CD8 T cells (data not shown) were PD-1+ in the blood post blockade.

PD-1 blockade was associated with a rapid induction of Ki-67 on circulating T_{CM} and CD28- CD95+ effector memory (T_{EM}) CD4 T cells **(Fig. 4.3A)** and total CD8 T cells **(Fig. 4.3B)** that peaked at 7-10 days post blockade. At the peak response, these levels were about 2-5 fold higher than their respective levels on day 0 for T_{CM} ($P = 0.001$) and T_{EM} CD4 ($P = 0.0002$), and CD8 T cells ($P = 0.001$) **(Fig. 4.3C)**. We observed a significant increase in the frequency of Ki-67+ CD4 and CD8 T cells in the rectal mucosa of PD-1 treated macaques, as well, with a 2-10 fold induction of proliferating CD4 T cells and a 2-15 fold induction of proliferating CD8 T cells (data not shown).

After the administration of PD-1 blockade, we observed minimal effects on both the magnitude and quality of SIV specific CD8 T cell responses at mucosal sites (data not shown) with a minor effect on the magnitude of the GagCM9+ CD8 T cell response in the blood as well **(Fig. 4.4A)**. PD-1 blockade was also associated with a significant increase in the frequency of

virus-specific-CD8 T cells that were undergoing active cell cycling *in vivo* **(Fig. 4.5A-B)**. 4 out of the 5 Mamu A*01 RM showed increased Ki-67 expression on GagCM9+ CD8 T cells, with a 2-6 fold increase (P = 0.02) compared to the saline treated control animals **(Fig. 4.5A-B)** and all Mamu A*01 animals treated with PD-1 blockade showed a 2-4 fold induction of granzyme B expression (cytolytic potential; P = 0.038) and a 2-8 fold increase in the expression of CXCR5 (lymphoid follicle homing potential; P = 0.03). Proliferation and function of GagCM9+ CD4 T cells did decrease with continuous ART and a reduction in antigen load **(Fig. 4.5C).** Importantly, *in vivo* PD-1 blockade also enhanced the functional quality of anti-viral CD8 T cells and resulted in an increased frequency of polyfunctional cells capable of co-producing interferon (IFN)- γ and tumor-necrosis factor (TNF)-α **(Fig. 4.4B)**. On the day of initiation of PD-1 blockade, the frequency of cumulative Gag, Env1, and Env2 specific CD8 T cells was low but measurable with a geometric mean of .2% SIV specific TNF-α+ IFN- γ+ CD8 T cells. Post blockade a geomean of approximately 0.6% (P = 0.009) of SIV specific CD8 T cells co-expressed TNF-α and IFN- γ with the peak response at day 10 post blockade and 2-8 fold induction in the frequency of cells co-producing cytokines. We also found a significant inverse correlation between the frequency of TNF-α+ IFN- γ+ SIV specific CD8 T cells at day 10 post blockade and the fold change in viral load at day 10 post ART initiation **(Fig. 4.9B)**.

More importantly, PD-1 blockade during the initiation of ART resulted in enhanced kinetics of viral suppression **(Fig. 4.6A-C)**. In the PD-1 blockade treated group, we observed a more rapid time to suppression compared to the saline alone treated group, **(Fig. 4.6B)** with a significant fold difference in plasma viral load (p = 0.02) of the treatment group at day 10 post ART compared to baseline **(Fig. 4.6A- 4.6B)**. Multiple studies have highlighted the importance of Th17+ CD4 T cells in maintaining mucosal homeostasis. These T cells that are enriched in the gastrointestinal tract (GI) and produce the cytokine IL-17, which is essential in the generation of an adaptive immune response against both bacterial and fungal pathogens (92). It has also been well established that Th17 cells are rapidly depleted during SIV/HIV infection (152, 153) and can

occur as quickly as day 4 post infection. We therefore, assessed the frequency of Th17+ CD4 T cells in the rectal mucosa and blood of both the anti-PD-1 treated and untreated macaques and found a significant increase in the frequency of Th17+ CD4 T cells in the blood of both PD-1 blockade treated ($P = 0.002$) and the saline treated macaques ($P < 0.0001$) **(Fig. 4.7A)**, with no significant difference in the induction of Th17 cells post ART between PD-1 treated and untreated RM. We did however, observe a significant increase in the frequency of Th17+ CD4 T cells in the rectal mucosa of the PD-1 blockade treated macaques ($P = 0.03$) that was not observed in the saline alone treated animals, with a higher fold induction of Th17 cells in the PD-1 blockade treated group ($P = 0.029$) compared to the saline treated macaques **(Fig. 7B)**. Multiple studies have demonstrated an inverse association between immune activation and the frequency of Th17 CD4 T cells (148, 261, 262). We find a significant inverse correlation between the frequency of proliferating CD4 T cells at the day of ART initiation and Th17 frequencies in the blood and rectal mucosa at 24 weeks post ART, suggesting that immune activation at the on-set of ART may effect Th17 reconstitution under suppressive ART **(Fig. 4.4C)**. Most individuals treated with ART are capable of maintaining suppressed levels of viral replication for prolonged periods of times, however there is still incomplete CD4 T cell recovery both in the systemic circulation in the GI tract of patients on long-term ART (154, 263). There are many factors that have been hypothesized to effect reconstitution of CD4 T cells under suppressive ART and these include residual immune activation, immunological senescence, and ongoing HIV replication, which inevitably lead to a poorer clinical prognosis (83). We determined the level of CD4 reconstitution in the blood and rectal mucosa of the anti-PD-1 treated and saline treated macaques. We found a significant increase in the reconstitution of CD4 T cells both in the periphery and in the rectal mucosa of all animals, irrespective of treatment **(Fig. 4.8A)**, and a significant increase in CCR5+ CD4 T cells in the blood of both treatment groups **(Fig. 4.8B)**. We did also observe an increase in the frequency of CCR5+ CD4 T cells in the blood of PD-1 treated animals at peak time-points post blockade, which may be a result of increased proliferation of

total and memory CD4 T cells, but we did not however observe an increase in CCR5+ CD4 T cells in the rectal mucosa **(Fig. 4.8B)**

During suppressive ART, the major molecular form of HIV/SIV is integrated viral DNA that persists as a latent form predominantly in memory CD4 T cells that constitute that major viral reservoir during long-term ART (71). Despite ART controlling viral replication in the plasma to levels below the limit of detection (in our study < 60 copies/ milliliter of plasma), ART is unable to eliminate these stable and persistent viral reservoirs. Recently, different subsets of memory CD4 T cells have been carefully studied to better understand the contribution of each memory CD4 T cell subset to the total viral reservoir under suppressive ART. Memory subsets that make up the CD4 T cell memory compartment include T_{CM} CD4 T cells, a less differentiated subset compared to T_{EM} CD4 T cell, and transitional memory CD4 T cells (T_{TM}), a subset that shows functional, phenotypic, and transcriptional characteristics in between T_{CM} and T_{EM} CD4 T cells. The presence of a more immature memory CD4 T cell population with stem cell-like properties (T_{SCM}), has recently been identified and more highly studied in mice, non-human primates, and humans (166, 264). These T_{SCM} cells have been shown to represent the earliest and most long lasting of the stages of memory CD4 T cell differentiation. Two important studies demonstrated that T_{SCM} CD4 T cells harbor high levels of integrated HIV-1 DNA during long-term ART (265) and this population of memory CD4 T cells remains extremely stable throughout the course of antiretroviral treatment (91). Thus to better access the effects of PD-1 blockade on manipulating the CD4 T cell viral reservoir generated under suppressive ART, we treated RM for 24 weeks with ART and then FACS sorted peripheral blood mononuclear cells on the basis of surface expression of CD45RA, CC chemokine receptor-7 (CCR7), the co-stimulatory molecule CD28, and Fas receptor (CD95). The following populations were sorted: T_{CM} (CCR7+ CD45RA- CD28+ CD95+), TEM (CCR7- CD45RA- CD28- CD95+), T_{TM} (CCR7- CD45RA- CD28+ CD95+), and T_{SCM} (CCR7+ CD45RA+ CD28+ CD95+). We then performed highly sensitive rT-PCR to quantify the copies of cell associated HIV Gag DNA found per million cells in each sorted subset

from the PD-1 treated and untreated RM at week 24 post ART. Our results indicate that the SIV reservoir is more highly enriched in memory CD4 T cells of a less differentiated phenotype, as it was observed that T_{SCM}, T_{CM}, and T_{TM} contained a greater number of cells harboring HIV cell associated DNA than the T_{EM} population **(Fig. 4.9A)**. Moreover, anti-PD-1 treated animals compared to saline control treated animals showed no significant difference in levels of cell associated Gag DNA found in any of the memory CD4 T cell subsets studied under suppressive ART. A quantitative viral outgrowth assay, which can assess replication competent DNA versus total viral DNA, should be conducted to specifically measure the impact of PD-1 blockade on the size of the viral reservoir (266). It is interesting to note that one animal that had very low levels of cell associated DNA in all memory CD4 T cell subsets was also the macaque that generated antibody responses against the infused antibody (data not shown). PD-1 expression was undetectable in this animal at the time-point when cell associated DNA was measured, despite infused antibody titers for this animal having waned 18 weeks prior. We are unable to fully understand at this time whether the antibody responses developed against the infused antibody resulted in depletion of PD-1+ CD4 T cells that ultimately led to a reduction in the total reservoir in this macaque. Further studies need to be conducted to measure cell associated DNA and replication competent DNA in lymphoid sites (peripheral lymph nodes and rectal mucosa) in addition to the blood to better understand the total reservoir status of all macaques.

Discussion

Our data are consistent with previous *in vivo* therapeutic PD-1/PD-L1 blockade studies demonstrating enhanced anti-viral immunity during chronic SIV infection (146, 179, 214, 267). This present study initiated PD-1 blockade 10 days prior to the initiation of ART, resulting in enhanced proliferation, effector function and polyfunctionality of anti-viral CD8 T cells. To date, there is a lack of information or supporting data on the clinical benefit of administering repeated PD-1 receptor blockade with the initiation of ART. Amancha *et al* studied the effect of blocking the PD-1/PD-L1 pathway during short term ART, administered 16-20 weeks post SIV infection with a 1 week overlap of rPD-1-Fc treatment (22) initiated at 19 weeks post infection. Administration of ART and soluble rPD-1-Fc showed enhanced survival and polyfunctionality of anti-viral CD4 and CD8 T cells, enhanced *ex vivo* proliferation, and a slight delay in viral rebound, but no differences were observed in the set-point viral loads exhibited post treatment interruption. Moreover, due to the timing of rPD-1Fc administration, the cumulative response of PD-1 blockade therapy may not have had a maximal impact, as rPD-1Fc was administered 4 weeks post the initiation of ART, when viral replication was significantly reduced. PD-1 expression is highly expressed on lymphocytes during chronic SIV/HIV infection (124, 194, 224), and expression of PD-1 is associated with high antigen load (208, 268). Targeting the PD-1 receptor or pathway during the window of time in which viral replication is greatest may result in a greater clinical benefit in reversing immune dysfunction associated with persistent PD-1 expression. Importantly, in our study we observed that PD-1 blockade administered prior to the initiation of ART resulted in more rapid suppression of viral replication with the introduction of ART and is the first study to show synergy between PD-1 blockade and viral suppression with ART during chronic SIV infection.

HIV/SIV infection leads to a significant depletion of CD4+ T cells in the GI tract, which persists despite the initiation of ART. It is now well appreciated that chronically HIV-infected

individuals experience a preferential loss of Th17 cells in the GI tract (56), and this process was not observed in non-pathogenic sooty mangabeys (SM) that were also characterized in this study. It is thought that non-pathogenic hosts such as SM may maintain Th17 frequencies in the GI tract, despite overall loss of mucosal CD4 T cells, as a means to prevent perturbed mucosal homeostasis, such as microbial translocation and systemic immune activation. A study by Macal *et al*. demonstrated that some HIV-infected individuals that were treated long-term (greater than 5 years) were able to reconstitute both Th17 and CD4 T cell frequencies in the GI tract and periphery (183). Restoration of Th17 CD4+ T cells in this study was associated with improved polyfunctional HIV specific T cell responses, despite continued CD4+ T cell proviral burden and residual immune activation that persisted in the GI tract. In our study we observed restoration of both CD4 T cell frequencies and Th17+ CD4 T cells in the rectal mucosa and periphery of PD-1 treated macaques, with a significant 3 fold higher induction of Th17+ CD4 T cells in the rectal mucosa of treated RM. Our data suggests that PD-1 blockade used as an adjunct therapy to ART may provide enhanced reconstitution of CD4 T cell and Th17 frequencies under suppressive ART that may contribute to a better clinical outcome during long-term therapy. A recent study d'Ettore *et al*. found that during eight months of combined anti-retroviral therapy (cART), the magnitude of intestinal CD4+ T cell reconstitution correlated with the reduction in plasma LPS (a marker frequently used to indicate microbial translocation and damage to the GI tract) and pre-treatment frequencies of activated CD4+ T cells predicted the magnitude of Th17 cell reconstitution (261). We found in our study that pre-ART frequencies of Ki-67+ CD4 T cells inversely correlated with Th17 frequencies after 24 weeks of ART **(Fig. 4.4C)**. Our data suggest that PD-1 blockade may modulate the levels of immune activation prior to or during ART allowing for decreased levels of immune activation and enhanced CD4 T cell reconstitution under suppressive ART.

Our study also reported that PD-1 blockade had a limited effect on the level of cell associated Gag DNA within memory subsets under suppressive ART. Recent studies have suggested that early initiation of ART therapy can reduce the size of the viral reservoir seeded

under ART and subsequently delay or reduce viral rebound after ART cessation in humans (185, 187, 269-271). Whitney *et al*. recently conducted a controlled study to better elucidate the effects of hyper-acute ART administration on the seeding of viral reservoirs and effectively demonstrated that post exposure prophylaxis of SIV, as early as 3 days post infection, can limit the size of the reservoir. Despite effectively limiting emergence of viral RNA and pro-viral DNA in the peripheral blood and lymphoid sites, hyper-acute administration of ART failed to eliminate the viral reservoir and did not prevent viral rebound despite a full 24 week course of suppressive ART. This data is consistent with clinical studies that have shown that most HIV-1 infected individuals that initiate ART during acute infection will still continue to rebound post treatment cessation (188, 271, 272). Although we did not find a difference in the level of cell associated Gag DNA further assays need to be conducted, such as the quantitative viral outgrowth assay, to better assess the size of the viral reservoir in treatment groups under suppressive ART. Furthermore, early ART initiation, particularly during primary infection, when reservoirs are thought to be seeded, may be required for PD-1 blockade to have a measurable effect on limiting reservoirs that are seeded and then continue to persist during acute infection. Hyper-acute ART initiation in RM in the study by Whitney and colleagues did highlight immunological consequences to early ART initiation such as the limited development of anti-viral CD8 T cell responses. Exposure to viral antigens is critical for developing a functional anti-viral immunity to SIV/HIV, and thus pre-existing immunity prior to early initiation of ART may be required for effective targeting of the viral reservoir. An improved understanding of the viral reservoirs seeded in mucosal and lymphoid sites in addition to systemic sites needs to be addressed in this study to better understand the complete effects of PD-1 blockade with the initiation of ART.

More recently, due to the limited success in generating an effective prophylactic vaccine for HIV, there has been a renewed interest into therapeutic immunomodulation under suppressive ART, which could potentially result in improved anti-viral immunity and targeted elimination of viral reservoirs. Currently, a second phase of this trial is underway to test the efficacy of PD-1

blockade in augmenting immune responses and manipulating the viral reservoir when administered under suppressive ART. Animals enrolled in this study will be discontinued from ART 2 weeks after 3 monthly infusions of PD-1 blockade and combined period of ART suppression of 36-40 weeks and viral resurgence will be assessed.

Our current study highlights for the first time, the synergy between PD-1 blockade and anti-retroviral therapy. This study demonstrates that PD-1 blockade administered in tandem with ART results in increased proliferation of total CD4 and CD8 T cell responses, as well as enhanced proliferation and function of SIV specific CD8 T cell responses. Moreover, we find that PD-1 blockade resulted in more rapid viral suppression with the initiation of ART. Moreover, our findings highlight the importance of designing studies to better identify timing of blockade in tandem with ART or combination therapeutic strategies that will more effectively target and eliminate the viral reservoir. Recent studies have shown beneficial effects of recombinant IL-21 in limiting inflammation, viral persistence, and improving immune reconstitution of Th17+ CD4 T cells in ART suppressed RM (273). Further studies need to be conducted to better understand complementation of rIL-21 with PD-1 blockade and other therapeutic vaccine strategies and an effort to achieve a functional cure for HIV.

Material and Methods

Study group. Twenty-seven Indian rhesus macaques (Macaca mulatta) infected with SIV were studied. Eleven macaques were used for the anti-PD-1 treatment group and sixteen macaques were used for the saline control group. All twenty-seven macaques were infected intra-rectally with SIVmac251 at a dose of 647 $TCID_{50}$ (1.25 x 10^7 copies of viral RNA). There were a total of 13 Mamu A*01 macaques, 5 were enrolled in the PD-1 blockade treatment group and 6 were enrolled in the saline control group. The criteria for enrolling macaques were based on a plasma viral load cut-off of between 10,000 and 100,000 RNA copies/mL of plasma at week 24-post infection. SIV RNA levels were determined using a qPCR (238). All animals were treated with a 4-drug ART regimen consisting of 3 reverse transcriptase inhibitors (AZT; 5 mg/kg administered orally twice daily, and PMPA; 20 mg/kg and FTC; 30mg/kg, administered subcutaneously once daily), and 1 protease inhibitor (Kaletra, 12 mg/kg of Lopinavir and 3 mg/kg of Ritonavir, administered orally twice daily. Macaques were housed at the Yerkes National Primate Research Center and were cared for under guidelines established by the Animal Welfare Act and the NIH 'Guide for the Care and Use of Laboratory Animals' using protocols approved by the Emory University IACUC.

In vivo antibody treatment. Macaques were infused with a primatized antibody (clone EH12-2132) in a 25mL solution of saline or 25 mL of saline alone. The anti-PD-1 antibody has a humanized EH12 heavy chain variable domain with rhesus macaque IgG4 (CH1-hinge-CH2-CH3 domains) and a humanized EH12 kappa variable domain with rhesus macaque kappa constant region. The clone EH12 binds to macaque PD-1 and blocks interactions between PD-1 and its ligands *in vitro* (data not shown). Antibodies were administered intravenously at 3 mg per kg of body weight on days 0, 3, 7, 10 and 14.

Isolation of mononuclear cells. Mononuclear cells were isolated from the blood and rectal tissue, and flow cytometry analysis was performed as described previously (224). Briefly, peripheral blood mononuclear cells were isolated from blood collected in CPT tubes and ACK lysed to remove residual red blood cells. Mononuclear cells from the rectum were isolated after tissue was digested for 2 hours in complete medium with 10% fetal bovine serum, 1% Penicillin/streptomycin, 0.05% Gentamycin, 1% Hepes, 200U/mL of Collagenase IV (Worthington, Lake Wood, NJ), and DNase I (Roche, Indianapolis, IN). Digested tissue was then passed through decreasing size needles (16-, 18-, and 20-gauge, fix to six times).

Antibodies. The following antibodies were used. FITC conjugated Perforin (clones: Pf-80/164; BDBiosciences), PE-conjugated CxCR5 (clone MU5UBEE; eBioscience), PerCP conjugated CD3 (Clone SP-34-2; BDBiosciences), Streptavidin-APC- conjugated to biotinylated Gag-CM9 Tetramer (courtesy of the laboratory of Rafi Ahmed), PE-TR conjugated CD28 (Clone CD28.2; eBioscience), BV605 conjugated CD95 (clone DX2; BDBiosciences), Brilliant Violet 421-conjugated CD279 (PD-1; Clone EH12.1; Biolegend), BV510 conjugated CD8 (Clone SK1; BDBiosciences), APC conjugated CCR5 (Clone 3A9, BDBiosciences), Live Dead-IR stain (Invitrogen), Pe-Cy7 conjugated Ki-67 (Clone B56; BDBiosciences) Brilliant Violet 650 conjugated CD4 (Clone L200; BDBiosciences), BV711 conjugated CD127 (Clone HIL-7R-M21), Alexa700 conjugated Granzyme B (clone GB11), APC conjugated IL-21 (Clone 3A3-N2; BDBiosciences), BV605 conjugated IL-2 (Clone MQ1-17H12; BDBiosciences), Alexa700 conjugated IFNγ (Clone B27; BDBiosciences), PE-TR conjugated TNF-α (Clone MAb11).

Intracellular cytokine staining. Fresh blood and rectal samples were suspended in RPMI medium (Gibco, Life Technologies) with 10% FBS (HyClone, Thermo Fisher Scientific), 100 IU/mL of penicillin, and 100 μg/mL of streptomycin (Lonza). Stimulations were conducted in the

presence of anti-CD28 antibody and anti-CD49d antibody (1μg/ml; BD Pharmingen). One million cells were stimulated with either 200ng/ml of PMA and 1ug/mL of Ionomycin, or pooled peptides spanning the entire SIV Gag protein (single pool of 125 peptides with each peptide at a concentration of 1.0ug/mL; NIH AIDS Research and Reference Reagent Program catalog number 6204), Env1 and Env2 in the presence of Brefeldin A (5 μg/mL; Sigma) and GolgiStop (0.5 μL/mL; BD Pharmingen) after 2 hours of stimulation for 4 hours at 37°C in the presence of 5% CO_2. At the end of stimulation, cells were washed once with FACS wash (PBS containing 2% FBS and 0.25g of sodium azide) and surface stained with Live/Dead IR, anti-CD8 and anti-CD4 at room temperature for 20 min. Cells were then fixed with cytofix/cytoperm (BD Pharmingen) for 20min at 4°C and washed with Perm wash (BD Pharmingen). Cells were then incubated for 30 min at 4°C with antibodies specific to CD3, IL-2, IFNγ, and TNF-α, washed once with Perm wash, once with FACS wash, and re-suspended in PBS containing 1% formalin. Cells were acquired on LSR-Fortessa with four lasers (205,288, 532, 633nm) and analyzed using the FlowJo software (Treestar Inc. CA). At least 50,000 events were acquired for each sample.

Phenotyping. Mononuclear cells isolated from the blood and rectum and were stained with LIVE/DEAD Near-IR Dead Cell stain (Life Technologies) at room temperature for 15 min in PBS to stain for dead cells. Cells were then washed with FACS wash and stained on the surface using antibodies specific to CD3, CD4, CD8, CD28, PD-1, CD95, CXCR5, CCR5, GagCM9 tetramer, and CD127, and then treated with 1x BD FACS Lysing solution for 10min at room temperature, permeabilized with 1x BD Permeabilizing solution for 10 min at room temperature, washed with FACS wash, stained with anti-Ki67, anti-Perforin, and anti-Granzyme B antibodies, washed 2x with FACS wash, and assessed by flow cytometry.

Anti-PD-1 ELISA. To measure the levels of infused PD-1 antibody, plates were coated with human PDCD1/PD-1 protein (Sino Biological, catalog number: 10377-H-8H-50), blocked and incubated with different dilutions of plasma to capture the infused anti-PD-1 antibody. Bound antibody was detected using anti-mouse IgG conjugated to HRP (pre-absorbed to human immunoglobulin, Southern Biotech). Known amounts of anti-PD-1 antibody captured in the same manner were used to generate a standard curve. To measure the levels of monkey antibody response against the anti-PD-1 antibody, plates were coated with anti-PD-1 antibody (5 $\mu g\ ml^{-1}$), blocked and incubated with different dilutions of serum to capture monkey antibody against the anti-PD-1 antibody. Bound antibody was detected using anti-human λ-chain-specific antibody conjugated to HRP (Southern Biotech). This detection antibody does not bind to the PD-1 antibody because only the constant regions of the heavy and light chains were humanized and the constant region of the light chain is ϰ. The amount of captured monkey immunoglobulin was estimated using a standard curve that consisted of known amounts of purified macaque immunoglobulin that had been captured using anti-macaque immunoglobulin.

Quantification of SIV RNA Viral Load. The SIV copy number in the plasma was determined by using a quantitative real-time PCR as previously described (274). All samples were extracted and amplified in duplicate and the mean of the two values were then reported.

Cell Sorting and quantification of SIV*gag* DNA. Peripheral blood mononuclear cells were isolated, processed, and stained with LIVE/DEAD Near-IR Dead Cell stain, anti-CD3, anti-CD4, anti-CD8 and anti-CD45RA, anti-CCR7, anti-CD28, and anti-CD95 for 25 minutes at 4°C and the T_{CM} (CCR7+ CD45RA- CD28+ CD95+), T_{EM} (CCR7- CD45RA- CD28- CD95+), T_{TM} (CCR7- CD45RA- CD28+ CD95+), and T_{SCM} (CCR7+ CD45RA+ CD28+ CD95+) CD4 memory T cell populations were sorted using a FACSAriaII (BD). In all sorting experiments, the grade of purity

on the sorted cells was >93%. Quantification of SIVmac gag DNA was performed as described previously (43). For cell number quantification, quantitative PCR was performed simultaneously for monkey albumin gene copy number. The sensitivity of the assay is fifty SIV DNA copies per 10^6 cells and samples with undetectable SIV DNA were assigned the value of half of the lower limit of detection.

Statistical analysis. Statistical analyses were performed using Prism (version 5.0d; GraphPad Software Inc.). Statistical significance (p-values) was obtained using paired-t tests (for comparisons between matched time-points for each animal), unpaired Mann Whitney t-tests for unmatched time-points and animals. A Log Rank Mantel Cox test was used for comparison of time to suppression between treatment groups. We also used a non-parametric spearman correlation analysis for all correlations shown. (240).

Acknowledgements

We thank the veterinary staff at Yerkes for animal care; CFAR virology core for viral RNA and qPCR analysis; and CFAR immunology core for help with flow cytometry; This work was supported by the NIH grants R01 AI074471, R01 AI071852 and RC2 CA149086 to RRA; YNPRC base grant, P51 RR00165; Emory CFAR grant, P30 AI050409.

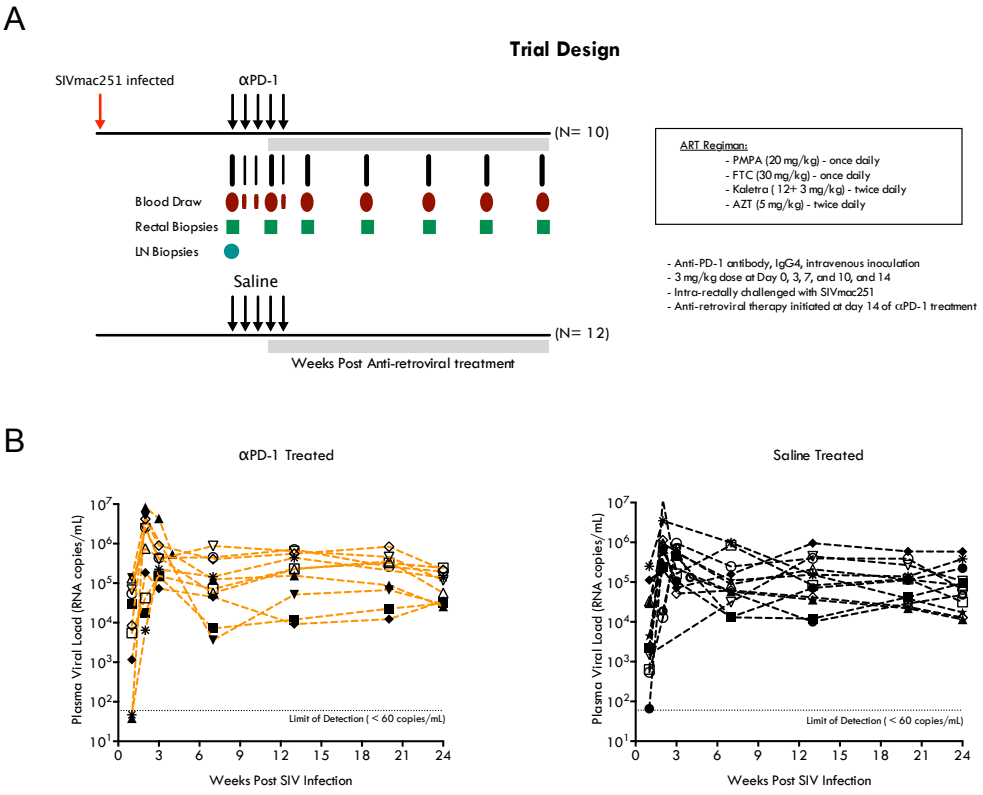

Figure 4.1

Figure 4.1. *In vivo* PD-1 ART study design. (A) 22 rhesus macaques were intra-rectally infected with SIVmac251 for 24-30 weeks and then either treated with saline (n = 12) or primatized anti-PD-1 antibody (n=10) at 3mg/kg dose on day -10, -7. -3. 0 and 4 days post ART. Whole blood and rectal biopsies were sampled at –day 10, 0, week 2, week 6, week 12, and week 24 post ART and 2 mL EDTA blood draws were collected at day all major and minor time-points post ART. (B) Kinetics of plasma viral loads in both cohorts of macaques prior to treated with saline or anti-PD-1 antibody.

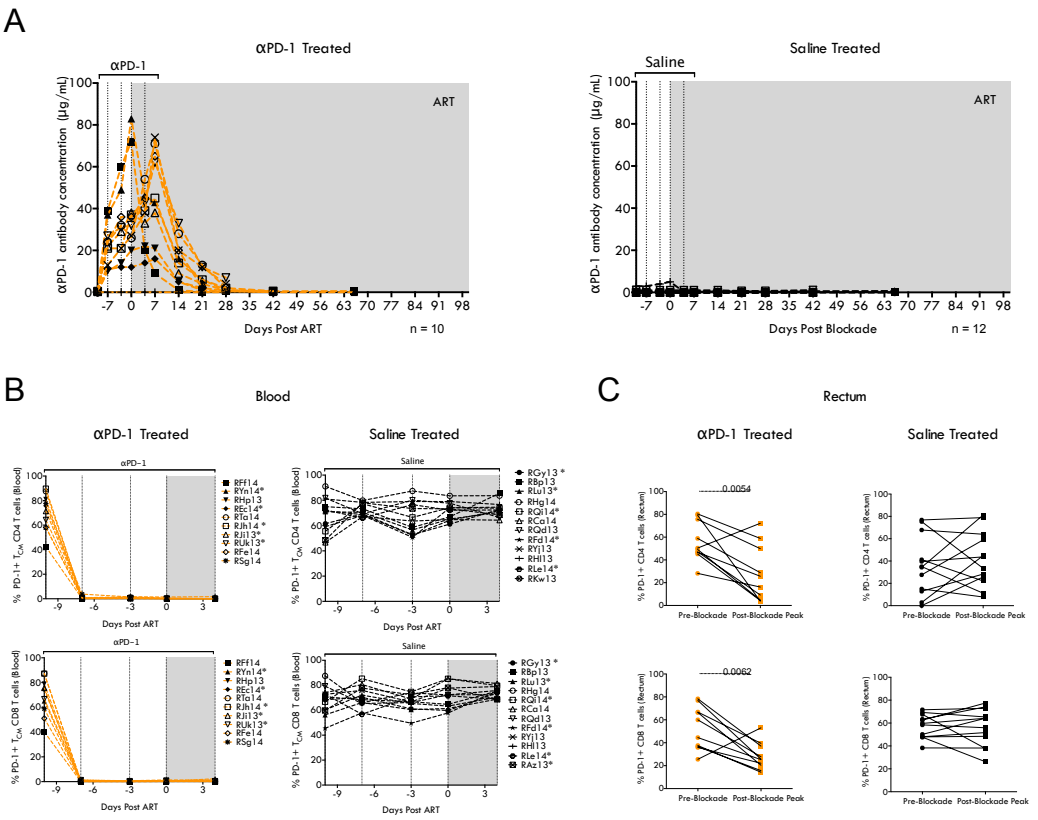

Figure 4.2

Figure 4.2. Rapid and discernable PD-1 blockade *in vivo*. (A) Plasma concentrations of anti-PD-1 antibody (B) Flow cytometric analysis in anti-PD-1 treated and saline treated macaques of the frequencies of detectable PD-1+ central memory (CD95+ CD28+; T_{CM}) CD4 and CD8 T cells in the blood, shown as a percent of T_{CM}+ CD4 and CD8 T cells over the 2 week course of anti-PD-1 or saline treatment (B), and PD-1+ CD4 and CD8 T cells in the rectal mucosa as a percent of live CD4 and CD8 T cells at baseline and day 10 post blockade (C) using the fluorescently conjugated EH12 clone of anti-PD-1 antibody used for the *in vivo* administration of PD-1 blockade. Paired t-tests were used for matched animals at matched time-points.

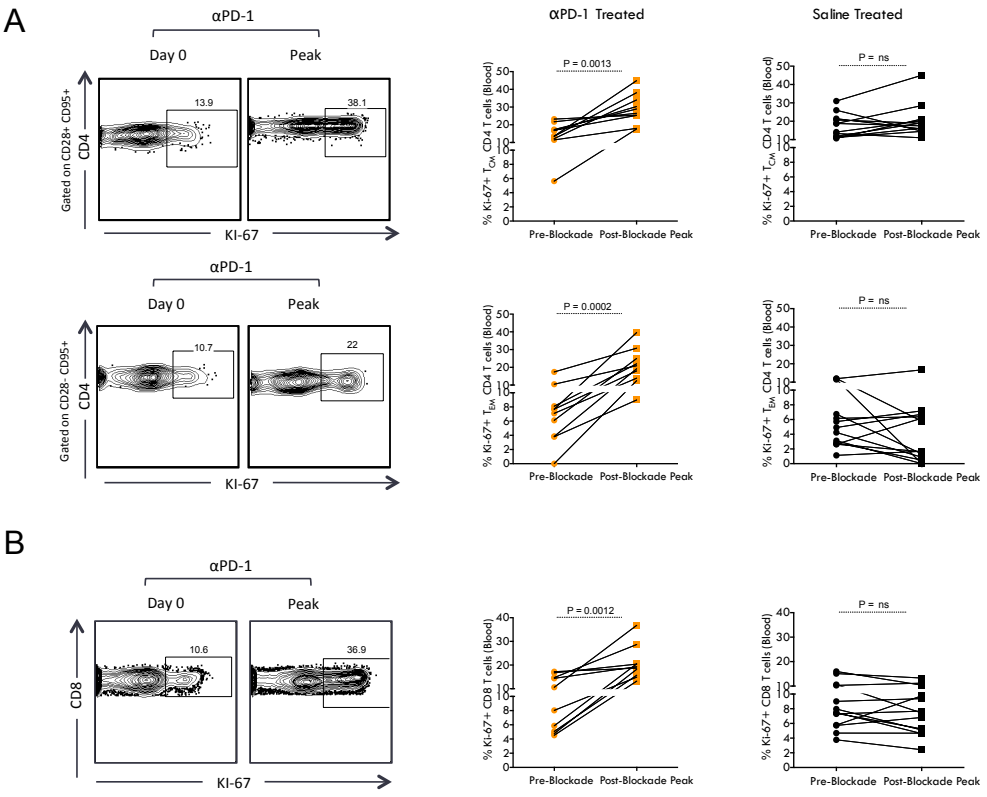

Figure 4.3

Figure 4.3. PD-1 blockade results in enhanced *in vivo* proliferation of CD4 and CD8 T cell. Representative flow cytometry (FACS) plots and corresponding scatter plots showing the frequencies of Ki-67+ central memory (CD95+ CD28+; T_{CM}) and effector memory (CD95+ CD28-; T_{EM}) CD4 T cells as a percent of respective memory CD4 T cells (A), and representative flow plots and frequencies of Ki-67+ CD8 T cells as a percent of CD3+ CD8+ T cells (B) in the blood of PD-1 treated macaques at day 0 and the respective peak time-point post PD-1 blockade (day 7-10 for PD-1 treated macaques and day 10 post saline infusion). Paired t-tests were used for matched animals at matched time-points.

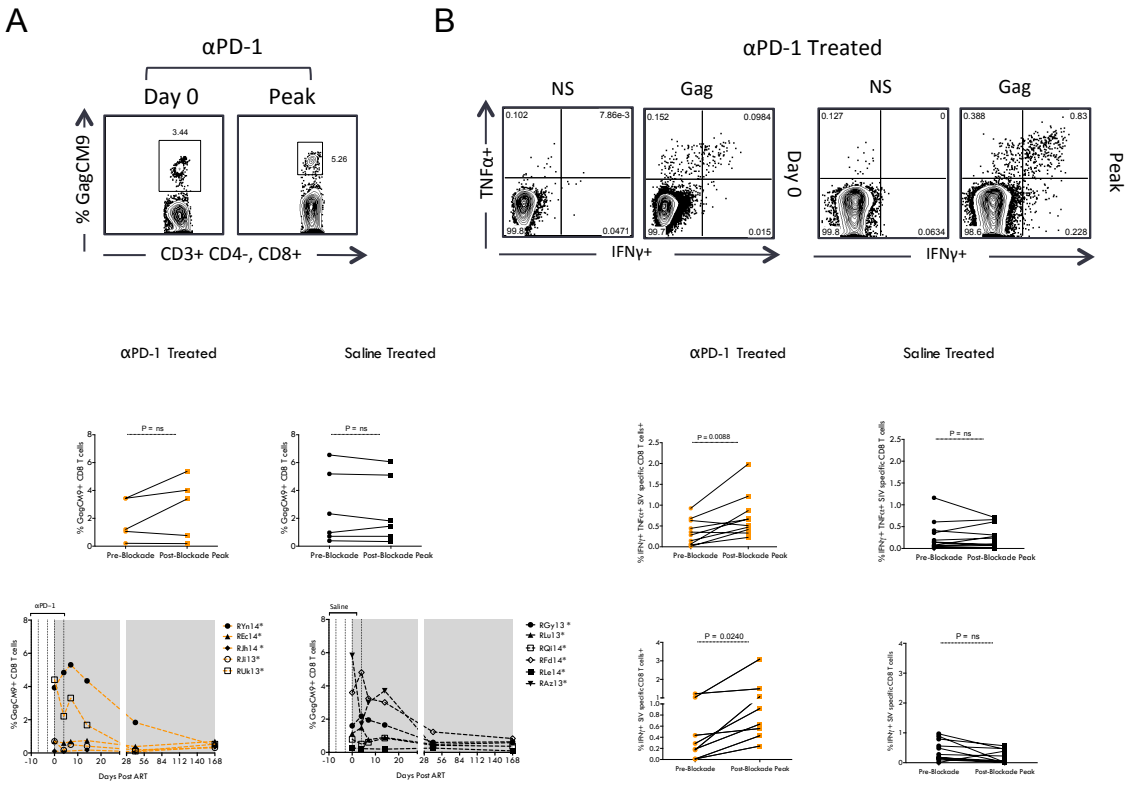

Figure 4.4

Figure 4.4. PD-1 blockade results in enhanced polyfunctionality of SIV specific CD8 T cells. (A) Representative flow plots of GagCM9+ CD8 T cells in the blood of an anti-PD-1 treated macaque at day 0 and day 10 post blockade and corresponding scatter plots showing the frequency of GagCM9+ CD8 T cells in the blood of anti-PD-1 treated and saline treated macaques at baseline and at the respective peak time-point post blockade (day 7-10 post PD-1 blockade, day 10 for saline treated animals) (top) and the kinetics of the frequency of GagCM9+ CD8 T cells after the administration of ART. (B) Representative flow plots showing CD8 T cells co-producing TNF-α and IFN-γ in response to SIV antigens (Gag, Env1, and Env2) at day 0 and at day 10 post PD-1 blockade and corresponding scatter plots showing the frequencies of double producing TNF-α+ IFN-γ+ CD8 T cells (top) and IFN-γ+ CD8 T cells in the blood of PD-1 treated and saline treated macaques. Paired t-tests were used for matched animals at matched time-points.

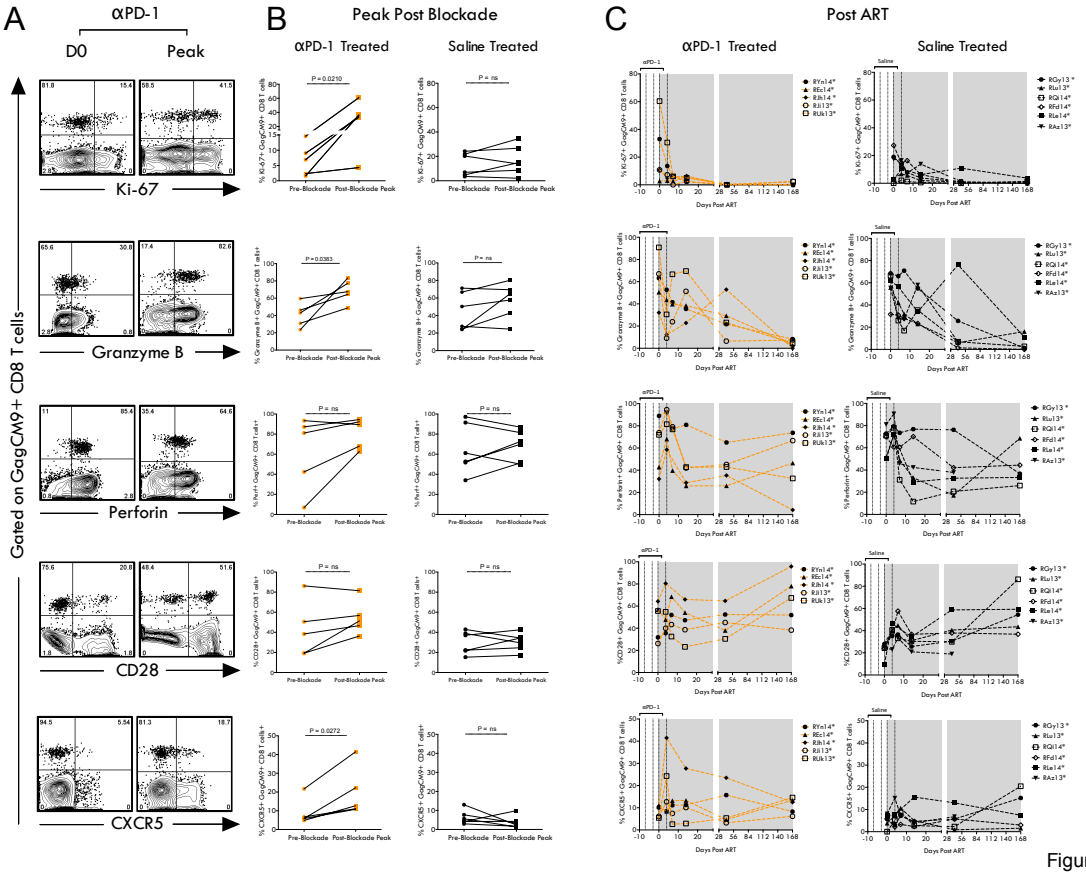

Figure 4.5

Figure 4.5. PD-1 blockade results in enhanced functional quality of CD8 T cells. (A) Representative FACS plots for a anti-PD-1 treated macaque showing the phenotype of GagCM9+ CD8 T cells at baseline and peak post PD-1 blockade in the blood and (B) the corresponding scatter plots showing the frequency of GagCM9+ CD8 T cells expressing Ki-67, Granzyme B, Perforin, CD28, and CXCR5. (C) Kinetics of the frequency of GagCM9+ CD8 T cells expressing Ki-67, Granzyme B, Perforin, CD28, and CXCR5 over time with the administration of ART. Paired t-tests were used for matched animals at matched time-points.

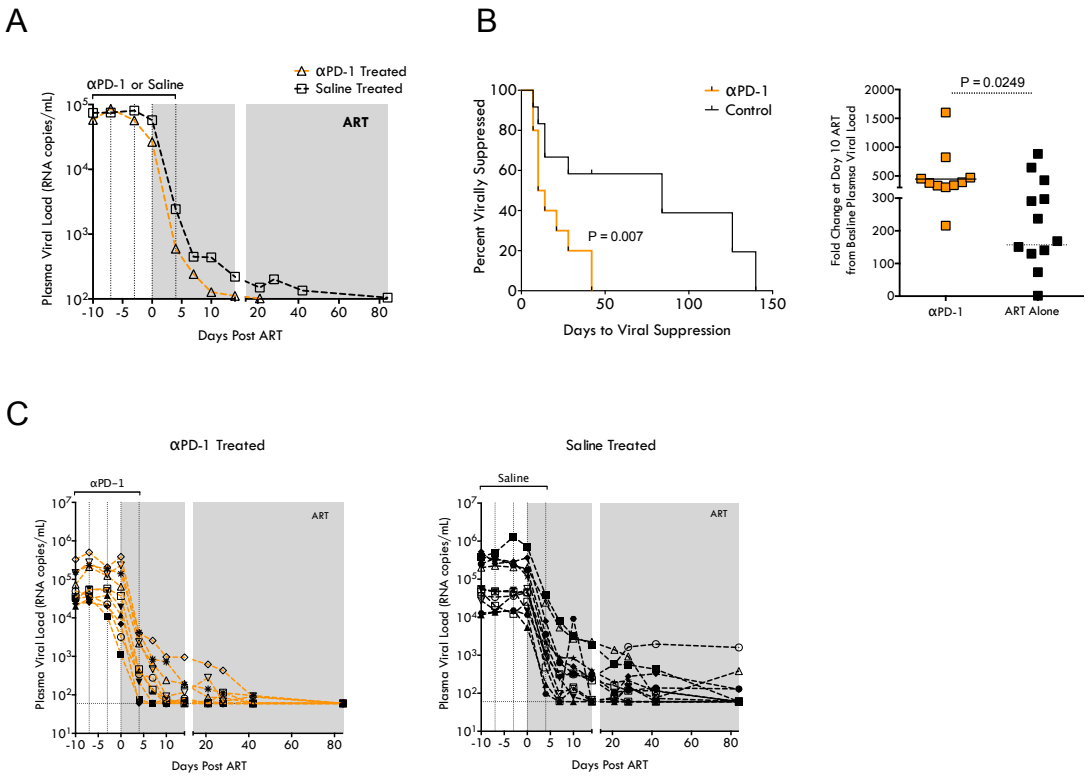

Figure 4.6

Figure 4.6. ***In vivo*** **PD-1 blockade synergizes with ART resulting in increased viral pull down.** (A) Plotted kinetics of plasma viral load post treatment showing the geomeans at each respective time-point post blockade of plasma viral loads for the anti-PD-1 treated and saline treated macaques. (B) Plot showing the percentage of macaques and the corresponding days to viral suppression for the anti-PD-1 treated and saline alone treated animals post ART initiation and a scatter plot showing the fold change in plasma viral load at day 10 post ART from baseline in each individual macaque treated with anti-PD- 1 or saline. (C) Kinetics of viral loads for each macaque in the anti-PD-1 treated and saline treated groups post treatment and the initiation of ART. Unpaired non-parametric Mann-Whitney test was used for statistical analysis between anti-PD-1 treated and saline treated macaques.

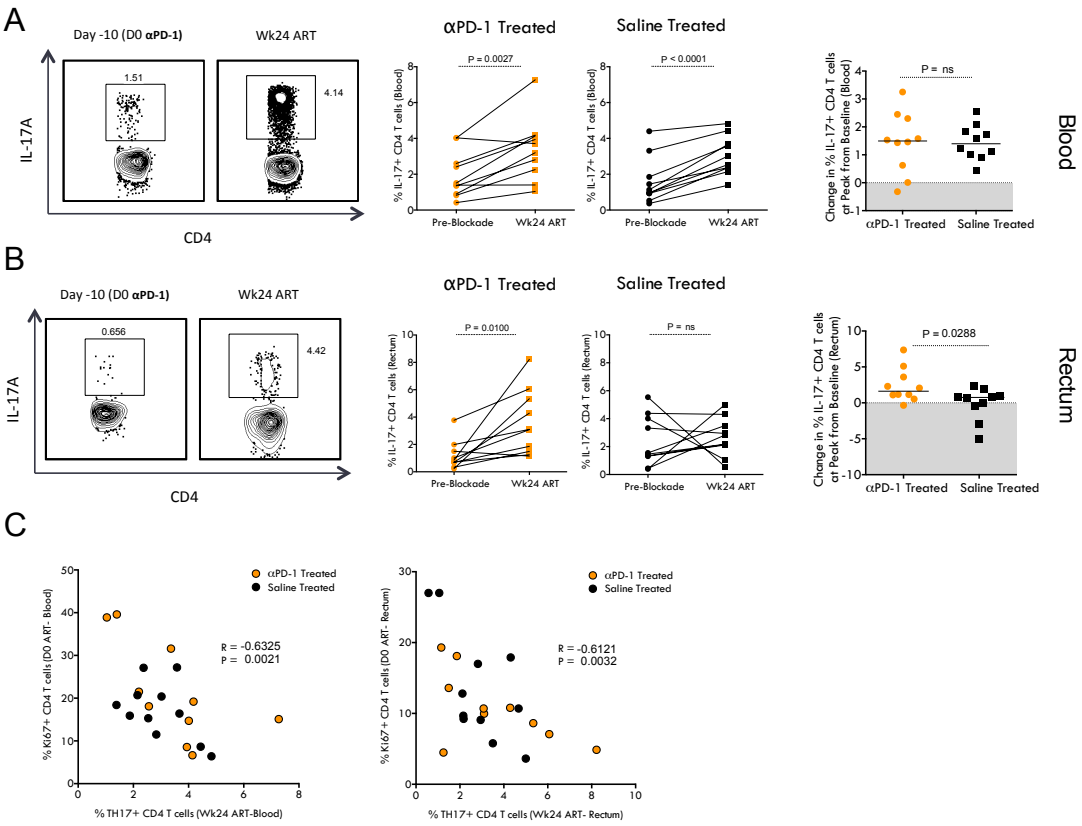

Figure 4.7

Figure 4.7. ***In vivo*** **PD-1 blockade enhances Th17 reconstitution in the rectal mucosa under suppressive ART.** Representative FACS plots of an anti-PD-1 treated macaque at baseline and week 24 post ART and corresponding scatter plots showing the frequency of IL-17A producing CD4 T cells and the fold change in IL-17+ CD4 T cells at week 24 post ART from baseline in the blood (A) and rectal mucosa (B) of anti-PD-1 treated and saline treated macaques. (C) Correlations between the frequencies of Th17+ CD4 T cells at week 24 of ART and proliferating (Ki-67+) CD4 T cells in the blood and rectal mucosa at day 0 of ART initiation.

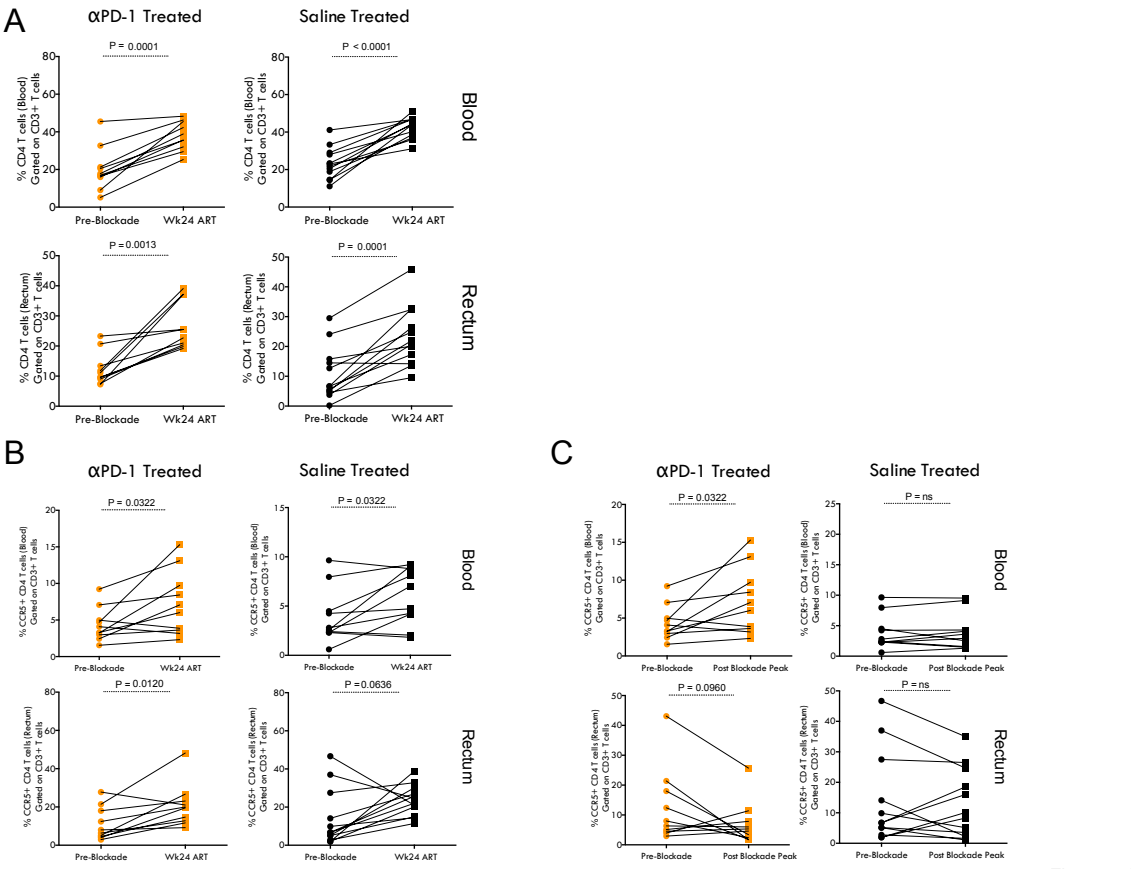

Figure 4.8

Figure 4.8. Partial reconstitution of CD4 T cells in the blood and rectal mucosa of ART treated macaques. (A) Scatter plots showing the frequency of CD4 T cells as a percent of CD3 T cells in the blood and rectal mucosa of anti-PD-1 treated and saline treated macaques, (B) the frequency of CCR5+ CD4 T cells as a percent of CD3+ T cells in the blood and rectal mucosa at baseline and week 24 post ART, and (C) the frequency of CCR5+ CD4 T cells in the blood and rectal mucosa of anti-PD-1 treated and saline treated macaques at baseline and the peak time-point post PD-1 blockade (D10 post anti-PD-1 treatment).

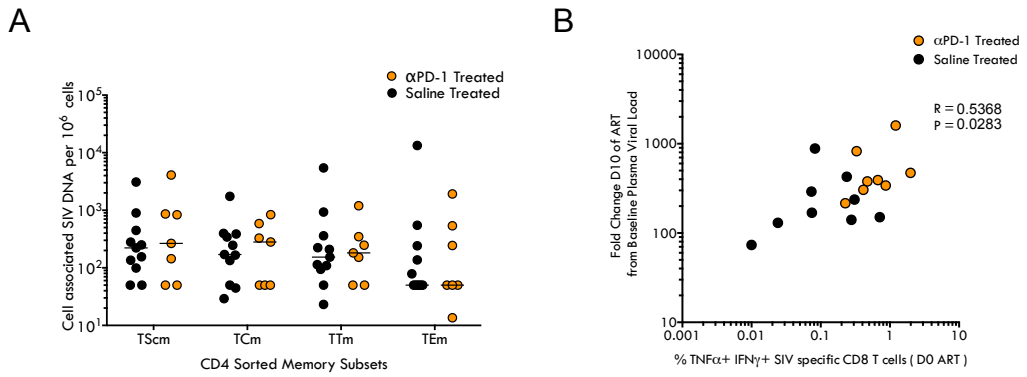

Figure 4.9

Figure 4.9. Levels of cell associated Gag DNA within memory CD4 T cell subsets in PD-1 treated and control groups during suppressive ART. (A) Scatter plots showing the copies of cell associated SIV gag DNA per 10^6 cells from FACS sorted T_{SCM} (CCR7+ CD45RA+ CD28+ CD95+), T_{CM} (CCR7+ CD45RA- CD28+ CD95+), T_{TM} (CCR7- CD45RA- CD28+ CD95+), and T_{EM} (CCR7- CD45RA- CD28- CD95+) memory CD4 T cell subsets in the anti-PD-1 treated and saline treated macaques. (B) Correlation between the frequency of SIV specific TNF-α and IFN-γ double positive CD8 T cells at day 0 of ART and fold change in plasma viral load from baseline after 10 days of ART.

Chapter 5: Discussion

The isolation and identification of HIV greater than thirty years ago significantly impacted the global scientific field and consequently the HIV/AIDS epidemic has become one of the most significant and highly studied global health burdens. The most important and critical medical advancement in the clinical management and treatment of HIV/AIDS has been the development of highly active anti-retroviral therapy (HAART), also referred to as anti-retroviral therapy (ART). The introduction of ART in the early to mid 90's significantly impacted the course of the HIV epidemic, dramatically improving both viral control and life for patients living with HIV. In 2011, an HIV Preventions Trials Network (HPTN) 052 study validated the profound effects early HIV treatment has on transmission rates, demonstrating that the risk of transmitting HIV to an uninfected partner was reduced by 96% when patients were treated with ART (275). The global community has worked tirelessly to develop an effective prophylactic vaccine capable of affording sterilizing immunity to HIV, but despite five large-scale vaccine efficacy trials conducted to date, only one trial, RV144, has demonstrated modest protection from acquisition of infection. Two immune correlates of protection were identified from this trial; the first showing a decreased risk of infection in vaccinated individuals that developed non-neutralizing IgG antibodies against the V_1V_2 loops of the Env protein of HIV and the second demonstrating an increased risk of infection in vaccinees that retained higher levels of plasma IgA. Moreover, it was shown the individuals that showed lower plasma IgA titers showed higher antibody dependent-cytotoxicity (ADCC) and NAb responses, suggesting that higher IgA antibody titers may antagonize IgG mediated antibody responses (134, 276, 277).

Despite the efficacy of ART in controlling HIV replication, treatment cannot eliminate viral reservoirs seeded early during primary infection in metabolically inactive cells. Viral rebound post treatment cessation is a direct consequence of latent but persistent virus maintained in these viral reservoirs (278, 279). With the lack of success in generating an effective

prophylactic vaccine and the currently 15 million and growing number of HIV infected individuals on life-long ART(8), the field has re-directed its focus towards modulating the immune response under suppressive ART in hopes of achieving what is now being referred to as a 'functional cure.' The broad definition of a functional cure is a state in which ART can be arrested in HIV infected individuals with limited to no viral resurgence due to host elicited immune control of viral replication. Immune modulation to achieve a functional cure has the potential to eliminate the unequivocal dependency on ART and reduce many of the co-morbidities associated with ART(280). Current strategies to achieve a functional cure include administration of therapeutic vaccine strategies during ART in tandem with latency reactivating drugs (LRA's; capable of purging the viral reservoir) and/or immunomodulators (check-point inhibitors to enhance anti-viral immune responses or cytokine based therapies). Even with long-term ART, complications to the immune system still persist, particularly in those individuals that have initiated ART at later stages of HIV infection (281, 282). These observations underscore the necessity to investigate the effects of novel anti-viral treatment regimens. Studies have shown that while ART facilitates CD4 reconstitution in the blood, there is only a limited improvement in the function of anti-HIV specific CD8 T cell responses and/or reconstitution of CD4 T cells at mucosal sites (82, 283-287). Therefore, strategies aimed at enhancing the immune response under ART will be crucial to developing a functional cure for HIV. Data from our lab has determined important aspects of lymphoid dynamics during chronic SIV infection. In particular, we identified a subset of PD-1^{hi} expressing T follicular helper cells (Tfh) in the lymph nodes (LN) and rectal mucosal of SIV infected rhesus macaques (RM). These Tfh CD4 T cells were enriched in cell associated viral RNA suggesting an active role in contributing to ongoing viral replication and production (158). Importantly, we identified a novel subset of SIV specific GC infiltrating CD8 T cells that correlated with enhanced viral control and reduced infection of Tfh cells. Utilizing PD-1 blockade therapy as a therapeutic adjuvant to ART demonstrated enhanced anti-viral CD8 T cells with increased expression of CXCR5, a chemokine receptor integral for homing to B cell follicles

of lymphoid sites. These findings underscore the importance of identifying cellular subsets at sites of ongoing viral replication and the mechanisms that drive the development and maintenance of these infected cellular subsets. These findings also demonstrate important insights into facets of the immune response that facilitate SIV/HIV pathogenesis and highlight the importance of identifying immunomodulators that can significantly enhance immune control during suppressive ART.

Early ART Initiation

The size of the viral reservoir under suppressive ART is one of the most reliable predictors of viral set-points post treatment cessation (67). Recent media coverage into three particular studies has brought attention to the importance of optimal timing for the initiation of ART and the rapid nature of viral reservoir seeding early after primary infection. Two recent reports of achieved virologic remission, the first referred to as the VISCONTI cohort and the second a French teenager, received ART within months of detectable HIV infection. The VISCONTI cohort involved 14 HIV infected adults who were treated within 10 weeks of infection and treatment was then interrupted 3 years later. This cohort has maintained extremely low levels of viral replication in the absence of therapy (271). The French teenager was HIV infected at birth, initially treated with a single drug, zidovudine, and once viral loads increased significantly, the infant then 3 months old at the time, was given a four drug combination ART regimen(288). When the child was 5-6 year old, the child was removed from therapy and remained healthy with undetectable levels of HIV for approximately 12 years. Unfortunately, in the third study, referred to as the 'Mississippi baby', despite hyper-acute initiation of ART within 30 hours postpartum, the infant experienced viral rebound approximately 2 years after treatment interruption (289). Although the child did inevitably exhibit viral rebound, the prolonged control of virus without treatment does suggest that very early administration of ART can significantly restrict the number of cells harboring HIV and supports the implementation of early ART in HIV

infected individuals to promote a reduced viral reservoir. One important difference between the Mississippi baby and the two other studies that achieved virological remission, was the potential immune response allowed to develop in the former two studies. The Mississippi baby was treated immediately after delivery and may not have had adequate time to develop functional anti-viral immunity against HIV, limiting any future immune response that could be elicited post treatment interruption to prevent viral replication once virus re-emerged. Whitney and colleagues provide insight into the timing of ART initiation in a more recent macaque model of SIV infection and early ART administration. This study demonstrated that initiation of ART as early as 3 days post infection was still unable to prevent the seeding of viral reservoirs following an intra-rectal SIV challenge (65). This study also showed that early initiation of ART limited priming of anti-viral CD8 T cell responses such that when ART was interrupted and viral resurgence occurred, there were potentially very limited SIV-specific CD8 T cells present to control viral replication. Structured treatment interruptions of ART have also been used as a therapeutic option to enhance anti-HIV immunity using the pulses of reemerging viremia as a source of antigen in both SIV-infected ART suppressed macaques (290, 291) and HIV-infected humans, but this strategy has also proved to be unsuccessful with minimal effects on decreasing set-point viremia post-interruption. One unexplored possibility to improve the treatment outcomes of hyper-acute ART initiation would be prophylactic vaccination prior to the initiation of ART. This strategy may provide the essential pre-existing immunity necessary to effectively target and limit viral replication post treatment interruption. A recent study assessed hyper-acute CD8 T cell responses in a cohort of high-risk uninfected women, known as the FRESH cohort (Females Rising Through Education, Support, and Health) and studied the magnitude and evolution of CD8 T cell responses and their association with plasma viral loads at set-point post infection (292). This particular study found that early after HIV infection (within 1-3 days following onset of plasma viremia), a massive and highly activated anti-viral CD8 T cell responses was generated. This response was confined to HIV specific antigens and not to other pathogens, and was associated

with enhanced viral control. Thus the generation of a profound early anti-viral CD8 T cells may be crucial in dictating immune control and treatment outcomes both during suppressive ART and post treatment interruption, in the context of host independent control of HIV in the absence of therapy. Our recent study discussed in this thesis, investigating the effects of PD-1 blockade as an adjunct therapy to ART, found enhanced SIV specific immune responses and more rapid viral suppression when PD-1 blockade was administered with the initiation of ART. One potential caveat to this PD-1 blockade and ART study that could affect treatment outcomes is that PD-1 blockade and ART were initiated as late as 30 weeks post SIV infection. Administering ART and PD-1 blockade this late after infection could potentially limit the overall effect of therapy, as viral escape mutations and drug resistance may have developed. It would be important in our study, to better understand to what extent CD8 T cell mutations and drug resistant mutations may have developed. It would also be important to test acute administration of PD-1 blockade and ART, 6-8 weeks post SIV infection and how differential timing of this therapeutic intervention and ART may impact viral suppression and immunological outcomes. Moreover it would be important to explore additional therapies that can increase the magnitude and function of anti-SIV immunity pre-ART initiation, which could potentially facilitate long-term viral control in the absence of ART and reduce the overall reservoir burden seeded under continuous ART. Therapeutic vaccinations may also play a significant role in achieving this due to both its feasibility and efficacy in significantly enhancing anti-viral immune responses.

Targeting the Tfh Viral Reservoir

Recent data has emerged highlighting particular memory CD4 T cell populations that highly contribute to the viral reservoir under suppressive ART. Multiple groups, including our own, have shown that T follicular helper cells (Tfh) are highly infected and actively contribute to ongoing viral replication and production in lymphoid sites during both chronic infection and under suppressive ART (88, 157-160, 169, 197), but it is not well understood what mechanisms

define the propensity of these cells to remain highly infected, to persist, and to resist the cytopathic effects of HIV. In an attempt to better understand what controls the fate of a productively HIV infected versus non-productively infected cell, Doitsh *et al*. determined that over 95% of quiescent lymphoid CD4 T cells die by pyroptosis through a caspase-1 mediated pathway and this pathway is triggered by abortive viral infection (49). Prior to this report being published, *in vitro* studies using HIV infected human lymphoid aggregate culture (HLAC) systems showed a significant loss of CD4 T cells after HIV infection mediated by both caspase 1 and caspase 3 cell death pathways (293). Caspase 3 activation leads to apoptosis without inflammation whereas pyroptosis, through caspase 1 activation, induces a highly inflammatory environment. Doitsh and colleagues demonstrated that in fact the majority of CD4 T cells that are lost during HIV infection die through a pyroptosis and caspase 1 mediated cell death pathway and these cells are not productively infected. Based on recent studies, including the data presented in this thesis, have demonstrated an aberrant accumulation of virally infected Tfh cells at lymphoid sites during chronic SIV/HIV infection. These cells decline slightly under ART but persist and continue to produce virus (88). In light of this recent data that most CD4 T cells that are depleted during HIV infection are non-productively infected cells and that Tfh cells in fact are not depleted but continue to persist during chronic infection, may suggest that Tfh cells and other PD-1+ CD4 T cells are intrinsically resistant to pyroptosis or caspase-1 mediated cell death. Further studies need to be conducted to better understand how PD-1, which is highly expressed on these Tfh cells, contributes to the longevity and maintenance of these viral reservoirs and whether there is a connection between PD-1 signaling and caspase 1 versus caspase 3 mediated cell death. It is known that signaling of PD-1 via its ligands PD-L1 and PD-L2 recruit SHP phosphatases to the PD-1 cytoplasmic tail and subsequently inhibit T and B cell activation (211). Tfh cells, expressing high levels of PD-1 may also retain a more quiescent intracellular environment due to the inhibitory effects of PD-1 signaling. Thus better understanding PD-1 signaling on Tfh cells in lymphoid sites, particular under conditions of ART, where latency is established, would be

crucial in better determining how to target and eliminate these cellular reservoirs.

Another recent hypothesis for the importance of Tfh as an active member of CD4 T cell reservoir under suppressive ART is the particular localization of this subset to germinal centers (GC) of lymphoid follicles. The germinal center and lymphoid follicles as reservoir sites for HIV were described as early as the 1990's (294), but Tfh cells and the contribution of this cellular subset to viral persistence were not studied until very recently. With the current understanding that Tfh cells are highly infected and contribute to viral replication, particularly at lymphoid sites, Fukazawa *et al*. sought to understand SIV replication in Tfh cells in the tissues of elite controller macaques (EC), capable of controlling viral replication to low or undetectable levels in the absence of ART compared to typical progressor macaques (TP). This study found that viral replication was largely restricted to Tfh cells in elite controllers compared to typical progressors, and this was mediated by anti-viral CD8 T cells effective at limiting viral replication in extra-follicular sites, but unable to clear virally infected Tfh cells within B cell follicles of lymphoid sites (171). CD8 T depletion of these EC macaques resulted in outgrowth of SIV to non-Tfh subsets, highlighting the importance of CD8 T cells in limiting ongoing viral replication in non-Tfh cells at extra-follicular sites but exposed the limitations of CD8 T cell mediated control at sites such as the germinal center, largely devoid of CD8 T cells. This study did show through *in situ* hybridization and immunofluorescence that a small but detectable frequency of CD8 T cells are found in the GC, but GC's serve largely as a 'sanctuary' for continued viral replication and production in EC macaques, with limited CD8 T cell infiltration (171). A recent study published by our lab further studied SIV infection at lymphoid sites in DNA/modified vaccinia Ankara (MVA) expressing gag, pol, and env vaccinated macaques subsequently challenged with SIVmac251 intra-rectally. Macaques were separated into 2 cohorts, a group of vaccinated viral controller RM with plasma viral loads less than 10^4 RNA copies/mL of plasma at set-point and a cohort of non-controller RM with plasma viral loads greater than 10^4 RNA copies/mL of plasma at set-point. Our data demonstrated an inverse association between PD-1^{hi} Tfh cells in the LN of

SIV infected macaques and the frequency of SIV specific CD8 T cells (158). Interestingly, in a more recent and extensive study by our lab, we found a population of SIV specific CD8 T cells that expressed CXCR5, the chemokine receptor important for homing to the B cell follicles of lymphoid sites (unpublished). These follicular CD8 T cells (Tfc) in animals that controlled viral infection were present at low but detectable frequencies both in the LN and peripheral blood pre-challenge, underwent an anamnestic expansion post SIV infection, and inversely correlated with both viral control and the frequency of infection of Tfh cells. These data demonstrate that CD8 T cells with the potential to infiltrate the GC may be a crucial component of the anti-viral CD8 T cell response at lymphoid sites. Moreover, we observed through *in situ* Gag-CM9 tetramer staining, a high frequency of infiltrating CD8 T cells in the LN, spleen, and rectal mucosa of vaccinated viral controllers (unpublished). Our data suggest that interventions such as therapeutic vaccinations during suppressive ART may be capable of eliciting Tfc with the potential to enhance viral control at lymphoid sites harboring cellular reservoirs. If a higher density of anti-viral CD8 T cells can be primed to traffic to the GC during ART and more effectively target virally infected Tfh cells in addition to infected CD4 T cells at extra-follicular sites, these cells may play a major role in limiting the established reservoir and achieving viral remission post treatment interruption. Further studies need to be conducted to better understand what adjuvants or vaccine regimens would more effectively prime and expand CXCR5+ follicular SIV specific CD8 T cells. Preliminary data from our lab suggests that TGF-β, in addition to IL-12 and IL-23 can induce a higher frequency of CXCR5+ CD8 T cells *in vitro* during a 5-day proliferation assay (unpublished). Addition of Resiquimod (R848), a TLR 7/8 agonist, in combination with TGF-β further augmented the CXCR5 induction observed on these CD8 T cells (unpublished). Resiquimod has been used as an effective adjuvant in multiple vaccine studies and is effective in enhancing type I interferon responses (295). Recent studies have also demonstrated the effects of a TLR7 agonist on destabilizing the latent viral reservoir during suppressive ART. Whitney *et al.* in collaboration with Gilead Sciences, Inc. carried out a controlled study in which SIV infected

macaques receiving ART were administered bi-weekly doses of an analogue of GS-9620, a TLR-7 agonist. This data presented at CROI 2015, demonstrated that administration of this drug induced transient plasma viremia, and reduced SIV DNA by 30-90% compared to control ART suppressed untreated macaques, and reduced viral set-points after ART interruption. Thus studies need to be conducted to test the effects of resiquimod on CXCR5 induction during SIV infection and under suppressive ART, as well as the possible benefits of expansion of anti-viral Tfc on treatment outcomes, particularly in the context of destabilizing the viral reservoir and improving viral control post ART interruption.

HIV Sanctuary – Exposing the Germinal Center Reservoir

The B cell follicles and in particular Tfh cells that reside in the germinal centers of secondary lymphoid organs have recently been more actively studied due to their ongoing contribution to viral replication and production during chronic SIV/HIV infection and during ART. In trying to develop strategies that can effectively target and destabilize the HIV viral reservoir, it is important to understand what mechanisms underlie the maintenance and stability of the GC that retains these virally infected Tfh. T cells increase their expression of the chemokine receptor CCR7 to coordinate interactions with dendritic cells (DCs) in the secondary lymphoid organs. CD4 T cells, once activated, will also increase the expression of the chemokine receptor CXCR5 that can respond to high densities of its cognate ligand CXCL13 or BCA-1 expressed mainly by reticular and endothelial cells in B cell follicles (296). The co-expression of CXCR5 and CCR7 promote positioning of these activated T cells at the border of the T cell B cell zones in secondary lymphoid regions (297-299). Further down-regulation of CCR7 allows for $CXCR5^{hi}$ CD4 T cells to be attracted to and migrate into the germinal centers of B cell follicles (300). Tfh cells also express high levels of the co-stimulatory molecule ICOS, which is a member of the CD28 family and is induced upon activation. Moreover, ICOS deficient mice have poor T dependent antibody responses and a defective GC. A paper by Bossaller *et al*. demonstrated a

profound decrease in circulating memory CXCR5+ CD4 T cells in humans deficient in ICOS, and patients with this deficiency showed an abrogated generation of GCs as well as almost absent frequencies of the CD57+ CXCR5+ GC specific population (301). These data suggests that ICOS is actively involved in the generation of CXCR5+ CD4 T cells and in GC formation and antibody responses and that potentially modulating the ICOS/ICOSL axis may have profound effects on the generation and stability of virus laden GCs, particularly in the context of SIV/HIV infection. A very recent study by Weber *et al*. investigated the effects of CD28 and ICOS, both co-stimulatory molecules important for T dependent B cell responses in the germinal center. Lack of appropriate co-stimulation results in very small GCs and a reduced number of Tfh cells in mice (302-305). Weber *et al*. found exclusive roles for CD28 and ICOS during the stages of Tfh development. Importantly they found that while CD28 regulated early expression of the master transcription factor of Tfh cells, Bcl-6, ICOS was essential in maintaining the Tfh phenotype by regulating the novel transcription factor Klf2, a transcriptional repressor of ICOS (306). Blocking ICOS resulted in a redistribution of Tfh cells back to the T cell zone and reversion of their phenotype to a non-Tfh effector cell phenotype, consequently leading to the collapse of the GC response. The contribution of ICOS to the integrity of the GC can possibly be modulated during long-term ART to help destabilize and eliminate the viral reservoir that persists at these GC sites. As mentioned earlier, GC's have been thought to serve as a viral sanctuary for infected Tfh cells that persist and contribute to ongoing viral production under ART. Fukazawa and colleagues showed that Tfh cells are the predominate population of virally infected cells at lymphoid sites during elite control of SIV and this can be attributed to a low frequency of anti-viral CD8 T cells infiltrating these dense viral sites (171). Our recent data suggest that germinal center infiltrating SIV specific CD8 T cells may be integral for promoting enhanced viral control at these secondary lymphoid sites (unpublished). Thus immunomodulation of the ICOS pathway may be a highly effective means of destabilizing the germinal centers as active reservoir sites during suppressive ART. Studies from our lab also reveal that frequencies of CXCR5+ SIV specific CD8 T cells can

be induced upon vaccination, thus a therapeutic intervention aimed at enhancing GC/B cell follicle infiltrating CD8 T cells in tandem with a strategy to remove the stability of the GC and re-locate virally infected Tfh cells to extra-GC/follicular areas, with greater exposure to anti-viral CD8 T cells, may serve as an important strategy to eliminate viral reservoirs and limit viral burden post treatment interruption. A blocking monoclonal antibody against ICOS-L (AMG 557; Amgen) has been successfully tested in a phase Ib study with systemic lupus erythematous patients and is currently being evaluated for the treatment of lupus arthritis (Sullivan, B.A., W. Tsuji, A. Kivitz, M. Weisman, D.J. Wallace, M. Boyce, M. Mackay, R.J. Looney, S. Cohen, M.A. Andrew, and et al. 2013. American College of Rheumatology/Association of Rheumatology Health Professionals Annual Meeting). Testing this reagent in a controlled SIV ART macaque study and/or developing small molecule agonists of Klf-2 would be critical to identifying a novel strategy to target and destabilize the GC viral reservoir under ART. These therapeutic interventions could also be tested in combination with PD-1 blockade therapy. We observed enhanced anti-viral immunity in our study in which PD-1 blockade was administered in tandem with the initiation of ART. Administering PD-1 blockade in tandem with ART to boost anti-viral immune response and more rapidly suppress viral replication with ART and then subsequently administering an ICOS-L blocking antibody or small molecule antagonist may generate a more exposed follicular environment allowing for SIV specific CD8 T cells to gain entry into these more excluded sites and eliminate virally infected CD4 T cell targets. Importantly, studies must be conducted to identify the optimal timing, dose, and the possible benefit of using currently tested immunomodulators such as PD-1 blockade in combination with vaccination and/or other targeted approaches aimed at eliminating the HIV reservoir.

Modulating Anti-viral Immunity under ART

Biological

Check-point inhibitors may also be critical in modulating the immune response under suppressive ART. PD-1 blockade has been highly effective in enhancing anti-viral CD8 T cell responses and SIV specific humoral immunity during chronic SIV infection. Macaques receiving *in vivo* PD-1 blockade also showed a transient decline in plasma viral load and pro-longed survival (214). Moreover, a study by Porichis and colleagues in 2011 demonstrated that *in vitro* blockade of PD-L1 restored HIV-specific CD4 cytokine function and was followed by an increase in total cellular proliferation; similar to what was observed in the *in vivo* PD-1 blockade study by Velu *et al*. These data suggest that targeted interference of PD-1 on CD4 T cells might be beneficial in restoring CD4 T cell function and frequencies in ART treated HIV infected individuals (223), leading to improved clinical outcomes. Recent studies have demonstrated that PD-1 and CTLA-4 are also important check-point inhibitors that are highly expressed on CD4 T cells enriched in the latent viral reservoir (307). Studies have tried to address whether PD-1 blockade given either after the initiation of ART or prior to ART interruption can effectively impact viral control post treatment cessation. One study in particular used a recombinant macaque PD-1 fused to a macaque Ig-Fc (rPD-1-Fc) in SIVmac239 infected rhesus macaques to assess the impact of blockade of the PD-1/PD-L1 signaling axis on viral control during ART and post ART interruption. This study found that although SIV specific responses were enhanced post treatment with rPD-1-Fc under ART, and there was a significant delay in viral rebound, virus still rebounded to similar pre-treatment levels indicating no measurable effect on the viral reservoir. Moreover, an unpublished study by Bristol-Myer Squibb, demonstrated that *in vivo* blockade of PD-L1 administered to SIV infected rhesus macaques durably suppressed with ART did result in a delay in viral rebound in 4 out of the 8 anti-PD-L1 treated macaques and a significant reduction in viral set-point post treatment interruption (unpublished, poster presented at CROI, 2014).

We sought to study the effects of PD-1 blockade during suppressive ART on anti-viral

immune responses and destabilization of the persistent viral reservoir. Our data demonstrated that PD-1 blockade administered prior to and during the first 4 days of ART resulted in enhanced proliferation and polyfunctionality of anti-viral CD8 T cells at peak time-points post anti-PD-1 treatment and more rapid kinetics of viral suppression. Preliminary data did not show a significant reduction in the cell associated DNA within the T_{SCM}, T_{CM}, T_{EM}, and T_{TM} memory CD4 T cell compartments after 20 weeks after suppressive ART and PD-1 blockade. However, more extensive analyses need to be carried out with matched animal samples pre- and post ART, to fully understand the effects of PD-1 blockade on the viral reservoir established during suppressive ART. Taken together our data highlights the potential use of check-point inhibitors to modulate viral replication and latency, but underscore the need to further refine what dose and frequency of check-point inhibitors will provide the greatest reduction in the viral burden under ART.

Therapeutic Vaccines

A number of vaccine modalities have been developed and tested over the past decade in a therapeutic setting during HIV and SIV infection, and some of these have been successful in inducing a strong anti-viral T cell immunity with a modest effect on viral control. These vaccine approaches are currently being explored in combination with approaches to "kick" the latent virus out of the persistent viral reservoirs, in an effort to develop strategies to achieve a functional cure for HIV. It is critical to test these therapeutic modalities alone and in combination with the aforementioned check-point inhibitors or latency reactivating drugs to identify which strategy is optimal in modulating the immune response under ART and in particular what combination approach will provide the best clinical benefit and inevitable dissolution of dependence from anti-retroviral drugs. Therapeutic vaccines for HIV infection should aim to elicit anti-viral CD8 T cells (CTLs), CD4 T cells, and neutralizing antibody as these immune responses work in concert to control viral replication. In addition to increasing the magnitude of these immune responses, it

will be important to generate poly-functional T cells (capable of producing multiple cytokines and performing effector functions), as these HIV specific T cells have been shown to be associated with long-term non-progression. It is also critical to generate broad cellular responses as HIV mutates very rapidly to escape immune pressure. Recent data has demonstrated that cytotoxic T lymphocytes (CTLs) effectively target HIV-1 Gag epitopes early after HIV infection and ART, but during chronic infection and when ART is initiated at later stages, greater than 98% of latent viruses carry CTL escape mutations that are incapable of being targeted by CTL's generated against common epitopes seen early after infection (189). These data suggest that CD8 T cell responses in chronically infected patients on ART must be broadly reactive against a spectrum of epitopes in order to target and eliminate the latent viral reservoir. Thus not only do therapeutic vaccines under ART need to generate a robust anti-viral CD8 T cell response, but the CTL response must target a spectrum of viral epitopes in order to eliminate the latent virus seeded in memory CD4 T cells during long-term suppressive ART. The HIV-specific CD4 T cell response is also important for maintaining the functional CD8 T cell and B cell response, in the context of both neutralizing and non-neutralizing antibody responses. However, HIV-specific CD4 T cells boosted during a therapeutic vaccine could also serve as potential targets for virus replication during continuous ART, and thus appropriate measures should be taken, such as the use of intensified ART regimens that include Maraviroc (a CCR5 antagonist) to limit the generation of additional CD4 T cell targets. The function of dendritic cells (DC) may be also critical for generating a protective cellular and humoral immune response, as chronic HIV/SIV infections are associated with impaired DC function (308). Thus, therapeutic vaccines may also need to use strategies such as adjuvants to enhance the function of innate immunity and improve cross-presentation of viral antigens to anti-viral CD8 T cells that can then target and eliminate virally infected and re-activated CD4 T cells expressing antigen under suppressive ART. Lymphoid follicles enriched in latent virus are also important sites that need to be strategically targeted by therapeutic interventions. Vaccine modalities and immunotherapies that can both

enhance and localize anti-viral CD8 T cells to these sites will be critical in targeting and eliminating viral reservoirs at lymphoid sites **(Fig.5.1)**.

The SIV/macaque model has been used to test the therapeutic potential of multiple vaccines to generate a strong anti-SIV T cell response and their influence on control of viral rebound after ART interruption. These include DNA vaccines with and without adjuvants (309), attenuated poxvirus vectors such as NYVAC (310) and MVA (Amara et al., unpublished results), and dendritic cells pulsed with autologous virus (311). The majority of these studies performed vaccinations under ART, while a few studies were performed in the absence of ART. Notably, the majority of these vaccines induced SIV-specific CD4 and CD8 T cell responses with improved function as measured by the production of cytokines such as IFNγ, TNFα and IL-2, and the expression of cytolytic molecules such as granzyme B and perforin. Earlier studies in which macaques were treated with ART very early (within the first few weeks) after SIV infection (312) revealed that ART alone provides some benefit in control of viremia after ART interruption similar to what has been observed in humans (313, 314). Some of these studies showed a modest effect (a log or lower) on control of reemerging viremia after ART interruption, with viral control maintained for only a few months. A recent study by Fuller and colleagues (315) used a particle mediated delivery of DNA vaccine with and without lymphotoxin as an adjuvant and showed a significant reduction in viral burden in the blood and jejunum of SIV-infected ART suppressed macaques, and durable protection from viral rebound post treatment cessation. Interestingly, the enhanced viral control was associated with greater breadth of SIV-specific T cell response in the gut. Experiments are underway to test the therapeutic potential of CMV based viral vector that has recently been shown to effectively control pathogenic SIV infection in the preventive setting (316).

Cytokine based therapies and Latency Reactivating Drugs (LRA's)

Cytokine based therapies and latency-reactivating drugs (LRA's) have also recently been explored for the potential to enhance immune responses and reactivate virus from its latent reservoir. Multiple groups have sought to understand the therapeutic effects of recombinant cytokine infusions and the possible effects on enhancing the immune response and triggering viral reactivation under ART. A study in which rIL-7 was administered to virally suppressed subjects showed a 70% increase in total number of circulating CD4 T cell with integrated viral DNA 4 weeks after therapy. The genetic diversity of the viral reservoir increased as well, indicating that this therapy is not suitable to generate a functional cure (317). IL-2 therapy was also considered promising and received a lot of attention as an immune restoration therapy in HIV infected individuals. Despite 2 internationally phase II trials, ESPRIT and SILCATT, recombinant IL-2 (rIL-2) failed to reduce the risk of HIV associated diseases or death when combined with ART therapy and neither therapy was found to have any clinical benefit (318). Recent studies have indicated that administration of IL-15 (rIL-15) during acute SIV infection induces stronger SIV-specific CD8 T cells but increases viral set point and accelerates disease progression (319). More promising results have been found using rIL-21 as a therapy. A study by Pallikkuth S et al prophylactic rIL-21 administration increased both perforin and granzyme B expression in CD8 T cells and increased the frequency of intestinal Th17 cells, which was associated with reduced levels of intestinal T cell proliferation, microbial translocation, and systemic immune activation during chronic infection (320). A more recent study published by Micci and colleagues(273) has shown promising results when using rIL-21 in combination with ART therapy. This study showed enhanced frequencies of both IL-22 and IL-17 producing CD4 T cells, reduced intestinal immune activation, and lower residual plasma viremia under ART. Although a variety of cytokines can be postulated to boost immune responses and conceivably aid in improving immune responses under ART, currently rIL-21 therapy has proved to be the best option in doing so. The improved mucosal immune function and decreased immune activation as

a result of rIL-21 therapy can be explored as a combination therapy under ART to achieve a functional cure for HIV.

One of the most significant barriers to eradication of HIV is the ability of HIV to integrate into the host genome and transition to a latent state, thus evading detection and elimination by the immune system. An approach to cure research that has gained more recent attention and focus is to find methods of inducing this latent virus to replicate, which may either cause death of the infected cell through cytopathic effects or elimination by HIV specific immune responses. There has been a number of latency reversing agents (LRAs) that have come to light from many anti-cancer studies. Two clinical studies have been conducted with the histone deactelyase inhibitory (HDACi) vorinostat, that have shown promise in forcing latently infected CD4 T cells to begin making viral RNA (321). An important *in vitro* study by Shan *et al*. assessed the cytopathic effects of reversing latency using the histone-deactelyase inhibitor (SAHA) and the contribution of cytolytic CD8 T cells to targeted killing of these virally reactivated CD4 T cells. This study revealed that despite efficient reactivation of resting latently infected CD4 T cells; these cells did not succumb to viral cytopathic effects(322). Furthermore autologous CD8 T cells were incapable of targeting and killing reactivated CD4 T cells, due to impaired effector function. An additional *in vitro* study by Jones RB and colleagues demonstrated that HDACi despite reactivating viral reservoirs impaired CTL mediated killing of HIV infected or pulsed target cells and decreased production of the effector cytokine IFNγ. Of the HDACi tested, romidepsin seemed to exert the greatest inhibitory effect on anti-viral function, compared to SAHA or panobinostat (323). Taken together, these data highlight the importance of generating functional anti-viral CD8 T cells that can effectively eliminate the viral reservoir. These results also support further investigating check-point inhibitors like PD-1 blockade in combination with LRA's to significantly reduce the viral burden under ART. Siliciano and colleagues sought to identify particular LRA's and their efficacy on independently reactivating the latent viral reservoir. The panel of LRA's studied included vorinostat, romidepsin, and panobinostat, along

with three other compounds with different mechanisms of action: disulfuram (acetaldehyde dehydrogenase inhibitor), JQ1 (bromodomain inhibitory) and Bryostatin (Protein kinase C agonist). Unfortunately their results demonstrated that none of the leading LRA's significantly affected the size of the latent reservoir *ex vivo* (324). More recent data presented at CROI 2015, suggests that combination LRA's have shown signs of promise but it is still unclear wither these will be safe to enter into human trials. A second paper published by Cillo and Colleagues comparing the activity of vorinostat and CD4 T cell activation showed that CD4 T cell activation through anti-CD3 and CD28 stimulation was about 20 times higher than that of vorinostat alone. This data suggests that the latent HIV virus is refractory to any currently used in vitro stimulus and we must direct our focus to identifying strategies that increase pro-viral gene expression without inducing global T cell activation to effectively eliminate the viral reservoir. Another more recent study by Wei and colleagues reported that the HDAC inhibitor romidepsin is capable of inducing virion production by latently infected CD4 T cells sampled from patients on ART therapy (325). Clinical trials are currently underway to access the activity of romidepsin in HIV positive ART treated individuals to more clearly understand its effects on purging viral reservoirs. A very recent study did report a proof-of-concept phase Ib/IIa trial to access the effects of pharmacologically induced activation of replication competent proviruses from latency in patients on long-term ART. Six aviremic HIV-1 infected adults received intravenous administration of a $5mg/m^2$ dose of romidepsin once weekly for three consecutive weeks, while being maintained on ART(326). Importantly, there were minimal adverse effects noted from this trial and both plasma HIV-1 RNA and HIV-transcription measured as copies of cell associated un-spliced HIV-RNA increased significantly from baseline at multiple time-points post the infusion of romidepsin. This study also found that romidepsin treatment did not hinder anti-viral T cell responses. This study establishes the potential to use romidepsin as treatment strategy for reversing HIV latency and eradicating the HIV-reservoir (326). Its important to note that despite romidepsin having a measurable effect on viral transcription under suppressive ART, there were minimal effects on

reducing the total viral reservoir in memory CD4 T cells, suggesting that this therapy alone may not be sufficient to fully deplete the viral reservoir. Taken together these studies suggest that treatment with HDACi despite mobilizing the latent viral reservoir may have unintended effects on viral immunity, and thus each LRA must be appropriately tested to understand the spectrum of effects it can have both on limiting anti-viral immune responses and promoting viral transcription from the latent reservoir.

Conclusions

A complete understanding of the cellular subsets and anatomical sites that contribute to the development and maintenance of the latent viral reservoir is essential to generating therapeutic strategies that can harness the immune response to target and eliminate HIV. Moreover, the interplay of different combination therapies under ART will be critical in defining the optimal treatment regimen to achieve a "functional cure." Cytokine therapies that can decrease immune activation and improve mucosal homeostasis may provide the best treatment options in terms of improving CD4 T cell immune reconstitution and an overall reduction in the prevalence of "end organ-disease". Vector derived immunizations that can boost both humoral and CTL based responses are paramount for developing an immune defense to target remerging virus driven by latency reactivating drugs or biologicals administered under ART. Furthermore, decreasing the total size of the viral reservoir with cellular and humoral based vaccinations and biologics **(Fig. 5.1)**(327), is central to achieving a functional cure for HIV, as a lower viral burden post treatment cessation faced with a functionally equipped immune response may inevitably provide host independent control of HIV.

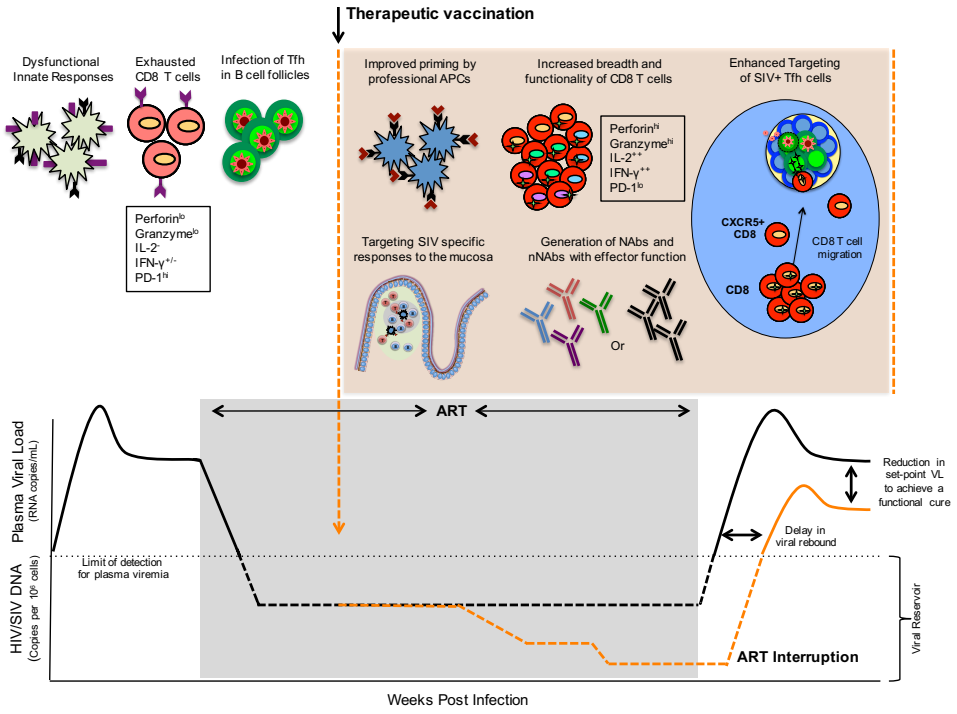

Figure 5.1. Potential mechanisms of viral control by therapeutic vaccines. ART therapy has allowed for profound control of viral replication in HIV infected individuals, but is unable to appropriately restore immune function to the levels seen prior to infection. Multiple arms of the immune response are compromised during chronic HIV infection and remain dysfunctional under highly suppressive ART including; impaired innate responses, exhausted anti-viral CD8 T cell responses, and productive infection of CD4 T cells including Tfh cells that are restricted mainly to the germinal centers of B cell follicles largely devoid of HIV specific CD8 T cells. Therapeutic vaccinations have the potential to beneficially modulate multiple immune parameters that are crucial to long term control of viremia and these include targeted priming of professional APCs, restoration of mucosal homeostasis, induction of bNAb, and expansion of highly functional CD8 T ells with the ability to infiltrate into sites of latent viral replication. These interventions can lead to delayed viral resurgence and a significant reduction in viral set-point post treatment interruption, facilitating host control of HIV(327). Keywords: APC (antigen presenting cells); Tfh (T follicular helper cells); bNAb (broadly neutralizing antibodies)

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