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Pragati Nigam Date

Influence of Regulatory and IL-17-secreting T Cells in the Pathogenesis of SIV Infection

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Doctor of Philosophy

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Pragati Nigam

M.S., Universidade Federal de Pernambuco, 2002

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ABSTRACT

INFLUENCE OF REGULATORY AND IL-17-SECRETING T CELLS IN THE PATHOGENESIS OF SIV INFECTION

PRAGATI NIGAM

Pathogenic HIV/SIV infections are characterized by severe CD4+ T cell depletion, hyperimmune activation and damage to gastrointestinal tract resulting in immune dysregulation. Understanding the early events at the site of virus exposure are critical to determine immune correlates for virus control and disease progression. CD4+ regulatory cells (Tregs) may influence viral control and disease progression by suppressing anti-viral immunity. However, these cells are infected and killed very early following SIV infection. Here, we demonstrate that pathogenic SIV infections utilize a novel immunosuppressive mechanism wherein they induce rapid expansion of CD8+ Tregs with suppressive capacity in colorectal tissue, one of the preferential sites of virus replication. The expansion of CD8 Tregs was not seen in non-pathogenic SIV infection of sooty mangabeys suggesting that these cells may be deleterious to the host and contribute to faster disease progression.

IL-17 is a pro-inflammatory cytokine that is important for protection against extracellular bacteria in the gut and maintenance of gut permeability. IL-17 producing CD4 T cells (Th17) have been well characterized and are depleted very early following SIV infection. It is increasingly becoming clear that a subset of CD8 T cells in humans and mice secrete IL-17 (Tc17), and the role of these cells is yet to be characterized for any infectious disease including HIV/SIV. Here we demonstrate that, in contrast to Th17 cells, the Tc17 cells are not depleted during the acute phase of infection, however are depleted during the end stage disease. We also demonstrate that Tc17 cells are not depleted in SIV-infected sooty mangabeys. These results suggest that Tc17 cells may compensate for the loss of Th17 cells during the acute and early chronic phases of pathogenic SIV infection and may play a role in prolonging disease progression.

In conclusion, our findings reveal important roles for gut resident CD8 Tregs and Tc17 cells in regulating SIV disease progression. Whereas CD8+ Tregs help the virus by constraining anti-viral immunity, Tc17 cells delay onset of disease by contributing to the maintenance of intestinal epithelial barrier.

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CHAPTER 1

Introduction to HIV

HIV epidemic

According to the UNAIDS, there were an estimated 33.4 million people living with Human Immunodeficiency Virus (HIV) in 2008, with 2.7 million new infections occurring that year. Although HIV infections were first described in populations of homosexual men with Kaposi's sarcoma and *Pneumocystis carinii* pneumonia(1-3), heterosexual transmission is the main route of dissemination of HIV infection worldwide and accounts for nearly 80% of HIV infections (unaids.org, (4). Despite the advances in understanding the viral infection and disease and the increase in antiretroviral therapy (ART) coverage, according to World Health Organization, there were still 2 million HIV-related deaths in 2008. HIV/AIDS was the leading cause of death by infection in low-income countries, and the second leading cause of death by infections worldwide.

HIV and Simian Immunodeficiency Virus (SIV) are positive single-stranded RNA viruses of the genus lentivirus, family orthoretrovirinae, order retroviridae. HIV uses the cell surface receptor CD4 for entry into cell (5). This induces conformational changes in the viral surface glycoprotein gp120 that permits binding to cell surface chemokine receptor type 5 (viral co-receptor CCR5) or CXC receptor 4 (CXCR4). Binding of CD4 and co-receptor triggers further conformational changes in the transmembrane glycoprotein gp41 facilitating fusion of the viral envelope with the cellular plasma membrane. Fusion of the viral particle with the cell is followed by uncoating of the viral

core and reverse transcription of the viral RNA genome into a DNA copy that is integrated into the host cell genome via the viral enzyme integrase. Viral proteins are produced from the integrated viral genome and are assembled into progeny virions at the plasma membrane, and continue the viral life cycle. It is possible for infected cells to remain latent, produce no/very little viral proteins, and escape immunosurveillance.

Signs and symptoms

HIV and SIV infections exhibit two phases of infection: acute and chronic phase. The virus is transmitted via exchange of bodily fluids, usually through sexual contact, but can also occur through sharing of needles, blood transfusion and mother-to-child transmission routes. The acute phase of infection may go unnoticed, but is often accompanied by flu-like symptoms, including fever, lymphadenopathy, pharyngitis, myalgia and malaise (acute retroviral syndrome). HIV and SIV establish persistent, systemic CD4+ T cell depleting infections within a few weeks. The viruses cross the mucosal barrier and establish at the point of entry by infecting target CD4+ T cells resident in the lamina propria. These viruses can also infect macrophages and dendritic cells. The virus and infected cells are disseminated first to the draining lymph nodes and later to other lymphoid tissue compartments through the bloodstream. During the acute phase, viremia levels may reach up to 10^7 viral copies/ml of plasma. After peak viremia, viral loads decline, possibly due to reduction in availability of target cells (6, 7) or generation of CD8+ T cell response (8, 9) that partially controls viral replication. The acute phase of HIV infection is accompanied by a rapid transient decrease in peripheral blood CD4+ T cell count and a rapid partial recovery of this loss. However, in the gut-

associated lymphoid tissues (GALT), a massive depletion of CD4+ T cells is observed by day 21-post infection, but does not rebound to pre-infection levels (10-15). The GALT is a major source of CCR5+CD4+ T cells (early targets of infection).

During the chronic phase of infection, viral loads reach a set point, where these are maintained between 10^3 to 10^6 viral copies/ml. This phase can last up to 10 years, and if left untreated, the infected individual will continue to gradually and progressively lose CD4+ T cells leading to collapse of immune function. During the chronic phase, HIV-infected individual will be susceptible to opportunistic infections and tumors, culminating in the development of Acquired Immunodeficiency Syndrome (AIDS), and eventually leading to death. HIV-positive patients are initially susceptible to oral *Candida* and to *Mycobacterium tuberculosis*, leading to development of candidiasis and tuberculosis. Later, latent herpes viruses may lead to worsening of herpes simplex eruptions, shingles, Epstein-Barr virus-induced B cell lymphoma (Kaposi's sarcoma). When CD4+ T cell counts are very low, the individuals are susceptible to toxoplasmosis, and cryptococcal infections. In the final stages of AIDS, infection with cytomegalovirus or *Mycobacterium avium* complex is prominent, and pneumonia caused by the fungus *Pneumocystis jirovecii* is common and frequently fatal. Antiretroviral treatment can significantly slow down progression to disease; however till today no therapy can cure this disease, and no vaccine has been able to prevent infection.

HIV and pathogenic SIV infections (i.e, in non-natural hosts, such as rhesus macaques and pig-tailed macaques) are characterized by a state of chronic generalized immune activation that is thought to be one of the important factors for determining disease progression (16-19). Immune activation can be measured by quantifying the

frequency of T cells expressing markers of activation (HLA-DR, CD38 and CD69) and proliferation (Ki-67); by determining the levels of activation-induced apoptosis of uninfected T cells; by determining the levels of T cell proliferation and levels of proinflammatory cytokines in the plasma (IFN-γ, TNF-α and IL-12) (16). The number of proliferating cells is increased in the intestine of HIV/SIV infected individuals (20). It has been shown that HIV proteins can target TNF receptor signaling, thus leading to apoptosis of uninfected bystander T cells and sustained viral replication in infected T cells and macrophages (21).

Pathogenic and non-pathogenic SIV infections

The immunopathogenesis of SIV infections can be studied by comparison of the two simian models of SIV infection: the non-pathogenic infections of natural hosts (sooty mangabeys and African green monkeys) and pathogenic infections (rhesus macaques and pig-tailed macaques). Pathogenic and non-pathogenic infections share some common features. In both, the peak of viral replication occurs during the acute phase of infection between one and two weeks post-inoculation (22). This is followed by a rapid decline in viremia and establishment of the set point. In both pathogenic and non-pathogenic infections, activated CD4+ T cells appear to be where viral replication occurs (23). However, CD4+ T cell counts progressively decline in blood over time in the majority of pathogenic infections, but only in a minority of non-pathogenic infections (24). But in both types of infection, CD4+ T cells are depleted in mucosal tissues (22). The discrepancy between peripheral CD4+ T cell counts and mucosal CD4+ T cell counts is a typical feature of non-pathogenic infections that is not observed in pathogenic infections.

Non-pathogenic SIV infections show lower levels of immune activation when compared to pathogenic infections (22, 25, 26). Likewise, they also have low levels of bystander apoptosis and cell cycle dysregulation (27). It is interesting to note that non-pathogenic infections do not demonstrate signs of microbial translocation from the intestinal lumen to systemic circulation (28). Another critical difference between the two simian models of SIV infections is the expression of CCR5 by CD4+ T cells. Only about 1-5% of CD4+ T cells in the blood and mucosal associated lymphoid tissues of sooty mangabeys are CCR5-positive in comparison to 10-20% in blood of rhesus macaque and greater than 50% in the mucosal tissues (29). Although the low levels of CCR5 expression on CD4+ T cells does not protect against infection, it may protect against immune activation as these cells may be less able to migrate to inflamed tissues and thus have less reservoirs for SIV replication and inflammation. It is also possible that the low level of CCR5 expression on CD4+ T cells in natural hosts may provide resistance from mother-to-child transmission.

Table 1: Features of pathogenic and non-pathogenic SIV infections

Characteristics of SIV infections	**SIV+ Rhesus macaques (Pathogenic)**	**SIV+ Sooty mangabeys (non-pathogenic)**
AIDS	Yes	No
Viral load	High	High
Peripheral CD4+ T cell loss	Yes	No
Mucosal CD4+ T cell loss	Yes	Yes
Chronic immune activation	Yes	No
Bystander apoptosis	Yes	No
Microbial translocation	Yes	No

Pathophysiology

The gastrointestinal mucosa provides an interface between a sterile internal environment and a contaminated external environment. The GI tract becomes a key participant during HIV/SIV infections, because it harbors the approximately 40% of all lymphocytes (30), and is the primary target of infection. Mucosal sites are not only primary sites of viral transmission, but also major sites for viral replication and CD4+ T cell destruction, independent of the route of transmission. Most of the CD4+ T cells located at the mucosal immune system are CCR5+ memory CD4+ T cells, which are the preferred targets of HIV/SIV infection (12, 14). During HIV/SIV infections, there are alterations in the intestinal structure and function (31-35). Intestinal immune dysfunction during HIV/SIV infection can result in structural changes to the intestinal mucosa, culminating in the breakdown of the intestinal epithelial barrier (36, 37). Normal function of mucosal surfaces requires intact epithelium, with intact tight junctions. Due to the massive depletion of activated memory CD4+ T cells at the gastrointestinal tract, there is disruption of the network that links the epithelial cells and the intestinal immune system. During HIV/SIV infections, there is down-regulation of genes involved in intestinal epithelial cell growth and renewal, as well as increased expression of inflammation and immune activation genes (38-40). There is also an increase in pro-inflammatory cytokine secretion that could facilitate mucosal damage (41-44). The altered intestinal epithelial permeability permits bacterial translocation to occur. These bacteria can bind to receptors expressed on dendritic cells and macrophages, leading to their activation and initiation of cytokine production that can result in activation of other immune cells, such as CD4+ T cells, which can serve as potential targets for the viruses. This further drives the

continuous and chronic stimulation of the immune system observed during pathogenic SIV and HIV infections (28, 45).

Protective factors for HIV infection

Defining the immune mechanisms that are responsible for controlling SIV/HIV replication during acute and chronic phases of infection is important for understanding the immunopathogenesis of AIDS. Many observations indicate that virus-specific cytotoxic T cells (CTLs) have a role in this process. CD8+ T cells from infected individuals have been shown to inhibit HIV replication *in vitro* (46). The control of initial burst of viral replication observed during the acute phase of infection coincides with the appearance of virus-specific CTL responses (8). Also, robust virus-specific CTL responses have been observed in HIV-positive individuals that have low viremia and persistent, non-progressive disease (47-49). Furthermore, the depletion of CD8+ T cells from non-natural hosts of SIV during both acute and chronic phases of infection resulted in a rapid and significant increase in viremia that was suppressed with the reappearance of SIV-specific CD8+ T cells (9, 50). Collectively this evidence suggests that cellular immunity is involved in controlling HIV replication.

HIV/SIV infections lead to extensive defects in the humoral arm of the immune system. The B cell defects can be attributed either to the direct or indirect effects of viremia. Although direct interactions between HIV and B cells have been reported (51), there is little evidence that HIV replication occurs in B cells *in vivo*. It has been demonstrated that HIV binds to B cells *in vivo* via interactions between the complement receptor CD21 (expressed on mature B cells) and complement proteins bound to HIV

virions that are circulating (52, 53). These immune-complexes may be providing stimulatory signals to B cells (52) or B cells may be facilitating cell-to-cell transmission of HIV (54). Some of the indirect effects of HIV viraemia on B cells include: B cell hyperactivity, lymphopenia and exhaustion. The hyperactivation of B cells by HIV is characterized by hypergammaglobulinemia, increased polyclonal B-cell activation, increased cell turnover, increased expression of activation markers, increased in the differentiation of B cells to plasmablasts, increased production of autoantibodies and an increase in the frequency of B cell malignancies. Ongoing HIV replication and disease progression can be represented by the loss of CD21 expression on peripheral blood B cells (55). A large proportion of $CD21^{low}$ B cells constitute exhausted tissue-like memory B cell population that are expressing putative inhibitory receptor Fc-receptor-like 4 (FCRL4) (56, 57). Other B cell inhibitory receptors CD22, CD72, CD85j and CD85k are also expressed by tissue-like memory B cells from HIV-viremic individuals providing further indication that tissue-like memory B cells could be an exhausted B cell compartment.

Non-viral T cells

Non-viral specific T cells may also play a role in the development of immunopathogenesis during HIV/SIV infections. Generally, viral infections activate the immune system generating large numbers of immune cells, pro-inflammatory cytokines and other effectors that are important to end the infection by eradicating infected cells and the virus. The initial expansion stage is followed by a contraction stage, during which regulatory T cells may play a role in maintaining the balance between an immune

response that is sufficiently robust to clear the infection and the immunopathological consequences of sustained immune activation and inflammation. During SIV/HIV infections, regulatory T cells may play one of two roles: They may mediate suppression of chronic hyper-immune activation, or they may suppress anti-viral cellular immune response. The first role may be beneficial, whereas the second role may be deleterious, leading to inability to control infection and faster progression to disease.

Another subset of non-viral specific T cells that may influence immunopathology during HIV/SIV infections are Th17 or Tc17 T cells. These cells are characterized by secretion of pro-inflammatory cytokine IL-17. IL-17 has been shown to function *in vivo* to promote recruitment of neutrophils to areas of bacterial infection, to induce proliferation of enterocytes and the production of antibacterial defensins (58-60). These cells are critical in the defense against microbes, especially at mucosal surfaces. Studies have demonstrated that in HIV/SIV infections, microbial products can translocate from the lumen of the gastrointestinal tract that is structurally and immunologically damaged during the course of infection into the systemic circulation. These microbial products can then contribute to immune activation during the chronic phase of infection (28, 61). Recent studies have shown that there is loss of Th17 T cells during HIV/SIV infections (62-65) and have suggested that depletion of these cells may accelerate the progression to AIDS.

During the advanced phase of HIV/SIV infections, neutropenia is observed and has generally been attributed as a result of malnutrition and wasting or as a side effect of medication (66). Chemotaxis or migration of neutrophils is also impaired in HIV infection (67). However with the knowledge that IL-17 is an important cytokine for

recruitment of neutrophils, and that Th17 T cells are depleted during the course of HIV/SIV infections, it is possible that the impairment of neutrophil function observed during chronic HIV infection may be a direct result of depletion of these cells.

Early events during HIV/SIV infection are critical in determining the course of disease progression. Viral-specific T cells generate an immune response to these infections and help control viral replication. However, non-viral specific T cells may also participate in modulating the generation of these immune responses, both during the acute and chronic phase of infections. The study of both viral-specific and non-viral specific T cells may help understand immunopathology associated with HIV/SIV infections and give an insight into how to generate more effective therapies. The main objective of the experiments reported in this dissertation is to study the influence of regulatory and IL-17 secreting T cells on disease progression during the course of HIV/SIV infection.

Lineage differentiation of T cells

CD4+ T cells are important mediators of adaptive immune responses. After interaction with antigen-presenting cells, naïve T cells receive signals by engagement of the T-cell receptor (signal 1), co-stimulatory molecules (signal 2), and a complex network of cytokine signals (signal 3) and undergo activation and differentiation into effector cells. In 1986 it was recognized that T helper (Th) cells could differentiate into two distinct CD4+ T cell subtypes with different cytokine profiles and functions within the immune system (68). Th1 cells typically produce IFN-γ and TNF-α. Their functions include macrophage activation, regulation of cellular immunity against intracellular infections and have a role in autoimmune disease and tissue damage associated with chronic infections. Th2 cells produce IL-4, IL-5 and IL-13. Their functions include providing humoral immunity against parasitic infections, and have a role in allergic diseases.

However, this dichotomy of T cell subsets was unable to completely explain the infections seen in certain clinical settings. In 2005, a distinct CD4+ T-cell lineage responsible for the production of IL-17 was described as Th17 (69). Th17 cells are characterized by the production of IL-17A, IL-17F, IL-22 and IL-26 (in humans). Although IL-17A was initially identified in 1995 (70), its importance in mediating inflammation during infections was first reported in 2000 (71), and the IL-23/IL-17 pro-inflammatory axis was described in 2003 (72). Another distinct subset of T cells is the regulatory T cells which play a role in tolerance to self-antigens. Regulatory T cells are characterized by the expression of high levels of CD25, forkhead family transcription

factor FOXP3 and the capacity of suppressing the activation of other T cells (73-75). The importance of regulatory T cells is demonstrated by mutations in FOXP3 that result in fatal autoimmune lymphoproliferative disease, such as Immune dysfunction Polyendocrinopathy Enteropathy X-linked syndrome (IPEX) in humans.

Table 2: T cell subset functions

T cell subset	**Functions**
Th1	Defense against intracellular pathogens; Macrophage activation
Th2	Defense against parasitic infections; Humoral immunity
Th17	Defense against extracellular bacteria and fungal infections
Treg	Tolerance

Naïve CD4+ T cells can differentiate into the four major lineages of T helper cells (Th1, Th2, Tregs, or Th17 T cells) by exposure to distinct cytokine profiles that enable the naïve T cell to commit to a differentiation pathway. Th1 cells are originated after exposure of the naïve T cell to IL-12, and engagement of transcription factors T-bet and STAT4. Similarly, naïve T cells differentiate into Th2 cells after exposure to IL-4, and engagement of STAT6 and GATA-3. Tregs are generated once naïve T cells have been exposed to TGF-β, and engagement of FOXP3. Th17 cells are generated when naïve T cells are exposed to a cytokine milieu consisting of TGF-β, IL-6 and IL-21, whereas IL-23 stabilizes the commitment of Th17 cells to this lineage. These polarizing cytokines act on newly primed cells to induce the expression of transcription factors RORγt and RORα, which induce Th17 differentiation (76, 77). STAT3 pathway is essential for the expression of RORγt and Th17 development (78, 79). IL-21 acts downstream of these

events to further amplify the generation of Th17 cells in an autocrine manner (80). Although *in vitro* studies have demonstrated that IL-23 is not necessary for the development of Th17 responses (81), *in vivo* studies in the mouse (82, 83) and in humans (84) show that IL-23 is critical for the maintenance of Th17 responses *in vivo*.

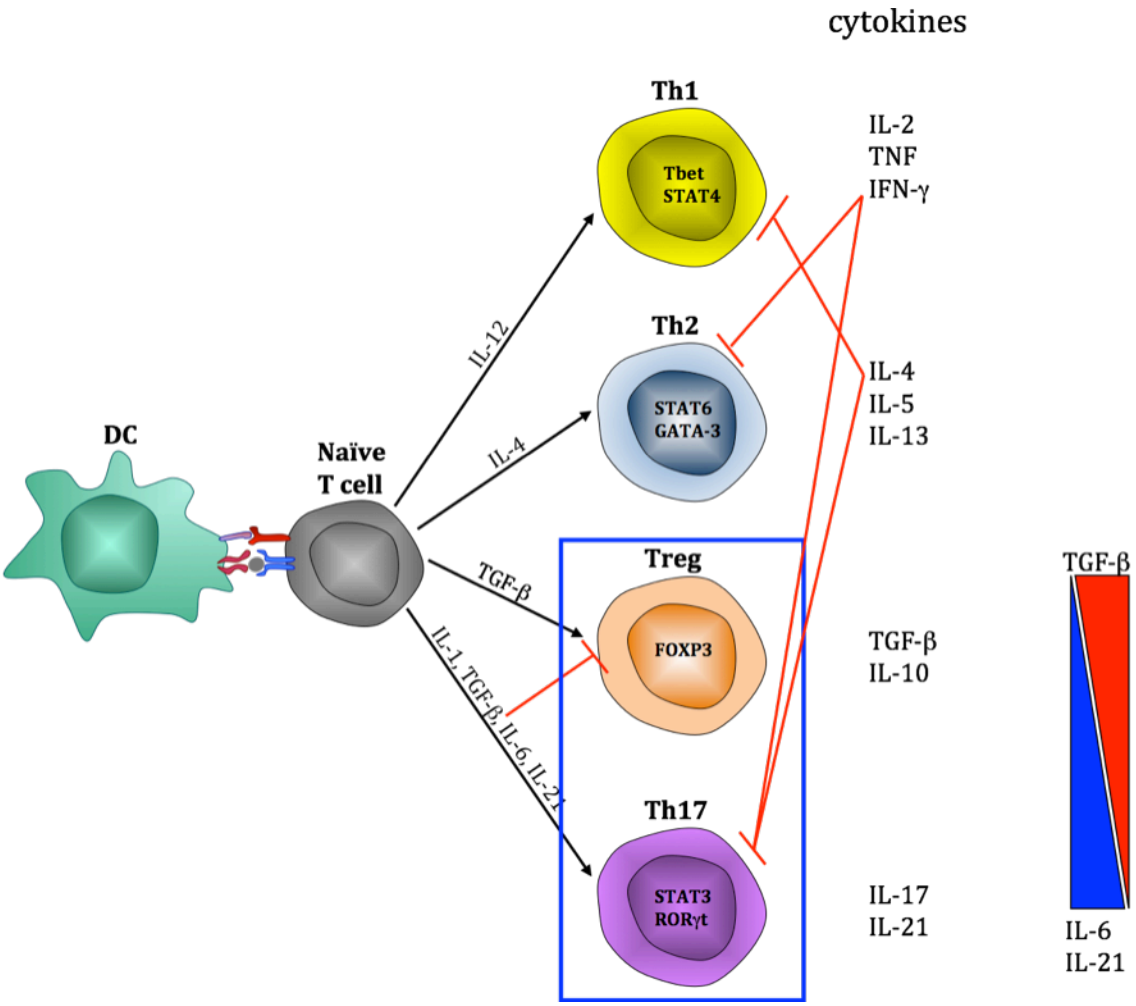

Figure 1.1: T cell differentiation: Naïve T cells commit to T cell lineage differentiation pathways by exposure to distinct cytokine milieu.

The generation of these Th cell subsets is tightly regulated. For example, IFN-γ can inhibit the differentiation of Th2 and Th17 cells, whereas IL-4 can inhibit the development of Th1 and Th17 cells. In Th1 cells, the IFN-γ-induced protein interferon

regulatory factor 1 (IRF1) binds to the FOXP3 proximal promoter and inhibits FOXP3 expression (85). In Th2 cells, IL-4 inhibits FOXP3 expression in peripheral naïve T cells by stimulating phosphorylation of STAT6 that binds and inhibits FOXP3 expression (86). The differentiation of regulatory T cells and Th17 cells is reciprocal, i.e, exposure to only TGF-β leads to the development of regulatory phenotype, whereas exposure to either IL-6 or IL-21 will inhibit Treg differentiation, and initiate a cascade of events that leads to generation of Th17 phenotype. The vitamin A metabolite retinoic acid (RA) inhibits Th17 cells and induces *de novo* generation of FOXP3+ regulatory T cells. RA induction of regulatory T cells form naïve CD4+ T cells may occur due to enhanced TGF-β-driven phosphorylation of SMAD3 along with inhibition of IL-6 and IL-23 receptor expression (87). RA has been shown to induce histone H4 acetylation of FOXP3 locus and FOXP3 expression in naïve CD4+CD25- T cells (88). RA has also been shown to induce expression of CCR9 and CD103 on regulatory T cells, and these regulatory T cells showed increased migration to gut-homing chemokine CCL25 (88, 89). Endogenous lipid mediators such as prostaglandin E2 released under inflammatory conditions can promote Th17 cell differentiation (90-93). This suggests that external mediators induced by various infections can influence the lineage commitment between Th1, Th2, Th17 and Treg pathways.

Regulatory T cell

Regulatory T cells were first described in the 1970s and were called suppressive T cells (94, 95). The concept that the extent of immune responses is limited by suppressor cells became popular, with the idea spreading to infectious diseases. However, this hypothesis was discredited in the 1980s due to inability to validate the cellular identity and molecular basis of immune suppression.

In 1995, Sakaguchi *et al*., showed that the IL-2 receptor α-chain CD25 could serve as phenotypic marker for CD4+ suppressor T cells, or regulatory T cells (96). Later, it was shown that the transcription factor forkhead box P3 (FOXP3) is critical for the development and function of CD4+CD25+ regulatory T cells (97-99). This revived the field of regulatory T cells, which has evolved thereafter rapidly. The classic regulatory T cells are thymus-derived CD4+CD25+FOXP3+ T cells, however several phenotypically distinct regulatory T cell populations have been identified (100-106), including natural regulatory T cells, type 1 regulatory T cells (Tr1) and T-helper 3 cells (Th3). Regulatory T cells are involved in the maintenance of peripheral tolerance.

Genetic defects in FOXP3 gene cause the scurfy phenotype in mice and IPEX syndrome (immune dysfunction, polyendocrinopathy, enteropathy, X-linked syndrome) in humans. Scurfy mice present a 2 bp insertion of aminoacids in exon 8, which results in a loss-of-function of FOXP3 gene and complete absence of Tregs leading to death at 3-4 weeks of age. 60% of IPEX show missense mutations mainly in exons 9, 10, and 11 (which form the forkhead domain), and display symptoms of nonfunctional Tregs. Scurfy mice exhibit various markers of disease, including ear thickening, scaling of eyelids, ears

feet and tail, and severe runting. Internally, these mice have lymphadenopathy, splenomegaly, hepatomegaly and massive lymphocytic infiltrates in skin and liver. Symptoms of patients with IPEX include eczematous dermatitis, nail dystrophy, autoimmune endocrinopathies and autoimmune skin conditions including alopecia and bullous pemphigoid.

Table 3: Regulatory T cell subsets

CD4+ Regulatory T Cells	
Regulatory T cell subset	**Suppressive mechanism**
CD4+CD25+FOXP3+	Cell-cell contact
Tr1 (FOXP3-)	IL-10 secretion
Th3	TGF-β secretion

CD8+ Regulatory T Cells	
Regulatory T cell subset	**Suppressive mechanism**
CD8+CD25+	TGF-β and CTLA-4
CD8+CD28-	ILT3 and ILT4 on dendritic cells
CD8+IL-10+	IL-10 secretion
CD8+FOXP3+	CTLA-4 and TGF-β

The FOXP3 gene is located on the short arm of chromosome X (Xp11.23 in humans and X 2.1 cM in mice). It possesses 11 coding and 3 non-coding exons. Immediately upstream to the FOXP3 transcriptional start site is a region that possesses several transcription factor binding sites (AP-1 and NFAT) known as the proximal promoter region. Other regulatory cis-elements are present between noncoding exons or far upstream of the transcriptional start site, and these are referred to as intronic enhancers or upstream enhancers, respectively.

Members of the forkhead family are characterized by the presence a forkhead domain (FKH) which has been shown to be necessary and sufficient for DNA binding (107). FOXP3 contains the following functional domains: FKH, Zinc finger domain, and leucine-zipper-like domain. At the amino terminal region, there is a proline-rich domain that has been named the repressor domain. Mutations have been found throughout FOXP3, including all of the domains mentioned, except for the zinc-finger. Mutations in the FKH domain affect DNA binding, whereas mutations in the leucine zipper have been found to affect FOXP3 homo and hetero-dimerization. Three missense mutations have been found in the repressor domain. The amino-terminal repressor domain has been shown to inhibit transcriptional activation by nuclear factor of activated T cells (NFAT) (108).

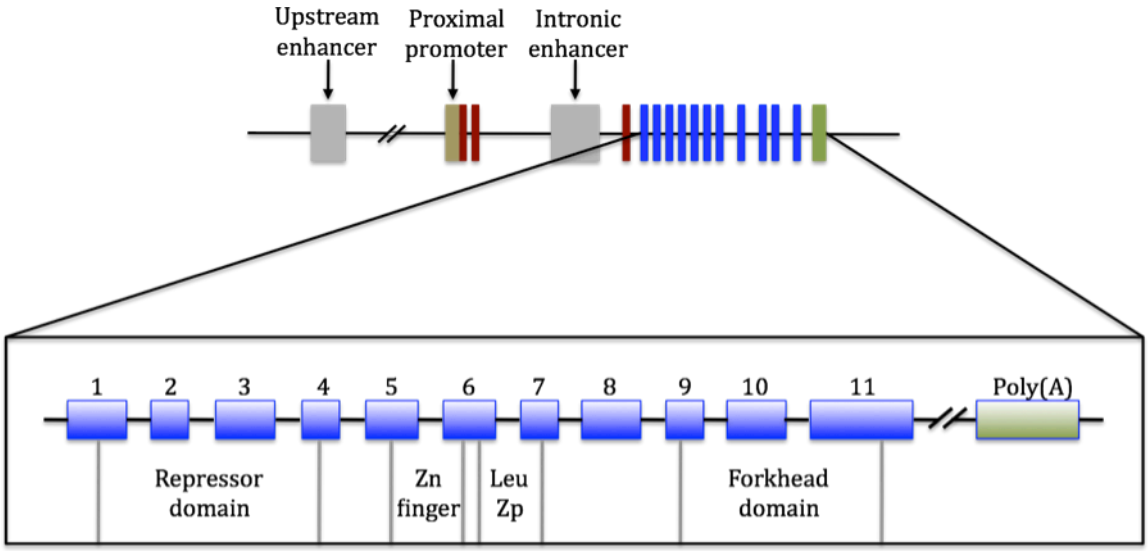

Figure 1.2: FOXP3 gene. Blue bars represent coding exons, and red bars represent non-coding exons.

FOXP3 protein is highly conserved in murine, feline, canine, bovine, macaque and humans (109). Western blot analysis shows that human cells express three isoforms

of FOXP3. The upper band is similar to the mouse FOXP3, whereas the lower band is unique to humans and lacks exon 2, which is part of the repressor domain. Exon 2 has been shown to interact and repress the function of retinoic acid-related orphan receptor-α (ROR-α) (110) and ROR-γt (111). The third isoform lacks both exon 2 and exon 7. Exon 7 encodes for a leucine zipper motif that acts as a dimerization structural element. Interestingly, it has been reported that the absence of exon 7 in natural FOXP3Δ2Δ7 abrogates suppressive function of regulatory T cells without affecting dimerization (112). FOXP3Δ2Δ7 could have a role in regulating the function of the other two isoforms, as it has been shown to inhibit FOXP3 and FOXP3Δ2 expression in a dominant negative manner. This isoform has also been shown to be over-expressed by certain malignant cells. However its role in cancer pathogenesis is yet to be determined.

Epigenetic modifications can take place either in chromosomal DNA or proteins that are linked with the chromosomal DNA. Functional units involved in epigenetics include methylation of CpG residues (5' position of cytosine of CpG) or covalent posttranslational modification of histones. The methylation status of the CpG residues in the proximal promoter region has an essential role in FOXP3 expression. Several studies indicate that only 5% of CpG residues in this region are methylated in regulatory T cells, when compared to 70% methylation of CpG residues in non-regulatory T cells (113, 114).

Acetylated histones are markers for open chromatin structure. Acetylation of core histone molecules can be achieved by histone acetyltransferase. Histone acetyltransferases are a class of enzymes that adds acetyl groups from ε-N-acetyl lysine amino acid on a histone at the amino terminus of the histone tail, whereas histone

deacetylases (HDACs) remove the acetyl groups on histones. The addition of acetyl groups to histones decreases the overall positive charge, thus decreases the histone affinity for negatively charged DNA, and allows for open conformation of chromatin, which in turn provides a platform for the binding of transcription factors (115). The N-terminal region of the FOXP3 protein is proline rich, and recruits the lysine acetyltransferase TIP60. TIP60 acetylates the FOXP3 protein, which binds to its target gene promoter, such as IL-2 to repress its transcription. The N-terminal region of FOXP3 can also recruit the deacetylase HDAC7, which deacetylates it, and inhibits FOXP3 function. HDAC inhibitors enhance FOXP3 expression in CD4+CD25- and CD4+CD25+ T cells, suggesting that they regulate both the gene and protein, and enhance the suppressive function of regulatory T cells (116, 117).

Regulatory T cell during the course of HIV/SIV infections

Regulatory T cells actively participate to modulate the outcome of various infections. During HIV/SIV infections, regulatory T cells may have a role in influencing viral control and disease progression. There are two possible outcomes of the impact of regulatory T cells during immunodeficiency viral infections: Regulatory T cells may suppress chronic hyper immune activation, and delay disease progression, or these cells may dampen the antiviral cellular immune responses and thus curtail viral control.

Various studies characterize the fate of CD4+ regulatory T cells during the course of HIV and SIV infections. These studies demonstrate that there is loss in the absolute numbers of CD4+ regulatory T cells following pathogenic immunodeficiency viral infections (118-120). They also show that there is a change in tissue distribution of regulatory T cells during HIV/SIV infections leading to accumulation of these cells in lymphoid tissue, the primary sites of viral replication (121, 122). Regulatory T cells can serve as reservoirs for HIV-1 *in vivo* (123). The direct infection by HIV-1 and the tissue redistribution can be possible explanations for the reduction in absolute numbers of regulatory T cells observed in progressive HIV-1 infection. Long-term non-progressors (LTNP) are untreated HIV-1 infected individuals that remain asymptomatic and maintain high CD4+ T cell counts for many years without antiretroviral treatment. These individuals also maintain high levels of regulatory T cells in peripheral blood, which correlated with low levels of CD4+ T cell activation (123). Likewise, HIV-resistant individuals have reduced levels of immune activation (as measured by CD69) and

elevated frequencies of CD4+ regulatory T cells when compared with HIV-negative control individuals (124).

Although there is decrease in absolute number of CD4+ regulatory T cells, there is an increase in the proportion of these cells in the lymphoid and gastrointestinal mucosal tissues in HIV-1 and SIV infections (64, 125-128). Consistent with this, a recent study demonstrates that mucosal regulatory T cells are less susceptible to productive SIV infection than non-regulatory T cells, and thus the rate of loss of CD4+ T cells is distinct from the loss of CD4+ regulatory T cells (129, 130). However, it has also been shown that during the early stages of infection, HIV infection rate of regulatory T cells is 10-fold higher when compared to naïve CD4+ T cells, but similar to infection rates of memory CD4+ T cells (131). CD4+CD25+ regulatory T cells isolated from HIV-infected individuals suppressed cellular proliferation and cytokine production by CD4+ and CD8+ T cells in response to HIV antigens/peptides *in vitro* via cell-contact dependent mechanism (132-134). The potent suppressive activity against HIV-specific cytolytic T cell function was observed even in individuals with high viral loads and/or low CD4+ T cell counts. Likewise, depletion of regulatory T cells from peripheral blood mononuclear cells enhances T cell responses to HIV-1 antigens (135). *In vitro* studies demonstrate that binding of HIV-1 to CD4+ regulatory T cells leads to an increase in their suppressor activities, and induces them to home to and accumulate in peripheral and mucosal lymphoid tissues by up-regulation of homing receptors CD62L and $\alpha 4\beta 7$ integrin (136).

Although many studies have evaluated the role of CD4+ regulatory T cells during the course of HIV/SIV infections, there is a discrepancy in the number of published studies on the role CD8+ regulatory T cells during immunodeficiency viral infections.

Since absolute numbers of CD4+ regulatory T cells decline during these immunodeficiency viral infections, it is possible that the virus may induce CD8+ regulatory T cells to suppress anti-viral immune responses. Studies have shown the presence of HIV-specific IL-10 positive CD8+ regulatory T cells (FOXP3-negative regulatory cells) in advanced stages of HIV infection (137, 138). These CD8+ regulatory T cells mediate suppression through direct cell-to-cell contact and were phenotypically distinct from other regulatory T cells (139).

Other studies have shown that HIV/SIV infections lead to expansion of FOXP3+ CD8+ T cells (106, 140, 141). In a group of cynomolgous macaques infected with SIVmac251, FOXP3+CD8+ T cells were induced a few days post peak viremia during primary infection. The number of these cells was positively correlated with viral load and inversely with CD4+ T cell activation (140). This indicates that large numbers of FOXP3+ CD8+ regulatory T cells may be markers of poor disease prognosis. However none of these studies evaluated the suppressive potential of FOXP3+ CD8+ regulatory T cells. Here, we have quantified and studied the anatomical distribution, phenotypic characteristics and functionality of FOXP3+CD8+ regulatory T cells following pathogenic SIV infection in rhesus macaques *in vitro* and *in vivo*

Th17 and Tc17 T cells

The classical Th1 and Th2 subsets have been recently joined by the Th17 lineage. If these subsets are not tightly regulated, their effector functions, mainly defined by cytokine production, are capable of leading to autoimmune and allergic inflammation, sometimes being fatal. IL-17 is a proinflammatory cytokine that functions in the clearance of extracellular pathogens. This is clearly observed in both IL-17R deficient mice, which show great susceptibility to lethal bacterial infections (142, 143) and in IL-17 deficient humans as part of the hyper-immunoglobulin E (IgE) syndrome (HIES), where patients are prone to recurrent infections (144, 145).

The IL-17 family of cytokines consists of 6 cytokines (IL-17A-IL-17F). The receptors belonging to the IL-17R family have distinct structural features and mediate signaling events that are unique from those triggered by other cytokine receptors. Whereas Th1 and Th2 cytokines induce activation mediated by Janus kinase (JAK) and signal transducer and activator of transcription (STAT) protein pathways, IL-17 induce signaling mediated by ACT1 and TNFR-associated factor 6 (TRAF6) which result in activation of nuclear factor-κB. NF-κB is associated with innate immune signaling (146). IL-17A was the first of IL-17 family members to be identified (147). The remaining members of this cytokine family (IL17B-IL-17F) were identified by genomic sequencing. IL-17A and IL-17F are the best-characterized cytokines of the IL-17 cytokine family. Both are covalent homodimers, but can also heterodimerize (IL-17A-IL-17F). It is interesting to note that IL-17A and IL-17F have distinct biological effects. Studies performed on IL-17A-/- mice and IL-17F-/- mice indicate that IL-17A has a more

important role in driving autoimmunity than IL-17F, possibly due to more potent strength of signaling (148, 149). IL-17E is produced mainly by mucosal epithelial cells. The overexpression of this cytokine stimulates the production of Th2 cytokines, and limits Th17 cell responses (150). The functions of IL-17B, -C and –D are poorly understood, but appear to have proinflammatory abilities.

IL-17 is a pleiotropic cytokine that acts on both immune and non-immune cells. The receptor for IL-17 is a type I transmembrane protein that is ubiquitously expressed on various organs, including, kidney, spleen and lung (70). Various cells express the receptor for IL-17, including leukocytes, epithelial cells, keratinocytes, fibroblasts, mesothelial cells, amongst others. They respond to IL-17R mediated signaling by the production of granulocyte colony-stimulating factor (G-CSF), MIP-2, IL-6 and IL-8, recruitment of neutrophils, and by other proinflammatory responses (151).

Th17 cells produce the cytokines IL-17, IL-22, IL-21 and TNF-α and primarily promote immunity to extracellular bacterial infections at mucosal surfaces. Initial studies with IL-17RA knockout mice demonstrated delays in neutrophil recruitment and dissemination of *Klebsiella pneumoniaie* infection, providing evidence of the importance of IL-17R signaling for neutrophil-mediated control of bacterial infections and for the host defense (142). The overexpression of recombinant adenovirus encoding IL-17 has been shown to reverse the disease phenotype in mice challenged with *K. pneumoniae* via the induction of chemokines, leukocyte recruitment and enhanced bacterial clearance and survival (152). IL-22 has been implicated in the induction of host cell antimicrobial peptides and defensins as well as epithelial repair functions in the respiratory epithelium that is needed for the early control of *K. pneumoniae* (153). Other studies also

demonstrate that Th17 cells have protective roles against extracellular bacterial infections in the gut mucosa. IL-17 has been shown to have a protective role against infections by *Citrobacter rodentium* (a naturally occurring mouse pathogen) by the induction of chemokines and antimicrobials (149, 154). However, exacerbated Th17 cell response can be deleterious to the clearance of infection. For example, there is evidence that suggests that infection by *Bordetella pertussis* (agent of whooping cough) can bias the host response to exacerbated production of Th17 cytokines (155, 156). The host bias towards Th17 cellular response after *B. pertussis* infection results in inflammation and destruction of the airways, leading to bronchiectasis and persistent cough. Although these studies provide evidence of the importance of Th17 cytokines for protection against various extracellular bacteria, it's becoming apparent that the need for balance between protection and pathology may define the outcome of infection.

Experimental autoimmune encephalomyelitis (EAE) is a CD4+ T cell-mediated disease that affects the central nervous system that is used as a model for multiple sclerosis (MS). Th17 cells have been implicated in the development of EAE. Evidence shows that mice that lack RORγt, IL-17 or even mice that are treated with IL-17 blocking antibodies are less susceptible to EAE than wild-type or untreated mice (76, 157-159). Furthermore, IL-17+ T cells have been found within lesions in brain tissues of patients with MS. It is well established that chemokine receptors provide specificity to cell trafficking in the steady state as well as during inflammation (160). In humans, CCR6 (chemokine expressed in Peyer's patches, liver and lungs), which is the receptor for CCL20 is expressed on Th17 T cells (161). One study shows that CCR6 knockout mice are highly resistant to the induction of EAE, but become susceptible once CCR6+ T cells

are transferred. Here, CCR6 was also required for the Th17 cells to enter the CNS through the choroid plexus (162).

Similar to the Th1/Th2 paradigm for CD4+ T cells, corresponding subsets of CD8+ T cells, Tc1 and Tc2 can be made in vitro by using polarizing cytokines (163). Likewise, IL-17 secreting CD8+ T cells with similar characteristics to the Th17 subset of CD4+ T cells can also be detected and are called Tc17 (164). Preliminary studies indicate that, in contrast to Tc1 cells, Tc17 cells may not express granzyme B, perforin or have cytolytic activity (164). Tc17 cells have been shown to be important for protection against lethal influenza challenge, which was accompanied by neutrophil influx into the lung. In one study, the majority of Tc17 cells were shown to co-produce IFN-γ, and to express CCR6 and CCR5 (165). In this report, we have characterized Tc17 and Th17 cells during the course of pathogenic SIV infection, and propose a role for these cells in mediating disease progression.

Th17 and Tc17 during HIV/SIV infections

Studies have demonstrated that during HIV and SIV infections microbial translocation can occur from the lumen of gastrointestinal tract into systemic circulation and contribute to immune activation that is observed during these viral infections (28, 61). The gastrointestinal tract has been shown to be structurally and immunologically compromised during pathogenic immunodeficiency viral infections (31-37). Various studies have shown that Th17 cells play an important role in antibacterial immunity particularly at mucosal surfaces, and that IL-17 can induce proliferation of enterocytes and production of antibacterial defensins (58-60). It has also been shown that there is a significantly higher number of Th17 cells in the intestinal surfaces than systemic tissues of healthy macaques (63). It is critical to study Th17 cells and Tc17 cells during the course of HIV/SIV infections.

Infection by *Salmonella typhimurium* in HIV-infected individuals can result in bacteremia that may be life-threatening. In a recent study of SIV-infected rhesus macaques, severe loss of mucosal Th17 cells was reported, resulting in blunted Th17 responses to *S. typhimurium* and increased bacterial dissemination (65). Pathogenic SIV infection results in significantly higher loss of Th17 than Th1 cells at mucosal sites of SIV-infected macaques. The frequency of Th17 cells at mucosal sites is inversely correlated with plasma viremia (63). Another study reports that while Th17 cells are detectable during the early phases of HIV-1 infection, these cell subsets were reduced to undetectable levels during the chronic phase of infection (62, 166). However, the depletion of Th17 cells appears to be specific to pathogenic infections, since infected

sooty mangabeys or African green monkeys maintain healthy frequencies of Th17 cells in the blood and in the gastrointestinal tract (62, 64).

Recent studies show that CD4+ T cells that express high levels of $\alpha4\beta7$ gut homing marker can bind to HIV and SIV with high affinity, thus making these cells more susceptible to infection (167). $\alpha4\beta7^{hi}$ CD4+ T cells were found to harbor most Th17 cells and were preferentially infected during acute phase of SIV infection (168). These cells had approximately 5 times more viral DNA than $\beta7^{-}$ CD4+ T cells (168, 169).

Highly active antiretroviral therapy for HIV infection provides suppression of viral replication to below detectable levels and results in the restoration of CD4+ T cells in the blood. However restoration of CD4+ T cells in intestinal mucosal surfaces is delayed and incomplete when compared to peripheral blood following HAART initiation (170-172). HAART is associated with substantial repopulation of $\alpha4\beta7^{hi}$ CD4+ T cells in the peripheral blood (173). One study demonstrated that when effective CD4+ T cell restoration is achieved in the gut-associated lymphoid tissue (GALT) of HIV-infected individuals during HAART, then there is an association with enhanced Th17 cell accumulation and polyfunctional anti-HIV cellular responses (174).

However, another study that evaluated effect of ART on restoration of Th17 cell response in macaques reports that the treatment failed to restore Th17 cell response in the blood, but it did not study intestinal Th17 cell response (173). This group also reports that SIV infection was accompanied with an increase in Tc17 cell responses suggesting an alteration in the ratio of Th17:Tc17 cells from a mainly Th17 phenotype to Tc17 phenotype. Despite restoration of CD4+ T cells in peripheral blood due to ART, there was persistence of high levels of Tc17 cells. We have conducted a study to characterize

the distribution, quality and function of Tc17 and Th17 cells in rhesus macaques prior to and after pathogenic SIV infection. We have also attempted to determine the effect of ART in the blood and gut mucosal tissue on these cell subsets.

Interplay between regulatory T cells and Th17 cells

Regulatory T cells and Th17 cells are intricately related. First, the development of both cell subsets is closely associated, whereby minute differences in exposure to cytokines can lead to commitment to one or the other pathway. Second, their functions are opposite, whereas regulatory T cells are inherently anti-inflammatory, Th17 cells are proinflammatory in nature. Third, regulatory T cells may directly interact with Th17 cells and influence the outcome of cellular immune response. Thus, the study of the dynamic interaction between these subsets is critical because it may influence the balance between immune activation and tolerance.

The development of regulatory T cells and Th17 cells are reciprocally regulated. Both cell types develop in the presence of TGF-β, however naïve T cells that are in presence of specific cytokine milieu may differentiate into either proinflammatory or anti-inflammatory T cells. It is interesting to study how the balance between these cell subsets is achieved, particularly at the intestinal mucosal surfaces. The constant exposure to luminal antigens could favor Th17 differentiation at the expense of regulatory T cells. The high amounts of Th17 cells at steady-state correlate with the inflammatory cytokines that are produced physiologically in the intestine (63, 175). However, the intestinal and airway mucosa are also highly effective at inducing regulatory T cells (176, 177).

Exacerbated Th17 cell response can drive autoimmune diseases such as rheumatoid arthritis. Studies have shown that addition of IL-10 to cell cultures in Th17 polarizing conditions can significantly decrease the percentage of Th17 cells (178). Another study shows that IL-10 can suppress Th17 cell development and promote

regulatory T cell development in patients with rheumatoid arthritis, thus treatment with IL-10 may be useful in the autoimmune diseases (179).

During pregnancy, the fetus is tolerated by the maternal immune system. The decidua (human pregnant uterine mucosa) is an active immunological tissue that contains a large population of maternal immune cells throughout the course of pregnancy. Regulatory T cells have been shown to be significantly enriched in the decidua and displayed a more suppressive phenotype (higher expression of FOXP3, and CTLA4) than in blood (180). Th17 cells were near absent in the decidua during the first trimester of pregnancy. This points to stringent control of the fetal-maternal interface in early pregnancy.

In MS and EAE, Th17 cells are the predominant pathogenic cell type. CD39 is an ectonucleotidase that cleaves ATP in a rate-limiting step to form AMP and has been recently described to be expressed on a subpopulation of regulatory T cells (181, 182). It has recently been demonstrated that although both CD39+ and CD39- regulatory T cells suppressed proliferation and IFN-γ production by responder T cells, only CD39+ regulatory T cells suppressed IL-17 production (183). These CD39+ regulatory T cells are impaired in patients with MS and might lead to inability to control IL-17 mediated autoimmune inflammation.

The level of immune activation that occurs after pathogenic SIV infection has been found to most accurately predict the rate of progression to disease (19, 184, 185). Chronic immune activation is observed in non-natural hosts of SIV infection (pigtailed and rhesus macaques) but not in natural hosts (sooty mangabeys and African green monkeys). Various studies have demonstrated the depletion of Th17 cells during

pathogenic SIV infection. One study shows that there is a loss of balance between Th17 and regulatory T cells in the blood, lymphoid organs and mucosal tissue of pathogenically infected pigtailed macaques, but not in African green monkeys. The loss of balance between these two cell subsets appears to be related to disease progression.

CHAPTER 2

Expansion of FOXP3+ CD8+ T cells with suppressive potential in colorectal mucosa following a pathogenic SIV infection correlates with diminished anti-viral T cell response and viral control

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Abstract

FOXP3+ CD8+ T cells are present at low levels in humans, however the function of these cells is not known. Here, we demonstrate a rapid expansion of CD25+ FOXP3+ CD8+ T cells (CD8+ Tregs) in the blood and multiple tissues following a pathogenic SIV infection in rhesus macaques. The expansion was pronounced in lymphoid and colorectal mucosal tissues, preferential sites of virus replication. These CD8+ Tregs expressed molecules associated with immune suppressor function such as CTLA-4 and CD39, and suppressed proliferation of SIV-specific T cells *in vitro*. They also expressed low levels of granzyme B and perforin suggesting that these cells do not possess killing potential. Expansion of CD8+ Tregs correlated directly with acute phase viremia and inversely with the magnitude of anti-viral T cell response. Expansion was also observed in HIV-infected humans but not in SIV-infected sooty mangabeys with high viremia, suggesting a direct role for hyper-immune activation and an indirect role for viremia in the induction of these cells. These results suggest an important but previously unappreciated role for CD8+ Tregs in suppressing anti-viral immunity during immunodeficiency virus infections. These results also suggest that CD8+ Tregs expand in pathogenic immunodeficiency virus infections in the non-natural hosts and that therapeutic strategies that prevent expansion of these cells may enhance control of HIV infection.

Introduction

Regulatory T cells have a fundamental role in suppressing autoimmune manifestations and maintaining tolerance in peripheral tissues (186-190). They exist in both CD4+ and CD8+ T cell compartments, however CD4+ regulatory T cells have been better characterized. Multiple subsets of CD4+ regulatory T cell have been defined. These include natural regulatory T cells, type 1 regulatory T cells (Tr1) and T-helper 3 cells (Th3)(100). Natural CD4+ regulatory T cells are characterized by constitutive expression of transcription factor Forkhead box P3 (FOXP3), and high levels of IL-2 receptor alpha chain (CD25) (99, 191, 192). These cells act in an antigen-nonspecific manner by a cell-to-cell contact-dependent mechanism (101, 193-195). In contrast, the Tr1 and Th3 cells do not constitutively express FOXP3 and act via secretion of cytokines such as IL-10 and TGF-β, respectively (103, 196).

FOXP3 is thought to be the most specific marker for CD4+ regulatory T cells. In mice, FOXP3 is strictly correlated with regulatory activity. However, human activated effector T cells have been shown to express FOXP3 following stimulation *in vitro* (197-200). These FOXP3 expressing activated T cells possess the classic phenotype of Tregs, however may (201) or may not (198, 199) possess suppressive function. Thus, expression of FOXP3 alone by human T cells is not indicative of their suppressive capacity.

FOXP3+ CD8+ T cells have not been reported in mice. However, a small fraction of CD8+ T cells in human blood express FOXP3 (202) and characterization of these cells ex-vivo has been difficult due to their low frequency (104). Currently it is not known how these cells develop *in vivo*. Many studies demonstrated the induction of FOXP3+ CD8+ T cells *in vitro* following polyclonal activation or peptide-specific stimulation of human

PBMC (105, 201, 203-206). These *in vitro* generated FOXP3+ CD8+ T cells have been shown to suppress T cell activation by inducing a tolerogenic phenotype in antigen presenting cells (105). Very little is known about the modulation of FOXP3+ CD8+ T cells *in vivo* following an infection or a disease. Similarly, very little is known about their suppressive potential *in vivo*. A recent study demonstrated the expansion of FOXP3+ CD8+ Tregs with suppressive potential at the tumor site in patients with colorectal cancer (207). The expansion of FOXP3+ CD8+ Tregs with suppressive potential has not yet been reported following an infection *in vivo*.

Regulatory T cells may play an important role in influencing viral control and disease progression during chronic HIV/SIV infections. On one hand, they may suppress anti-viral cellular immunity and thereby diminish viral control. On the other hand, they may suppress hyper-immune activation, a strong predictor for rapid disease progression, and thus may prolong survival. Several studies evaluated the fate of CD4+ regulatory T cells during HIV (121, 122, 135, 208, 209) and SIV (118, 210-212) infection and demonstrated a loss of these cells following infection suggesting that these cells may not contribute for suppression of anti-viral immunity or hyper-immune activation. While these studies characterized CD4+ regulatory T cells, there is very little data available on the fate of CD8+ regulatory T cells during HIV and SIV infections. It is possible that HIV/SIV infections induce CD8+ regulatory T cells to suppress anti-viral immunity. Indeed, a few recent studies demonstrated a low level expansion of FOXP3+ CD8+ T cells in the blood of SIV-infected cynomologous macaques and HIV-infected humans (106, 140, 141). However, these studies did not evaluate the suppressive capacity of these cells. In addition, it is important to study the fate of regulatory T cells in the lymphoid

tissue and gut (the major reservoirs of HIV/SIV) and their influence on anti-viral T cell response and hyper-immune activation *in vivo*. In view of the depletion of CD4+ regulatory T cells during SIV infection, we hypothesize that CD8+ regulatory T cells capable of suppressing anti-viral immune responses may be induced following HIV/SIV infection.

In this study, we conducted a thorough evaluation of the magnitude of CD25+ FOXP3+ CD8+ T cells (CD8+ Tregs) in multiple tissues including intestinal mucosa following SIV infection in rhesus macaques and studied their influence on anti-viral T cell response *in vitro* and *in vivo*. Our results demonstrate the expansion of CD8+ Tregs with suppressive capacity in blood and multiple tissues following infection. The expansion was higher at the preferential sites of virus replication such as lymphoid and colorectal tissue, than in blood. Importantly, the early expansion of these cells correlated inversely with acute phase anti-viral CD4+ and CD8+ T cell response. Expansion of these cells was not observed in SIV-infected sooty mangabeys despite these animals having high viremia. These results demonstrate an important but previously unappreciated role for CD8+ Tregs in suppressing anti-viral immunity during immunodeficiency virus infections in their non-natural hosts and suggest a direct role for hyper-immune activation and an indirect role for viremia in their induction.

Materials and Methods

Non-human primates and SIV infection

Young adult rhesus macaques and sooty mangabeys from the Yerkes breeding colony 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 Institutional Animal Care and Use Committee. Rhesus macaques were infected with SIVmac251 either intravenously or intrarectally at a dose of 100 or 1000 TCID50, respectively. Dr. Nancy Miller at NIH provided the challenge stock. All animals were infected under these conditions. Some of the SIV-infected macaques were treated with anti-retroviral drugs PMPA (20mg/kg), FTC (30mg/kg), Kaletra (Lopinavir, 12mg/kg; Ritonavir 3mg/kg) and AZT (5mg/kg) at about 18 weeks following infection. Sooty mangabeys were housed in colonies of 50-60 animals and SIVsm is endemic in this population. None of the animals used in the study were experimentally infected.

Human subjects

PBMC obtained from uninfected (HIV negative) and untreated HIV-infected individuals were used for analyses. The characteristics of the HIV-infected individuals have been presented previously (213). All of the HIV-infected individuals were recruited at the Emory Center for AIDS Research Clinic Research Core. HIV negative individuals were employees of the Emory University. The Emory University Institutional Review Board approved this study. Signed informed consents were obtained from all individuals before enrollment in the study.

Cell isolation from blood and rectal biopsies.

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood according to the standard Ficoll-Hypaque separation procedures as described before (214). Lymphocytes from pinch biopsies from the rectum were obtained as described before (214). Briefly, 10 to 20 pinch biopsies were collected in complete RPMI and washed two times with ice-cold Hank's buffered salt solution. Biopsies were digested with 200 units/ml of collagenase IV (Worthington, Lake wood, NJ) and DNase I (Roche, Indianapolis, IN), passed through decreasing sizes of needles (16-, 18-, and 20-gauge, five to six times with each needle), and filtered through a 100μm filter. Cells were washed twice with RPMI and resuspended in complete RPMI for analysis.

Staining for FOXP3 positive cells

FOXP3+ CD25+ T cells were measured directly ex vivo. Two million PBMCs or one million cells from colorectal mucosa were re-suspended in 100 μl of RPMI plus 10% FBS. Cells were stained with LIVE/DEAD marker (Invitrogen, Carlsbad, CA), followed by surface staining with antibodies against CD3 (APC-Cy7, clone SP34-2, BD Pharmingen, San Diego, CA), CD4+ (PerCP, clone L200; BD Pharmingen, San Diego, CA), CD8+ (Qdot 655, clone SK1; BD Bioscience, San Jose, CA), CD25 (PE, clone 4E3; Miltenyi Biotech, Auburn, CA), CD95, (Pacblue, clone DX2; Caltag, Carlsbad, CA) and CD28 (PE-Cy7, clone CD28.2; eBioscience, San Diego, CA) for 30 minutes at 4^{o}C. Cells were washed with PBS with 2% FBS, and fixed with 1X fix/perm buffer (Biolegend, San Diego, CA) for 20 minutes at room temperature. Cells were then washed once with Permeabilization solution (Biolegend, San Diego, CA), and incubated in this solution for 15 minutes. Cells were washed once with PBS with 2% FBS, and

incubated for 30 minutes with monoclonal antibody to FoxP3 (Alexa Fluor 488, clone 206D; Biolegend). Cells were then washed twice with PBS with 2% FBS, and resuspended in 1% formalin in PBS. Samples were acquired on LSRII (BD Immunocytometry systems, San Jose, CA) and analyzed using FlowJo software (Treestar, Inc., Ashland, Oregon). Lymphocytes were identified based on live cells, followed by scatter pattern and CD3+, CD8+-, CD4+ cells were considered as CD4+ T cells and CD3+, CD8+, CD4+- cells were considered as CD8+ T cells (Fig. 1A). The gates for FOXP3+ cells were defined based on the lack of FOXP3 expression on non-CD3+ cells within the same sample.

For analysis of the expression of intracellular Ki-67 (clone B56, BD Pharmingen, San Diego, CA), surface CD127 (clone R34.34; Beckman-Coulter, Fullerton, CA) and surface Cytotoxic T Lymphocyte Antigen-4 (CTLA-4) (clone BNI3; BD Pharmingen, San Diego, CA), intracellular Granzyme B (clone GB11,BD Biosciences, San Diego, CA), intracellular perforin (PF344, Mabtech, Cincinnati, OH) a similar procedure was used except that PE anti-CD25 Ab was replaced with antibody against the respective marker. For analysis of tetramer positive cells, cells were stained with antibodies against human CD3, CD8+ and FOXP3, and Gag CM9 tetramer (215).

Intracellular cytokine staining analysis

Intracellular cytokine production was assessed as previously described with few modifications (216). Briefly, two million PBMCs were stimulated in 200 μl of RPMI with 10% FBS in a 5 ml polypropylene tube. Peptide pools (15mer overlapping by 11) specific for SIV Gag and Env were used for stimulations at a final concentration of 1

μg/ml. Staphylococcal enterotoxin B (SEB) was used as a positive control at 1 μg/ml. Stimulations were performed in presence of anti-CD28 and anti-CD4+9d antibodies (1 μg/ml; BD Pharmingen, San Diego, CA). Cells were incubated at 37°C in the presence of 5% CO_2 for 6 hours. Brefeldin A (10 μg/ml) was added after 2 hours of incubation. At the end of stimulation, cells were washed once with phosphate-buffered saline (PBS) containing 2% FBS, surface stained for 30 min at 4°C with anti-human CD4+ (clone L200; BD Pharmingen, San Diego, CA), anti-human CD3 (clone SP34-2, BD Pharmingen, San Diego, CA) and anti-human CD8+ (clone SK1; BD Biosciences, San Jose, CA). Cells were fixed with cytofix/cytoperm (BD Pharmingen, San Diego, CA) for 20 min at 4°C and permeabilized with 1X permwash (BD Pharmingen, San Diego, CA). Cells were then incubated for 30 minutes at 4°C with anti-human IFN-γ Ab, washed twice with 1X Permwash, once with 2% FBS in PBS and resuspended in 1% formalin in PBS. Approximately 500,000 lymphocytes were acquired on the LSRII (BD Immunocytometry systems) and analyzed using FlowJo software (Treestar, Inc., San Carlos, California). Lymphocytes were identified based on their scatter pattern and CD3+, CD8+-, CD4+ cells were considered as CD4+ T cells, and CD3+CD8+CD4+- cells were considered as CD8+ T cells. These CD4+ or CD8+ T cells were then gated for cytokine positive cells.

Suppressor Assay

PBMCs were CFSE stained according to previously described protocol (217). Cells were then incubated with CD4+ microbeads (Miltenyi Biotec, Auburn, CA)) for 15 minutes at 4°C and were negatively selected on LD columns (Miltenyi Biotec, Auburn, CA). Column bound CD4+ cells were reserved at 4°C for later usage. Cells from the

flow through of LD columns (CD4+ depleted cells) were incubated with CD25 microbeads (Miltenyi Biotec, Auburn, CA) for 15 minutes at 4^{o}C and were positively selected on MS columns (Miltenyi Biotec, Auburn, CA). The column bound fraction was considered as CD8+ Tregs, and the flow through of MS columns (CD4+ depleted and CD25 depleted cells) was mixed with CD4+ T cells (column bound fraction of LD columns) in same proportion as prior to depletion. This cell population contained all cells except CD8+ Tregs (CD8+ Treg depleted cells). Stimulations with Gag and Env peptide pools, and SEB were set up for the following subsets: Total cells, CD8+ Treg depleted cells, and where possible, CD8+ Treg depleted cell fraction reconstituted with CD8+ regulatory T cells. This reconstitution generally yielded a ratio of 1:0.08 (Total CD8+ Treg depleted cells: CD8+ Tregs). Stimulation was conducted for 5 days. Cells were then surface stained for LIVE/DEAD marker (Invitrogen), anti-CD3 (APC-Cy7, clone SP34-2; BD Pharmingen, San Diego, CA), anti-CD4+ (PerCP, clone L200; BD Pharmingen, San Diego, CA), anti-CD8+ (Qdot 655, clone SK1; BD Bioscience, San Jose, CA), and intracellularly for anti-Ki-67 (PE, clone B56; BD Pharmingen, San Diego, CA). Proliferation status was assessed by CFSE dilution, and expression of Ki-67 nuclear protein.

Quantitation of SIV RNA Plasma Load

The SIV copy number was determined using a quantitative real-time PCR as previously described (216). All specimens were extracted and amplified in duplicates, and the mean results are reported.

Statistical Analysis

The Wilcoxon signed-rank test was used to compare the frequency of FOXP3+ T cells between blood and colorectal tissue and between different time points after SIV infection. The Wilcoxon rank-sum test was used to compare the frequency of FOXP3+ CD8+ T cells in humans and sooty mangabeys. The Spearman's rank correlation coefficient (r_s) or the Pearson's product moment correlation coefficient (r) was used to assess the relationship between viral load, percentage of FOXP3+ cells and percentage of anti-viral T cells. If the data followed the parametric assumptions, Pearson's method was used, otherwise Spearman's method was used. A two-sided $p<0.05$ was considered statistically significant. Statistical analyses were performed using software program S-PLUS 7.0.

Results

FOXP3+ CD8+ T cells are present at low levels in blood and colorectal tissue of normal macaques

We measured the steady state levels of CD4+ and CD8+ T cells that co-express FOXP3 and CD25 in blood and colorectal mucosal tissue of normal (SIV negative) macaques to understand the relative levels and distribution of regulatory T cells. FOXP3+ CD25+ CD4+ T cells (referred to as 'CD4+ Tregs' hereafter) were readily detected in peripheral blood as well as colorectal mucosa (Fig. 2.1). The frequencies of CD4+ Tregs were generally similar in the two compartments with a geometric mean frequency of 4.8% of total CD4+ T cells (Fig. 2.1A and 2.1B). In contrast to CD4+ T cells, only a small fraction of CD8+ T cells co-expressed FOXP3 and CD25 (referred to as 'CD8+ Tregs' hereafter) (Fig. 2.1A and 2.1B). The frequencies of these CD8+ Tregs in normal macaques had geometric mean of 0.2% in blood and 0.12% in the colorectal mucosal tissue. These results demonstrate that in normal macaques CD8+ Tregs are present at much lower levels than CD4+ Tregs (Fig. 2.1B), and suggest that the latter play a greater role in maintaining T cell homeostasis and tolerance to self-antigens. In addition, the differences in frequencies could also point to differences in mechanisms utilized by the two cell types.

Rapid expansion of CD8+ Tregs following SIVmac251 infection: Higher expansion in colorectal mucosa than blood

To understand the role of CD8+ Tregs in SIV infection, we studied the frequencies of these cells both in blood and colorectal mucosa of 17 rhesus macaques that

were infected with SIVmac251. Analyses were performed before and at 2, 3, 6, 12 and 24 weeks following infection. The frequencies of CD8+ Tregs expanded in both blood and colorectal mucosal tissue following SIV infection. In blood, CD8+ Tregs peaked between 2-6 weeks, and ranged from 0.4% to 1.5% of total CD8+ T cells (Fig. 2.2B). Peak levels were approximately 2.5-fold higher than their pre-challenge levels ($p=0.003$), and remained fairly constant over the next 18 weeks (data not shown). Similar or higher increases were also observed for the absolute number of CD8+ Tregs in blood following SIV infection (Fig. 2.2C).

A greater expansion of CD8+ Tregs (4.3-fold) was observed in the colorectal mucosa than in blood following SIV infection ($p<0.001$) (Fig. 2.2A and 2.2B). In the colorectal mucosa, CD8+ Tregs generally peaked at week 6 and then remained fairly constant during the next 18 weeks (data not shown). The peak levels of these cells ranged from 0.5% to 6.7% of total CD8+ T cells and were approximately 14-fold higher than the levels observed prior to infection ($p<0.001$). A similar pattern was also observed for the absolute number of CD8+ Tregs in the colorectal mucosal tissue (Fig. 2.2D).

In order to assess whether expansion of CD8+ Tregs is a reflection of global T cell activation that occurs during viral infections, we compared the magnitude and kinetics of expansion of CD8+ Tregs and total activated CD8+ T cells (CD8+ CD25+) both in blood and colorectal mucosal tissue (Fig. 2.2C and 2.2D). In colorectal tissue, the expansion of total activated CD8+ T cells occurred by 2 weeks whereas in blood occurred by 6-12 weeks (Fig. 2.2D). These levels were generally 10-fold higher compared to the magnitude of CD8+ Tregs in the respective tissue. In addition, the expansion of total

activated CD8+ T cells peaked later in blood and earlier in colorectal tissue compared to CD8+ Tregs.

To further understand if FOXP3+ CD8+ T cells are generated following activation *in vitro*, we stimulated PBMCs for five days with anti-CD3 and anti-CD28 and stained for FOXP3 and CD25 (Fig 2.2E). As expected, we observed that the majority of CD8+ T cells express CD25 by the end of stimulation. However, only a small fraction (0.65%) of these CD25+ T cells expressed FOXP3, suggesting that the activation of macaque CD8+ T cells through TCR alone does not induce FOXP3 expression on these cells *in vitro*. Collectively, these results demonstrate that expansion of CD8+ Tregs occurs independent of total activated CD8+ T cells and suggest that CD8+ Tregs may not represent a subset of activated CD8+ T cells.

To further assess the expansion of CD8+ Tregs in HIV infection, we compared the levels of these cells in blood of HIV infected and uninfected individuals (Fig. 2.2F). CD8+ Tregs were present at low levels (geometric mean frequency of 0.014% of total CD8+ T cells) in HIV-negative humans. However, these cells were readily detectable and were 14-fold higher (geometric mean frequency of 0.2% of total CD8+ T cells; $p<0.001$) in HIV-infected individuals. These results demonstrate that CD8+ Tregs also expand following HIV infection in humans.

Selective expansion of CD8+ Tregs in intestinal mucosa following SIV infection

We next determined the distribution of CD8+ Tregs in various tissues of SIV negative and SIV-positive macaques (Fig. 2.3A and 2.3B). Tissues analyzed included blood, gut associated lymph nodes, spleen, thymus, liver, genitalia and various intestinal

mucosa. In normal macaques, low levels of CD8+ Tregs were detected in blood, lymphoid tissues, liver and genitalia, with highest levels detected in lymph nodes (approximately 1.5% of total CD8+ T cells). Similarly, in SIV-infected macaques, CD8+ Tregs were present in blood, lymphoid tissues, liver and genitalia (Fig. 2.3A and 2.3B). However, the levels of these cells tended to be higher in the lymphoid tissue of SIV-infected macaques than uninfected macaques.

Interestingly, CD8+ Tregs were present mostly below our detection limit (<0.01%) in intestinal mucosal tissue of normal macaques (Fig. 2.3B). However, in contrast to SIV negative macaques, CD8+ Tregs were readily detected in intestinal mucosal tissue of SIV-infected macaques, with the highest levels in the large intestine (colon and rectum, ranging from 0.5-6% of total CD8+ T cells, $p<0.05$) (Fig. 2.3B). These results demonstrate a selective expansion of CD8+ Tregs in the intestinal mucosal tissue of rhesus macaques following SIV infection.

CD8+ Tregs express molecules associated with regulatory function

We next sought to characterize CD8+ Tregs during chronic SIV infection (weeks 12-24 post SIV infection) for the expression of phenotypic markers that predict their function (Fig 2.4A, and 2.4B) such as Cytotoxic T Lymphocyte Antigen-4 (CTLA-4) and ATP ectonucleotidase (CD39). At 24 weeks post infection, about 40% of CD8+ Tregs expressed CTLA-4, which is known to participate in down-regulating immune responses (218) (Fig. 2.4A and 2.4B). This phenotype was stable even one year post infection (data not shown). Approximately 30% of CD8+ Tregs expressed CD39 (Fig. 2.4A and 2.4B), which degrades ATP into cAMP, and has been identified as a regulatory T cell marker

(Fig 2.4A and 2.4B) (181, 182). ATP is critical for actively proliferating cells and thus degradation of this molecule creates a microenvironment that is not conducive for proliferation of effector cells. To assess whether CD8+ Tregs have cytolytic capacity, we characterized these cells for expression of granzyme B and perforin. CD8+ Tregs expressed lower levels of granzyme B and perforin than FOXP3-ve CD8+ T cells, suggesting that these cells may not possess killing potential (Fig. 2.4A and 2.4B). Similar to human CD4+ Tregs (219, 220), CD8+ Tregs in rhesus macaques also expressed low levels of CD127 (Fig. 2.4A and 2.4B). We next characterized the proliferation status of CD8+ Tregs *in vivo* by staining for intranuclear expression of Ki-67 (Fig. 2.4A and 2.4B). Consistent with their expansion following infection, approximately 60% of CD8+ Tregs were actively proliferating at 12 weeks post infection.

To study whether these CD8+ Tregs produce IFNγ, we stimulated cells isolated from mesenteric lymph nodes with PMA/ionomycin and co-stained for expression of IFNγ and FOXP3 (Fig. 2.4C). About 10% of total FOXP3+ cells co-expressed IFNγ that was marginally lower than the levels expressed by FOXP3- CD8+ T cells demonstrating that these CD8+ Tregs can produce IFNγ following stimulation (Fig. 2.4C). It is important to note that the mean fluorescence intensity of FOXP3 on IFN-γ producing cells was lower compared to IFNγ- FOXP3+ cells. However, when cells were stimulated with a SIV Gag peptide pool, none of the FOXP3+ CD8+ T cells co-expressed IFNγ. Furthermore, none of the FOXP3+ CD8+ T cells were positive for Gag-CM9 tetramer that binds to CD8+ T cells specific for the immunodominant epitope CM9 in Mamu A*01 positive animals (215) (Fig. 2.4D). Collectively, these data suggest that these CD8+ Tregs may not be specific for SIV antigens.

CD8+ Tregs suppress anti-viral T cell response *in vitro*

To assess whether the CD8+ Tregs are capable of suppressing T cell proliferation, we depleted these cells and compared proliferation of total and CD8+ Treg depleted cells in response to either SIV antigens or a mitogen, SEB (Fig 2.5A). Selective depletion of CD8+ Tregs was achieved by a sequential depletion of CD4+ cells followed by CD25+ cells and reconstitution with CD4+ cells as outlined in Fig. 2.5A. This procedure yielded a near complete depletion of CD8+CD25^{high} Tregs (Fig. 2.5B). Selective depletion of CD8+ Tregs either from PBMC or cells obtained from colonic lymph nodes resulted in enhanced proliferation (2-33 fold; $p<0.05$) of CD8+ T cells in response to stimulation with SIV antigens (Gag or Env) suggesting that these CD8+ Tregs have suppressive potential (Fig 2.5C and 2.5D). In two of the five samples tested, proliferation prior to CD8+ Treg depletion was below our detection level (0.01%). However, after depletion of CD8+ Tregs, proliferation was clearly detectable at about 2% of total CD8+ T cells (Fig. 2.5D). It is important to note that this enhancement in proliferation was observed despite the presence of CD4+ Tregs in the culture. This enhancement in proliferation in the absence of CD8+ Tregs was not consistently observed following stimulation with SEB (Fig 2.5C and 2.5D).

To determine a direct evidence that CD8+ Tregs exert suppression, in some experiments we added these cells back to CD8+ Treg depleted cells at a CD8+ Treg:total PBMC ratio of 1:12.5 and evaluated their ability to suppress proliferation of CD8+ T cells in response to stimulation with SIV Gag peptide pool. Addition of CD8+ Tregs resulted in near total loss of proliferation of CD8+ T cells ($p<0.05$) (Fig. 2.5D), indicating that these cells are highly potent at suppressing anti-viral specific responses.

CD8+ Tregs suppress anti-viral T cell response *in vivo*

To assess whether CD8+ Tregs had a potential role in suppressing the expansion of anti-viral T cells *in vivo*, we compared the magnitude of SIV-specific cellular immune response and CD8+ Tregs early following infection in blood (Fig. 2.6A). We observed an inverse correlation between the frequency of SIV-specific IFN-γ producing CD8+ ($p=0.043$) or CD4+ T cells ($p<0.01$) and the levels of CD8+ Tregs at 2-3 weeks post infection (Fig. 2.6A).

To further understand the relationship between CD8+ Tregs and anti-viral CD8+ T cell response in the colorectal tissue, we compared the magnitude and kinetics of expansion of CD8+ Tregs and CD8+ T cells specific for the immunodominant Gag-CM9 epitope in Mamu A*01+ animals (Fig. 2.6B). In agreement with the suppression of anti-viral T cells in blood, we also observed an inverse relationship for the kinetics of expansion and contraction between CD8+ Tregs and the Gag CM9-tetramer positive T cells in the colorectal tissue. Importantly, in 3 of the 4 animals studied, the magnitude of CD8+ Tregs was comparable to the magnitude of Gag CM9-tetramer-specific CD8+ T cells suggesting that ratios of responder:Treg ratios similar to those frequently used for *in vitro* suppression assays can be achieved *in vivo*. Similar results were also observed in an additional 6 Mamu A*01+ rhesus macaques (data not shown). These results suggest an important role for CD8+ Tregs in suppressing virus-specific T cell response during early stages of SIV infection in macaques.

High levels of CD8+ Tregs correlate with high levels of viremia in rhesus macaques

In order to comprehend the relationship between the levels of CD8+ Tregs and viral load during SIV infection, we compared the levels of these cells and viral load during chronic phase of SIV infection (week 10 or 12 post infection) (Fig. 2.7A). A direct correlation between the plasma viral load and levels of CD8+ Tregs in blood ($p<0.001$) was observed (Fig. 2.7A), suggesting that high levels of viral RNA induce high levels of CD8+ Tregs following infection. This correlation was true for both percent CD8+ Tregs (Fig. 2.7A) and absolute number of CD8+ Tregs (data not shown).

In contrast to CD8+ Tregs, and as shown previously (118, 210-212), a decline in the absolute number of CD4+ Tregs was observed following SIV infection (Fig. 2.7B). This depletion was observed both in peripheral blood ($p<0.01$) and colorectal mucosa ($p<0.001$) and had occurred early following infection (Fig. 2.7B). Furthermore, depletion of CD4+ regulatory T cells was greater in colorectal mucosa than in blood (Fig. 2.7B). Depletion was also observed in other tissues such as gut associated lymph nodes, spleen and small intestine (data not shown). Consistent with the loss of CD4+ Tregs, about 25% of these cells expressed the viral co-receptor CCR5 (data not shown). An inverse correlation was observed between viremia and levels of CD4+ Tregs in colorectal tissue at 10-12 weeks post infection ($p<0.01$) (Fig. 2.7C). Collectively these results suggest that the loss of CD4+ Tregs might occur due to the cytopathic effects of viral infection as well as possible direct killing by the virus, and indicate that CD4+ Tregs play minimal role in modulating antiviral T cell responses during SIV infection.

To further understand the influence of viral load on CD8+ Tregs, we evaluated their levels in SIV-infected macaques before and after anti-retroviral therapy (ART) (Fig.

2.7D). Suppression of viral load with ART resulted in reduction in the frequency and absolute numbers (data not shown) of CD8+ Tregs in the colorectal mucosa ($p<0.001$). In contrast, suppression of viral load with anti-retroviral therapy resulted in an increase in the frequency of CD4+ Tregs in the colorectal mucosa ($p=<0.01$), indicating that reduction in viral load due to ART treatment can restore CD4+ Treg levels. This may be a reflection of total CD4+ T cell reconstitution that occurs after ART treatment. Interestingly, in the blood, ART did not influence the frequency or absolute number of either total CD8+ or CD4+ Tregs (Fig. 2.7D). However, we observed a significant reduction in the frequency of FOXP3+ central memory CD8+ T cells following ART (data not shown).

No expansion of CD8+ Tregs in SIV-infected Sooty Mangabeys

To better understand whether the expansion of CD8+ Tregs is a common feature of immunodeficiency viral infections, we sought to quantify these cells in SIV-infected sooty mangabeys (Fig. 2.8). Sooty mangabeys rarely progress to disease despite the presence of high viremia. The lack of disease progression in SIV-infected sooty mangabeys has been attributed to the absence of high levels of hyper-immune activation, which is normally seen in SIV-infected rhesus macaques and HIV-infected humans (221). We assayed 8 sooty mangabeys chronically infected with SIVsm that maintained persistent set point viral loads for more than 5 years (Fig 2.8A). In SIV-negative sooty mangabeys, CD8+ Tregs were present at low levels (<0.2% of total CD8+ T cells) both in the rectum and blood (Fig. 2.8B). Interestingly, the levels of these cells were similar between the SIV-infected and uninfected sooty mangabeys (Fig. 2.8B) demonstrating that

CD8+ Tregs did not expand during chronic SIV infection of the natural host. These results demonstrate that expansion of CD8+ Tregs is not observed in all immunodeficiency virus infections and suggest that hyper-immune activation in addition to level of viremia may influence their expansion.

Discussion

Our study evaluating the magnitude of FOXP3+ CD8+ Tregs following a pathogenic SIVmac251 infection in rhesus macaques clearly demonstrates a rapid expansion of these cells in the colorectal mucosal tissue, a preferential site of virus replication. These CD8+ Tregs highly expressed molecules associated with immune suppressive function and suppressed proliferation of virus-specific T cells *in vitro*. In addition, our results demonstrate a role for these cells suppressing virus-specific T cell response early following infection *in vivo*. Collectively, these results suggest an important role for CD8+ Tregs in regulating anti-viral immunity and viral control following a pathogenic immunodeficiency virus infection.

Our study is the first to demonstrate suppressive capacity of CD8+ Tregs in an infectious disease setting. The *in vivo* generated CD8+ Tregs following SIV infection suppressed anti-viral T cell responses *in vitro*. In agreement with their suppressive capacity *in vitro*, we also observed an inverse correlation between the frequency of CD8+ Tregs and anti-viral T cells during the acute phase of infection suggesting their suppressive role *in vivo*. We did not consistently observe suppression of T cell responses following *in vitro* stimulation with SEB. This may be due to higher ratios of SEB-specific T cells:CD8+ Tregs in comparison to relatively lower ratios of SIV-specific T cells:CD8+ Tregs.

One of the interesting aspects of our study is that the expansion of CD8+ Tregs peaks in blood (wk 2) earlier than in colorectal tissue (wk 6). This was true whether the animals were infected via intravenous or intrarectal route (data not shown). In addition, a significant proportion of CD8+ Tregs in blood expressed β7 integrin that is needed for T

cell trafficking to gut (data not shown). Based on these results, we propose that CD8+ Tregs are expressed in local lymph nodes and migrate to gut through blood.

In contrast to the enhancement observed in the frequency and number of CD8+ Tregs, the number of CD4+ Tregs declined following SIV infection. In addition, an inverse correlation was observed between the number of CD4+ Tregs and plasma viral RNA, suggesting that these cells might have been infected and killed by the virus. Consistent with this hypothesis, previous studies, (209, 222) as well as our study demonstrated that CD4+ Tregs express viral co-receptor CCR5 and can be infected by the virus. Our results suggest that because human and simian immunodeficiency viruses infect CD4+ Tregs, these viruses may use CD8+ Tregs as a mechanism to mediate suppression of anti-viral immunity during acute and chronic phases of infection.

Regulatory T cells are known to exert their suppressive effects through various mechanisms. These include cell-to-cell contact dependent mechanisms using molecules such as CTLA-4 (190, 194, 223) and glucocorticoid-induced tumor necrosis factor receptor (GITR) (224), degradation of ATP (225); secretion of cytokines such as IL-10 (226) and TGF-β (227); and sequestration of IL-2. The CD8+ Tregs described in our study expressed CTLA-4 and CD39. CTLA-4 binds to the same ligands as co-stimulatory molecule CD28 and inhibits T cell activation. CD39 is an ectoenzyme that converts ATP into cAMP. Expression of CD39 by CD4+ Tregs has been shown to be associated with their suppressive function (181, 182). We are yet to quantify the levels of cAMP harbored by these CD8+ Tregs. However, our results suggest that CD8+ Tregs in SIV-infected macaques may use CTLA-4 and CD39 dependant mechanisms for their suppressive function. CD8+ Tregs generated *in vitro* have been shown to suppress T cell

proliferation using their cytolytic activity (228). This raises the possibility that CD8+ Tregs in our study might be cytolytic. But only a small fraction of CD8+ Tregs in our study expressed molecules associated with cytolytic function such as granzyme B and perforin suggesting that killing may not be a preferred mechanism of their suppressive function.

Understanding the mechanisms by which SIV infection induces these CD8+ Tregs could have therapeutic benefits. It has been shown that SIV/HIV induces increased expression of indoleamine 2,3-dioxygenase (IDO), an immunosuppressive enzyme that is capable of inducing CD4+ Tregs (229). This increase in IDO expression is detected in lymphoid and intestinal mucosal tissues as early as 7 days after SIV infection (121, 122, 125, 212). Similarly, TGF-β has also been shown to induce expression of FOXP3 by mouse naïve CD8+ T cells following stimulation with anti-CD3 and anti-CD28 (230). SIV infection has been shown to increase expression of TGF-β (212). Collectively, these results suggest that IDO and TGF-β may be playing a role in the induction of CD8+ regulatory T cells in rhesus macaques. We observed a direct correlation between the frequency of CD8+ Tregs and viral load suggesting that viral load influences the magnitude of these cells either directly or indirectly. Analysis of CD8+ Tregs in normal and SIV-infected sooty mangabeys revealed no increase in these cells either in blood or rectum. These results strongly suggest that the observed increase in SIV-infected rhesus macaques may be due to indirect effects of high viral load. SIV-infected rhesus macaques and sooty mangabeys differ significantly for the level of hyper-immune activation (231), with the former inducing much higher levels than the latter. In addition, SIV has been shown to induce IFN-α production by plasmacytoid DC from rhesus macaques but not

sooty mangabeys (232). It is possible that activation of pDC's and/or hyper-immune activation may be responsible for induction of CD8+ Tregs in the rectum of SIV-infected rhesus macaques.

The origin and specificity of these CD8+ Tregs is not clear. Consistent with their expansion *in vivo*, a significant proportion of CD8+ Tregs expressed the proliferation marker Ki-67. This suggests that the expansion in the number of CD8+ Tregs could be due to the division of existing cells rather than induction of this phenotype in Treg precursors. Recent studies have demonstrated that human CD8+ T cells express FOXP3 following stimulation with anti-CD3 in the presence of either IL-2 or IL-15 but not IL-7 *in vitro* (203). This raises the possibility that the CD8+ Tregs in SIV-infected animals could be SIV-specific. However, the failure to produce IFNγ and bind to an immunodominant epitope-specific tetramer, and inverse relationship with expansion of anti-viral T cell response suggest that these CD8+ Tregs might not be specific for the virus. It is possible that virus-specific Tregs may produce immunosuppressive cytokines such as TGF-β (233) and IL-10 in response to SIV antigen stimulation. It is also possible that the CD8+ Tregs express CD8+ alpha-alpha rather than CD8+ alpha-beta and thus may not bind tetramers. In the absence of these analyses it is difficult to conclude their SIV antigen specificity.

We observed that the expansion of CD8+ Tregs during SIV infection occurs in the lymphoid compartments and in the large intestine. The expansion of CD8+ T regs in large intestine might indicate that these cells are suppressing anti-viral T cells at the preferred site of viral replication, whereas expansion in lymphoid compartments indicates

that these cells may also be suppressing generation of new anti-viral T cells at site of T cell activation.

It has been suggested that high levels of regulatory T cells during acute HIV/SIV infection could be advantageous for the host by suppressing the hyper-immune activation that has been shown to play a role in progression to disease (231, 234, 235). However, prior reports of FOXP3+ CD8+ T cells in HIV or SIV infections (141, 202, 222) did not demonstrate suppressive activity of these cells, leaving open the question whether FOXP3 expression on CD8+ T cells is a true marker of regulatory activity or activation of CD8+ T cells. Our data suggests that hyper-immune activation is inducing CD8+ Tregs capable of suppressing SIV-specific CD4+ and CD8+ T cell responses and that the high levels of CD8+ Tregs present during acute infection may be deleterious rather than advantageous to the host. In conclusion, our results demonstrate a rapid expansion of FOXP3+ CD8+ Tregs at the preferential sites of virus replication following pathogenic SIV infection and provide evidence that these cells contribute to the suppression of anti-viral T cell responses and poor viral control. Our results suggest that therapeutic approaches that block the induction of these cells may improve viral control.

Figure legends

Figure 2.1. FOXP3 positive T cells in normal rhesus macaques. (A) Gating pattern for CD4+ and CD8+ FOXP3+ cells in peripheral blood (PBMC) and colorectal mucosa. T cells were identified based on live/dead cell marker exclusion, followed by side scatter (SSC) and CD3, followed by forward scatter (FSC) and CD3. CD3+CD4+CD8+- cells were identified as CD4+ T cells and CD3+CD4+-CD8+ cells were identified as CD8+ T cells. FOXP3+CD25+CD4+ T cells were identified as CD4+ Tregs and FOXP3+CD25+CD8+ T cells were identified as CD8+ Tregs. (B) Summary of the frequency of FOXP3+ CD4+ and CD8+ T cells as a percent of respective total cells in blood and colorectal mucosa of normal macaques. The horizontal lines represent geometric mean for the group. PBMC, Peripheral blood mononuclear cells; gut, cells from rectal biopsies. These experiments were repeated at least twice, with 8 macaques or more per experimental group.

Figure 2.2. Expansion of CD8+FOXP3+ T cells post SIV infection in rhesus macaques. (A) Representative FACS plots for detection of CD8+CD25+FOXP3+ T cells following SIV infection in peripheral blood and colorectal mucosa. CD8+ T cells were gated as described in Fig. 1 and analyzed for expression of FOXP3 and CD25. The percentages on the FACS plots represent the frequency of FOXP3+ CD25+ CD8+ T cells as a percent of total CD8+ T cells. (B) Summary of the frequency of CD8+CD25+FOXP3+ T cells expressed as a percentage of total CD8+ T cells. Each symbol represents an individual macaque (n=17). (C) Summary of absolute number of CD8+CD25+FOXP3+ or CD8+CD25+ T cells per milliliter of blood. (D) Summary of

number of CD8+CD25+FOXP3+ or CD8+CD25+ T cells per 100,000 lymphocytes in gut following SIV challenge. (E) Representative FACS plot for detection CD8+CD25+FOXP3+ T cells after culture for 5 days with or without anti-CD3 and anti-CD28 stimulation (left); Summary of CD8+CD25+FOXP3+ and CD8+CD25+FOXP3- T cells post stimulations (right). (n=3). (F) Representative FACS plot for detection of CD8+CD25+FOXP3+ T cells in uninfected and HIV-1 infected humans. Cells were gated as described in Fig 1 (left); Summary of frequency of CD8+CD25+FOXP3+ T cells in uninfected and HIV-1 infected humans (right) (n=10). Each symbol represents an individual animal in the experimental groups. Error bars represent means ± SEM. **Indicates a p value of less than 0.01 and *** indicates a p value of less than 0.001.

Figure 2.3. Expansion of CD8+ Tregs in various tissues of normal and SIV-infected rhesus macaques. (A) Representative FACS plots for CD8+CD25+FOXP3+ T cells in indicated tissue samples. (B) Summary of frequency of CD8+CD25+FOXP3+ T cells in tissue samples of uninfected (n=3) and SIV-infected rhesus macaques (n=7). Data from SIV-infected macaques was obtained during chronic phase of infection (16-90 weeks). Error bars represent means ± SEM. * Indicates a p value of less than 0.05.

Figure 2.4. Characterization of CD8+ Tregs. (A) Histograms showing expression of CTLA-4, CD39, Granzyme B, Perforin, Ki-67 and CD127 for CD8+ FOXP3+ (CD8+ Tregs) and CD8+ FOXP3- (CD8+ non-Tregs) during chronic (weeks 12-24) SIV infection. (B) Summary of expression CTLA-4, CD39, granzyme B, perforin, Ki-67 and CD127. Open bars represent marker expression on CD8+ Tregs and filled bars represent

marker expression on CD8+ non-Tregs. There are at least 5 macaques or more per experimental group (C) Co-expression of FOXP3 and IFN-gamma on cells isolated from mesenteric LN stimulated with PMA/ionomycin or Gag peptide pool. Cells shown represent CD8+ Tcells. Bar graphs represent IFN-gamma positive cells on either CD8+ non-Tregs (black bars) or CD8+ Tregs (white bars) following stimulation with PMA/ionomycin or Gag peptide pool (n=4). (D) Co-staining for FOXP3 and Gag-CM9 tetramer. CD8+ Positive cells were gated as described in Fig. 1 and analyzed for FOXP3 and tetramer. Numbers on the graphs represent the respective positive cells as a percent of total CD8+ T cells (n=12). * Indicates a p value of less than 0.05.

Figure 2.5. Suppressive potential of CD8+ Tregs *in vitro*. (A) Flow charts depicting the method used to either deplete or isolate isolate CD8+ Tregs from total cells. Briefly, CD4+ cells were isolated from total cells by using CD4+ microbeads and LD column purification. Column bound CD4+ cells were reserved for later usage. CD25+ T cells were isolated from the flow through of LD columns (CD4+ depleted cells) by using CD25 microbeads and selected on MS columns. The column bound fraction was considered as CD8+ Tregs, and the flow through of MS columns (CD4+ depleted and CD25 depleted cells) was mixed with CD4+ T cells (column bound fraction of LD columns) in same proportion as prior to depletion. This cell population contained all cells except CD8+ Tregs (CD8+ Treg depleted cells). CD8+ Tregs were added back to CD8+ Treg depleted cells for reconstitution of total cells. (B) Representative FACS plots demonstrating purity of cell depletion/isolation. (C) Representative FACS plots of CD8+ T cells depicting proliferation in response to stimulation to Gag peptide pool, or SEB

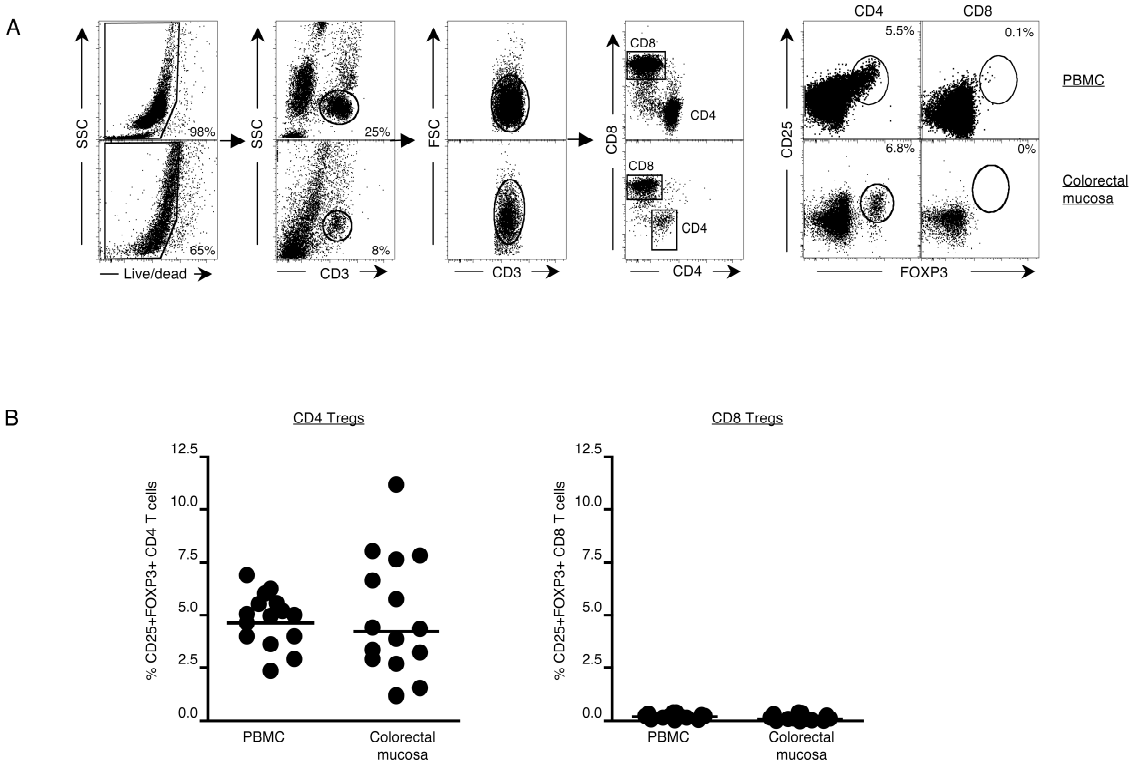
with and without CD8+ Treg depletion. (D) Summary of suppressor assay results for individual macaques. Each symbol represents an individual macaque. Closed symbols represent PBMCs and open symbols represent cells isolated from colonic lymph nodes. Experiments were repeated at least twice.

Figure 2.6. **Association between CD8+ Tregs with anti-viral T cell response in SIV-infected rhesus macaques *in vivo*** (A) Association between peak (wk 2 or wk 3) anti-viral specific CD8+ or CD4+ T cell response with CD8+ Tregs post SIV infection in blood (n=22). (B) Relationship between CD8+ Tregs and CM9-Gag tetramer-positive T cells in Mamu A*01 macaques in colorectal mucosa.

Figure 2.7. Relationship between CD8+ and CD4+ Tregs with viral load in SIV-infected macaques, and effect of ART on regulatory T cells. (A) Correlation between frequency of CD8+ Tregs and plasma viral RNA during chronic infection (wk 10 or 12 post SIV) (n=17). (B) Number of CD4+CD25+FOXP3+ T cells per milliliter of blood (left); number of CD4+CD25+FOXP3+ T cells per 100,000 lymphocytes in colorectal mucosa (right) (n=17). (C) Correlation between absolute numbers of CD4+ Tregs and plasma viral RNA during chronic infection (wk 10 or 12 post SIV) (n=17). (D) Influence of ART on CD8+ and CD4+ Tregs in blood and colorectal mucosa and viral load in blood. Each symbol represents an individual macaque (n=6). *** Indicates a p value of less than 0.001. ** Indicates a p value of less than 0.01.

Figure 2.8. **CD8+ Tregs in Sooty mangabeys.** (A) Plasma viral load in sooty mangabeys during the course of five years. (B) Frequency of CD8+ Tregs in blood and colorectal mucosa of normal (n=8) and SIV infected Sooty mangabeys (n=8).

Figure 2.1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12.5
10.0
7.5
5.0
2.5
0.0
PBMC
Colorectal mucosa

Figure 2.2

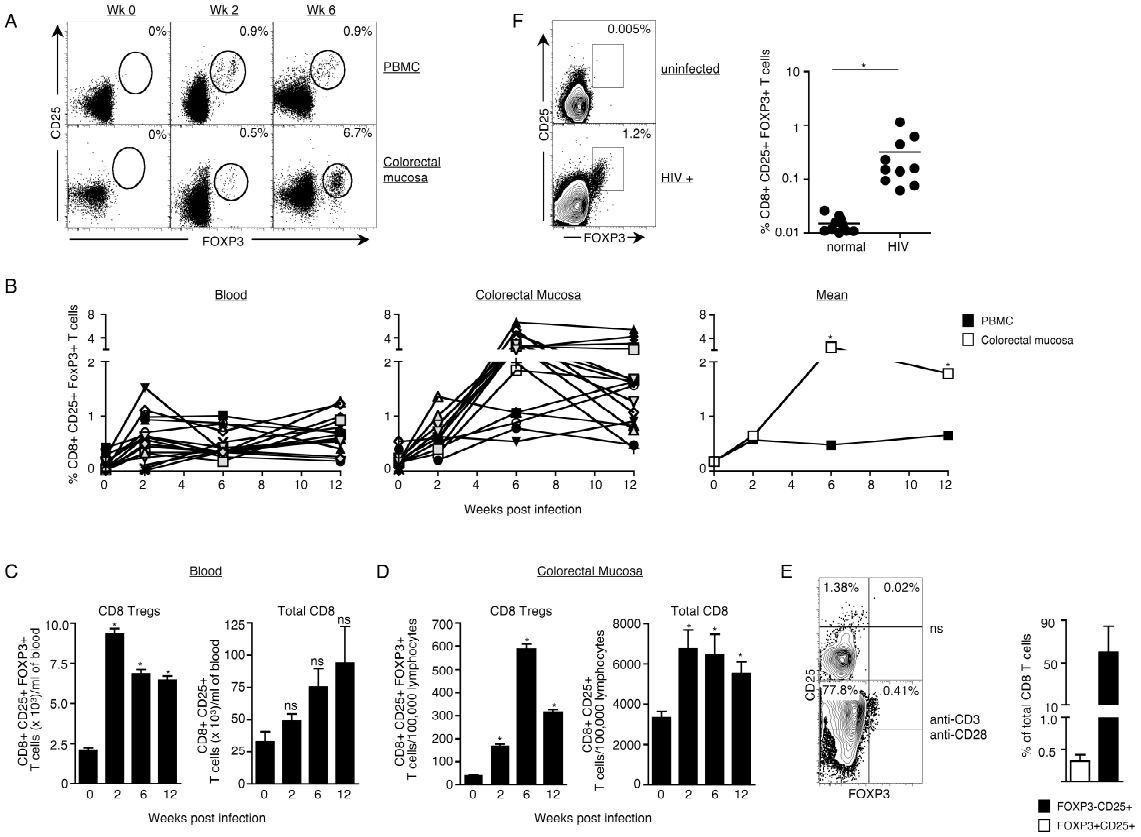
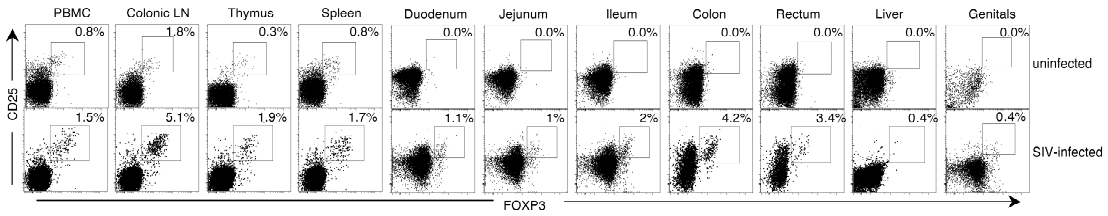

A
Wk 0
Wk 2
Wk 6
0%
0.9%
0.9%
PBMC
0%
0.5%
6.7%
Colorectal mucosa
CD25
FOXP3
F
0.005%
uninfected
1.2%
HIV +
CD25
FOXP3
% CD8+ CD25− FOXP3+ T cells
normal
HIV
B
Blood
Colorectal Mucosa
Mean
PBMC
Colorectal mucosa
% CD8+ CD25− FoxP3+ T cells
Weeks post infection
C
Blood
CD8 Tregs
Total CD8
CD8+ CD25+ FOXP3+ T cells (x 10⁶)/ml of blood
CD8+ CD25+ T cells (x 10⁶)/ml of blood
ns
Weeks post infection
D
Colorectal Mucosa
CD8 Tregs
Total CD8
CD8+ CD25+ FOXP3+ T cells/100,000 lymphocytes
CD8+ CD25+ T cells/100,000 lymphocytes
Weeks post infection
E
1.38%
0.02%
ns
77.8%
0.41%
anti-CD3
anti-CD28
CD25
FOXP3
% of total CD8 T cells
FOXP3-CD25+
FOXP3+CD25+

Figure 2.3

A


PBMC
Colonic LN
Thymus
Spleen
Duodenum
Jejunum
Ileum
Colon
Rectum
Liver
Genitals
0.8%
1.8%
0.3%
0.8%
0.0%
0.0%
0.0%
0.0%
0.0%
0.0%
0.0%
uninfected
1.5%
5.1%
1.9%
1.7%
1.1%
1%
2%
4.2%
3.4%
0.4%
0.4%
SIV-infected
CD25
FOXP3

B

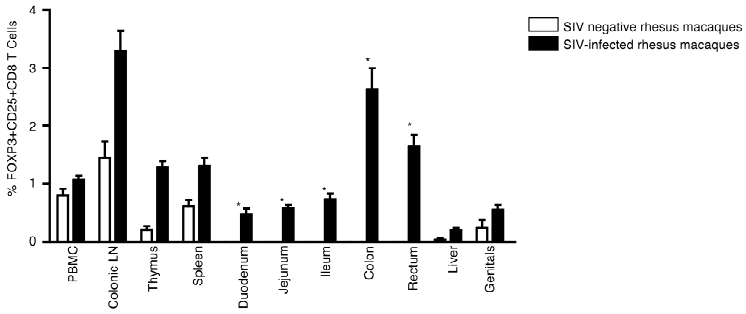

% FOXP3+CD25+CD8 T Cells
SIV negative rhesus macaques
SIV-infected rhesus macaques
PBMC
Colonic LN
Thymus
Spleen
Duodenum
Jejunum
Ileum
Colon
Rectum
Liver
Genitals

Figure 2.4

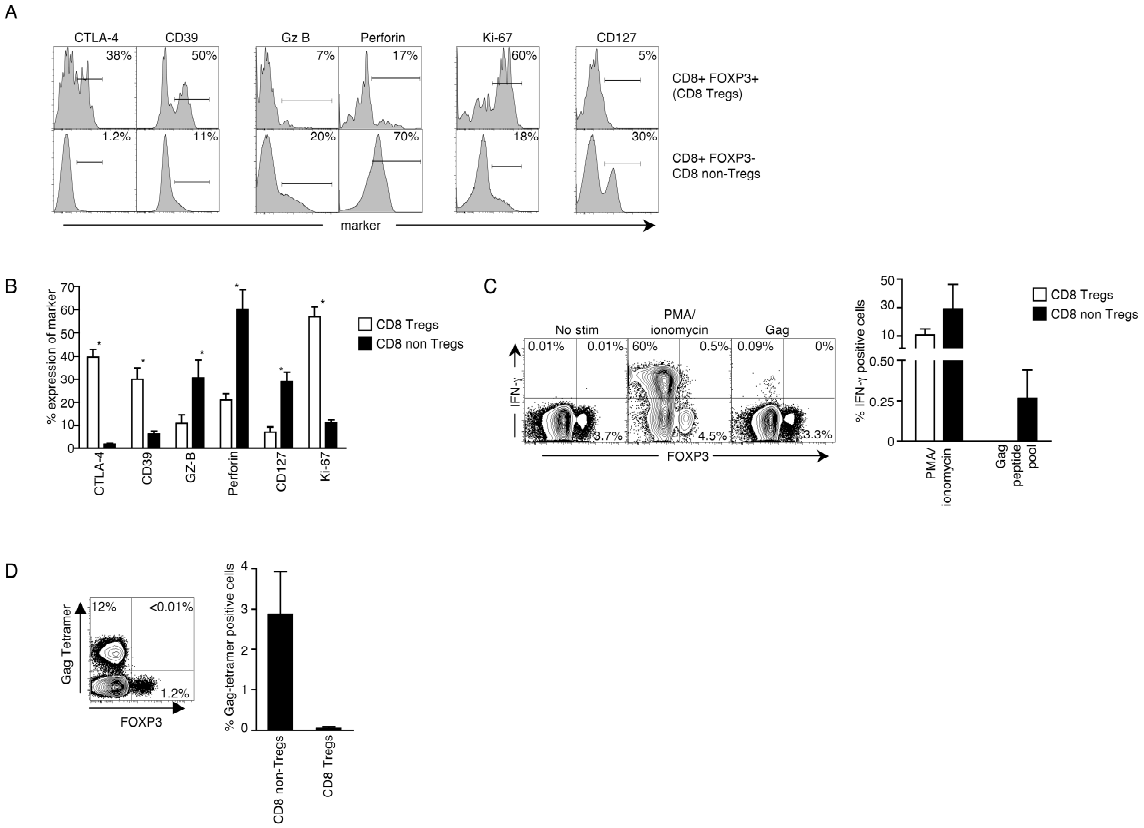

A
CTLA-4
CD39
Gz B
Perforin
Ki-67
CD127
38%
50%
7%
17%
60%
5%
1.2%
11%
20%
70%
18%
30%
CD8+ FOXP3+
(CD8 Tregs)
CD8+ FOXP3-
CD8 non-Tregs
marker
B
% expression of marker
CD8 Tregs
CD8 non Tregs
CTLA-4
CD39
GZ-B
Perforin
CD127
Ki-67
C
No stim
PMA/
ionomycin
Gag
0.01%
0.01%
60%
0.5%
0.09%
0%
IFN-γ
0.7%
4.5%
3.3%
FOXP3
% IFN-γ positive cells
CD8 Tregs
CD8 non Tregs
PMA/
ionomycin
Gag
peptide
pool
D
Gag Tetramer
12%
<0.01%
1.2%
FOXP3
% Gag-tetramer positive cells
CD8 non-Tregs
CD8 Tregs

Figure 2.5

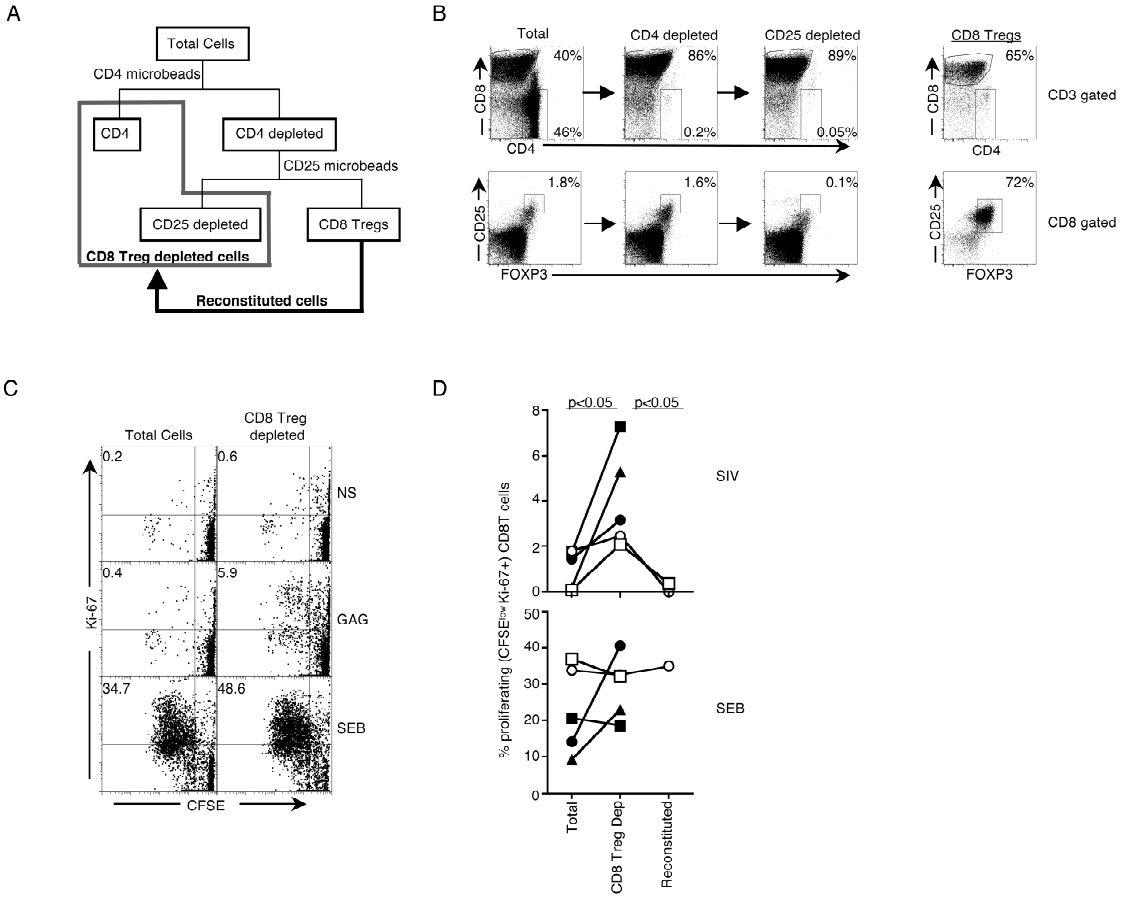

A
Total Cells
CD4 microbeads
CD4
CD4 depleted
CD25 microbeads
CD25 depleted
CD8 Tregs
CD8 Treg depleted cells
Reconstituted cells
B
Total
CD4 depleted
CD25 depleted
CD8 Tregs
40%
86%
89%
65%
46%
0.2%
0.05%
CD8
CD4
CD3 gated
1.8%
1.6%
0.1%
72%
CD25
FOXP3
CD8 gated
C
Total Cells
CD8 Treg depleted
0.2
0.6
NS
0.4
5.9
GAG
34.7
48.6
SEB
Ki-67
CFSE
D
p<0.05
p<0.05
SIV
SEB
% proliferating (CFSE^low Ki-67+) CD8T cells
Total
CD8 Treg Dep.
Reconstituted

Figure 2.6

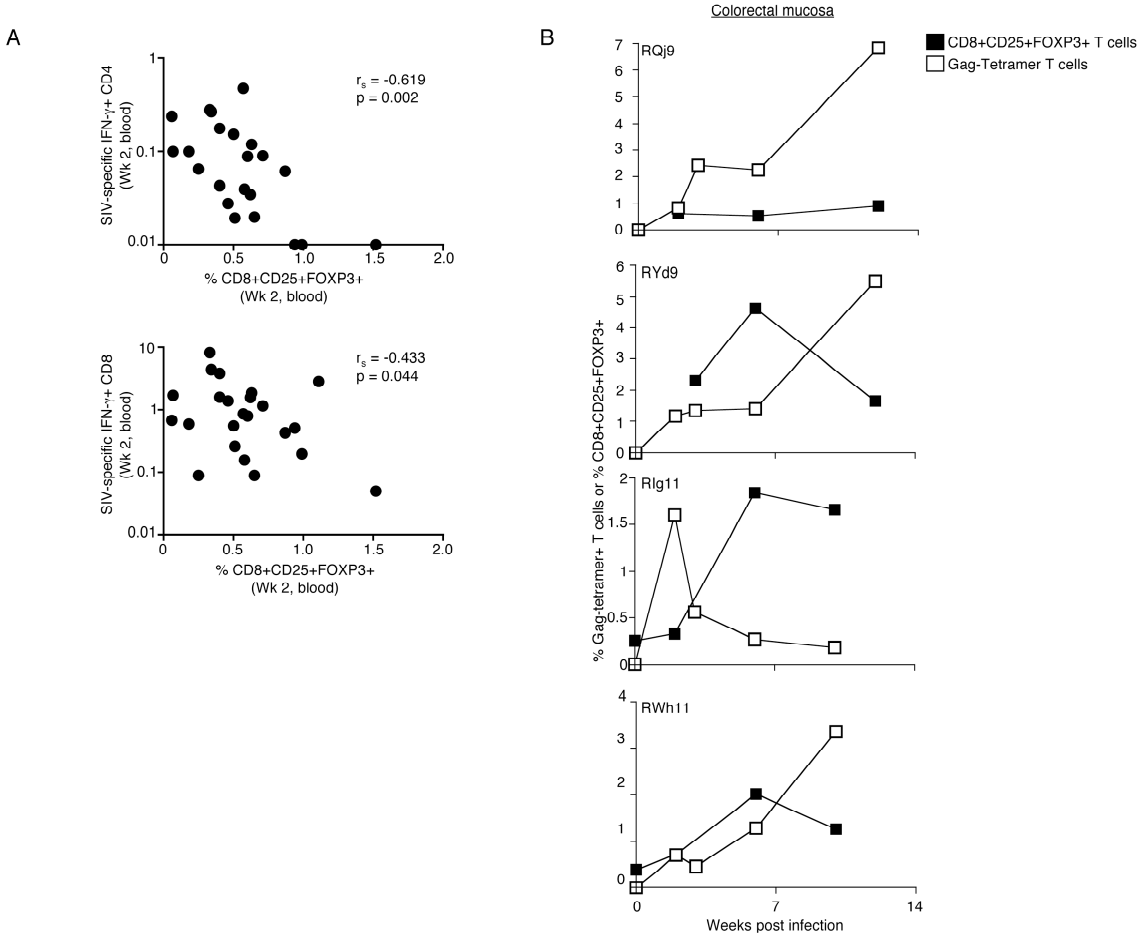

A
SIV-specific IFN-γ+ CD4 (Wk 2, blood)
r_s = -0.619
p = 0.002
% CD8+CD25+FOXP3+ (Wk 2, blood)
SIV-specific IFN-γ+ CD8 (Wk 2, blood)
r_s = -0.433
p = 0.044
% CD8+CD25+FOXP3+ (Wk 2, blood)
B
Colorectal mucosa
CD8+CD25+FOXP3+ T cells
Gag-Tetramer T cells
RQj9
RYd9
RIg11
RWh11
% Gag-tetramer+ T cells or % CD8+CD25+FOXP3+
Weeks post infection

Figure 2.7

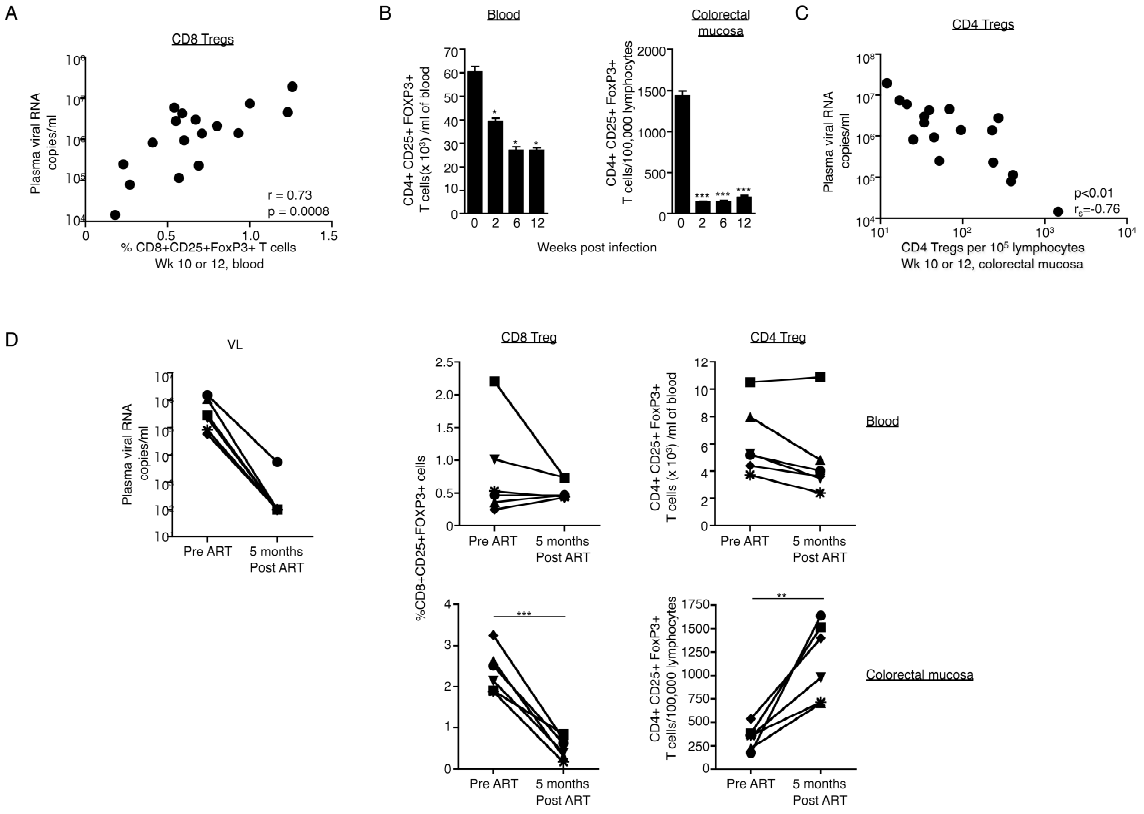
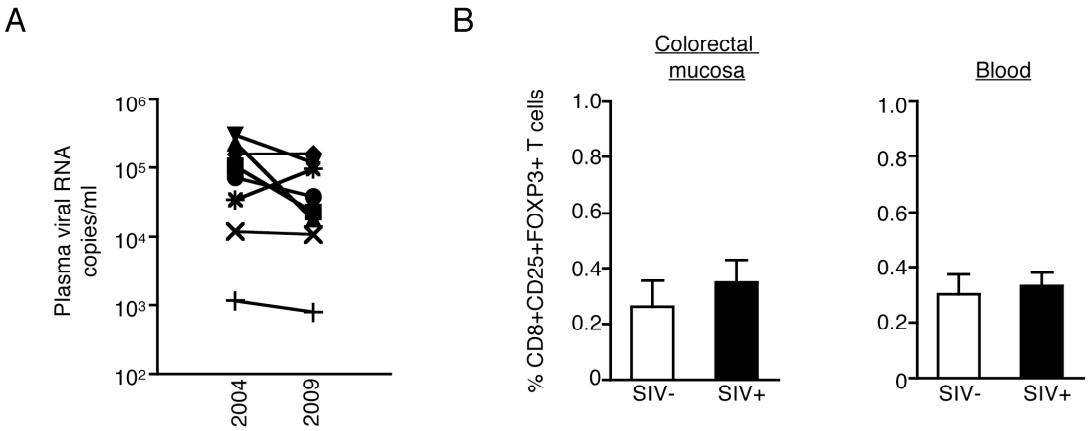

A
CD8 Tregs
Plasma viral RNA copies/ml
r = 0.73
p = 0.0008
% CD8+CD25+FoxP3+ T cells
Wk 10 or 12, blood
B
Blood
Colorectal mucosa
CD4+ CD25+ FOXP3+ T cells(x 10^6) /ml of blood
CD4+ CD25+ FoxP3+ T cells/100,000 lymphocytes
Weeks post infection
C
CD4 Tregs
Plasma viral RNA copies/ml
p<0.01
r_s=-0.76
CD4 Tregs per 10^5 lymphocytes
Wk 10 or 12, colorectal mucosa
D
VL
Plasma viral RNA copies/ml
Pre ART
5 months Post ART
CD8 Treg
CD4 Treg
%CD8-CD25+FOXP3+ cells
CD4+ CD25+ FoxP3+ T cells (x 10^6) /ml of blood
Blood
CD4+ CD25+ FoxP3+ T cells/100,000 lymphocytes
Colorectal mucosa

Figure 2.8


A
B
Plasma viral RNA copies/ml
10^6
10^5
10^4
10^3
10^2
2004
2009
Colorectal mucosa
Blood
% CD8+CD25+FOXP3+ T cells
1.0
0.8
0.6
0.4
0.2
0
SIV-
SIV+

CHAPTER 3

Loss of IL-17 producing CD8 T cells during late chronic stage of pathogenic SIV infection is associated with disease progression

Abstract

Progressive disease caused by pathogenic SIV/HIV infections is marked by systemic hyperimmune activation, immune dysregulation, and profound depletion of CD4+ T cells in intestinal tissues and gut-associated lymphoid tissues. IL-17 is a proinflammatory cytokine that functions at mucosal sites in protective immunity against extracellular bacterial infections and maintenance of the permeability of mucosal barrier. While IL-17 secreting CD4+ (Th17) and CD8+ (Tc17) T cells have been reported, very little is known about the latter subset for any infectious disease. Here, we characterized the anatomical distribution, phenotype and functional quality of Tc17 and Th17 cells in healthy and SIV-infected rhesus macaques. Our study confirms the predominance of Th17 and Tc17 cells in the small intestine over peripheral blood and lymphoid tissues. A greater proportion of Tc17 cells co-produce TNF-α and IL-2 than Tc1 cells. Surprisingly, both Tc17 and Th17 cells expressed higher levels of CTLA-4 than Tc1 and Th1 cells respectively, and only a minority of Tc17 expressed granzyme B. This suggests that Tc17 cells have a more regulatory than cytotoxic role. The depletion of Th17 cells occurs early following SIV infection but the depletion of Tc17 cells only occurs at the end-stage disease. Thus, Tc17 cells may compensate for the loss of Th17 cell during acute and chronic phases of pathogenic SIV infection. Anti-retroviral therapy (ART) was able to replenish Tc17 and Th17 cells in gut mucosa. However ART induced preferential replenishment of Th1 cells over Th17 cells. Depletion of Th17 and Tc17 cells is specific to pathogenic infections, as it was not seen in sooty mangabeys. Our results suggest that Tc17 cells have a critical role in regulating disease progression during pathogenic SIV infections.

Introduction

The gastrointestinal (GI) mucosa provides an interface between a sterile internal environment and a contaminated external environment. The GI tract becomes a key participant during HIV/SIV infections, because it harbors approximately 40% of all lymphocytes in the body (30), and nearly 90% of the memory CD4+ T cells in the GI tract are depleted within days following HIV/SIV infection. This early loss of memory CD4+ T cells compromises the ability of the host to generate secondary immune responses to pathogens and is thought to play a critical role in the progression to disease (10, 15, 64).

In addition to the loss of memory CD4+ T cells, HIV/SIV infections are also associated with impaired structure and function of gut mucosal tissue (31-35) culminating in the breakdown of the intestinal epithelial barrier (36, 37). Normal function of mucosal surfaces requires intact epithelium, with intact tight junctions. During HIV/SIV infections, there is down regulation of genes involved in intestinal epithelial cell growth and renewal, as well as increased expression of genes related to inflammation and immune activation (38-40). There is also an increase in pro-inflammatory cytokine secretion that could facilitate mucosal tissue damage (41-44). This altered intestinal epithelial permeability is believed to permit bacterial translocation leading to hyperimmune activation that has been shown to distinguish pathogenic and non-pathogenic SIV infections in non-human primates (28, 45).

Th17 cells secrete interleukin 17 (IL-17) and regulate permeability and microbial translocation at the gut mucosa. These cells can secrete two isoforms of IL-17, IL-17A and IL-17F, that are potent activators of neutrophilic inflammation at the gut mucosal

tissue. In addition, Th17 cells produce IL-22 that plays a critical role in host defense and epithelial-barrier function. Recent studies have demonstrated that there is loss of Th17 cells during HIV/SIV infections (62-65) and suggested that the depletion of these cells may accelerate the progression to AIDS. However, very little is known about the influence of HIV/SIV infections on the functional quality of Th17 cells during chronic infection. Furthermore, although these cells are depleted soon after infection, it takes several months to a year for disease development suggesting the existence of a compensatory immune mechanism.

It is increasingly becoming clear that a subset of CD8+ T cells in humans and mice secrete IL-17, and these cells are yet to be characterized thoroughly (164, 165, 236-239). Importantly, very little is known about their anatomical distribution, phenotype, functional quality and their role in the host defense. Similarly, very little is known about the modulation of these cells during chronic HIV/SIV infection and no information is available on the status of these cells in the colorectal tissue, one of the major sites of HIV/SIV replication during chronic infection.

Here, we characterized the anatomical distribution, phenotype and functional quality of IL-17 producing CD8+ (Tc17) and CD4+ (Th17) T cells in healthy rhesus macaques. Similar characterizations were also performed in SIV251-infected rhesus macaques during the acute and chronic phases of infection to understand the effect of pathogenic SIV infection on these cells. In addition, we sought to determine if the frequency of these cells could be restored following anti-retroviral therapy (ART) in blood as well as colorectal mucosal tissue, and if the depletion of IL-17 secreting T cells is specific to pathogenic infections.

Materials and Methods

Non-human primates and SIV infection

Young adult rhesus macaques and sooty mangabeys from the Yerkes breeding colony 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 Institutional Animal Care and Use Committee. Rhesus macaques were infected with SIVmac251 either intravenously or intrarectally at a dose of 100 or 1000 TCID50, respectively. Dr. Nancy Miller at NIH provided the challenge stock. Some of the SIV-infected rhesus macaques were treated with anti-retroviral drugs PMPA (20mg/kg), FTC (30mg/kg), Kaletra (Lopinavir, 12mg/kg; Ritonavir 3mg/kg) and AZT (5mg/kg) at about 18 weeks following infection. Sooty mangabeys were housed in colonies of 50-60 animals and SIVsm is endemic in this population. None of the sooty mangabeys used in the study were experimentally infected.

Cell isolation from blood, rectal biopsies and tissues.

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood according to the standard Ficoll-Hypaque separation procedures as described before (214). Lymphocytes from pinch biopsies from the rectum were obtained as described before (240). Briefly, 10 to 20 pinch biopsies were collected in complete RPMI and washed two times with ice-cold Hank's buffered salt solution. Biopsies were digested with 150 units/ml of collagenase IV (Worthington, Lake wood, NJ) and DNase I (Roche, Indianapolis, IN), passed through decreasing sizes of needles (16-, 18-, and 20-gauge,

five to six times with each needle), and filtered through a 100μm filter. Cells were washed twice with RPMI and resuspended in complete RPMI for analysis. Small pieces from lymphoid tissues (Axillary LN, colonic LN and spleen) were processed in medimachine (Becton Dickinson) using 50μm medicons and filtered through 50μm filcons in order to obtain a homogenous suspension of cells that were then ressuspended in complete RPMI. Cells were pelleted and lysed with ACK lysing buffer for 7 minutes, and then washed twice with RPMI and resuspended in complete RPMI for analysis. A small fraction of duodenum, jejunum, ileum, colon and rectum were cut into small pieces (1-2 mm^2) and were then quickly washed with 1mM dithiothreitol (DTT) in Hank's buffered salt solution. The sections were then placed in 2mM EDTA solution with 2% FBS in HBSS for 20 minutes with rotation at 37^oC. Intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs) were processed separately, and pooled at the end of cell isolation. The cells from the supernatant were pelleted and resuspended in cRPMI and placed on ice (These cells IELs). The remainder of tissues was then processed as the pinch biopsies from rectum and cells (LPLs) were collected in complete RPMI. IELs and LPLs were then pooled for further analysis.

Intracellular cytokine staining analysis

Intracellular cytokine production was assessed as previously described with few modifications (216). Briefly, two million cells were stimulated in 200 μl of RPMI with 10% FBS in a 5 ml polypropylene tube. Peptide pools (15mer overlapping by 11) specific for SIV Gag and Env were used for stimulations with each peptide at a final concentration of 1 μg/ml. Phorbol 12-myristate 13-acetate (PMA) and Ionomycin were used at 25

ng/ml and 0.5 μg/ml, respectively. Stimulations were performed in presence of anti-CD28 and anti-CD49d antibodies (1 μg/ml; BD Pharmingen, San Diego, CA). Cells were incubated at 37°C in the presence of 5% CO_2 for 6 hours. Brefeldin A (10 μg/ml) was added after 2 hours of incubation. At the end of stimulation, cells were washed once with phosphate-buffered saline (PBS) containing 2% FBS, stained with LIVE/DEAD marker (Invitrogen) followed by surface staining for 30 min at 4°C with anti-human CD4 (clone L200; BD Pharmingen, San Diego, CA), anti-human CD3 (clone SP34-2, BD Pharmingen, San Diego, CA) and anti-human CD8 (clone SK1; BD Biosciences, San Jose, CA). Cells were then fixed with BD FACS Lyse solution (BD Pharmingen, San Diego, CA) for 10 min and permeabilized with BD Perm 2 solution (BD Pharmingen, San Diego, CA). Cells were then incubated for 30 minutes at 4°C with anti-human IFN-γ Ab (clone B27; BD Pharmingen, San Diego, CA), IL-17 (clone eBio64DEC17; ebioscience, San Diego, CA), IL-2 (clone MQ1-167H12; BD Pharmingen, San Diego, CA) and TNF-α (clone MAb11; ebioscience, San Diego, CA). Cells were then washed twice with 2% FBS in PBS and resuspended in 1% formalin in PBS. Approximately 500,000 lymphocytes were acquired on the LSRII (BD Immunocytometry systems) and analyzed using FlowJo software (Treestar, Inc., San Carlos, California). Lymphocytes were identified based on their scatter pattern and CD3+, CD8-, CD4+ cells were considered as CD4 T cells, and CD3+CD8+CD4- cells were considered as CD8 T cells. These CD4 or CD8 T cells were then gated for cytokine positive cells. For phenotypic analysis, PMA/ionomycin stimulated cells were co-stained for surface expression of CTLA-4 (clone BNI3; BD Pharmingen, San Diego, CA), β7 (clone FIB504; BD

Pharmingen, San Diego, CA), CCR6 (Clone 11A9, BD Biosciences, San Diego, CA), and intracellular Granzyme B (clone GB11,BD Biosciences, San Diego, CA)

Quantitation of SIV RNA Plasma Load

The SIV copy number was determined using a quantitative real-time PCR as previously described (216). All specimens were extracted and amplified in duplicates, and the mean results are reported.

Statistical Analysis

The statistical significance of difference in the means ± SEM of cytokine secretion by T cell subsets and expression levels of cell surface markers was calculated with the Student's t-test (two-tailed). P values of less than 0.05 were considered statistically significant. Statistical analyses were performed using software program Prism 4.

Results

Tc17 and Th17 cells are predominant in the small intestines of normal macaques

We measured the frequencies of IL-17 and IFN-γ secreting CD4+ and CD8+ T cells in various lymphoid and intestinal tissues of normal (SIV negative) rhesus macaques to understand the relative levels and distribution of these cells. We stimulated cells isolated from respective compartments with PMA/ionomycin and quantified the levels of IFN-γ single producers (hereafter called Th1 for CD4+IFN-γ+ or Tc1 for CD8+IFN-γ+ T cells), IL-17 single producers (hereafter called Th17 or Tc17 for IL-17-secreting CD4+ and CD8+ T cells, respectively), and IFN-γ plus IL-17 double producers (hereafter called either CD4+ double-positive or CD8+ double-positive T cells) using an intracellular cytokine assay and multicolor flow cytometry (Fig 3.1A and 3.1B). All three subsets were present in all the tissues studied with IFN-γ+ being the most dominant subset and IFN-γ+ IL-17+ being the least dominant subset. The mean frequency of Th17 cells in different compartments ranged from as low as 1.7% to as high as 13.9% (Fig 3.1B). These were the lowest in spleen and highest in duodenum. There was predominance of Th17 T cells in the small intestine. Similarly, the mean frequency of Tc17 T cells in different compartments ranged from as low as 0.7% to as high as 12.6% and were predominant in the small intestine with the highest levels in duodenum (Fig. 3.1B). Tc1 T cells were detected in every compartment studied, with mean frequencies of 6.5% to 21.6% of total CD4+ T cells. Likewise, the mean frequencies of Tc1 T cells ranged from 25.9% to 40.6% of CD8+ T cells in the compartments studied. Double-positive CD4+

and CD8+ were also detected in every compartment, albeit at much lower levels and with predominance at the intestinal mucosa.

To understand the relative proportions of Th1 over Th17 cells in different compartments, we calculated the ratio between these cell subsets in each compartment (Fig 3.1B). We observed that for the majority of compartments studied, there was a predominance of Th1 over Th17 T cells. In blood, there were about 4 Th1 cells for every Th17 cell whereas in spleen, there were about 12 Th1 cells for every Th17 cell. However, in both the duodenum and jejunum, there were only about 1-2 Th1 cells for every Th17 cells. Similarly, for CD8+ T cells, we observed predominance of Tc1 over Tc17, however the ratios were about 10 fold higher than the ratios of CD4+ T cells. Consistent with the predominance of Tc17 cells in the small intestine, the ratios of Tc1:Tc17 cells were the lowest (ranging from 9.4 to 11) in this compartment (Fig. 3.1B).

Higher proportion of Tc17 are polyfunctional than Tc1

We next characterized Th17 and Tc17 cells for their ability to co-produce TNF-α and IL-2 (polyfunctionality) and compared them with Th1 and Tc1 cells, respectively (Fig. 3.2). It is important to study these cytokines since TNF-α has been shown to influence the permeability of intestinal epithelial barrier by acting on the tight junctions and by synergizing with IL-17 in inducing chemokine production by epithelial cells, and IL-2 is a cytokine that drives proliferation and effector T cell differentiation. It is well known that Th1 and Tc1 cells co-produce TNF-α and IL-2, however it is not known whether Th17 and Tc17 cells can co-produce these cytokines.

A significant proportion of Tc17 and Th17 cells in the blood (Fig. 3.2) and gut mucosal tissue (data not shown) were polyfunctional. Based on the co-expression of TNF-α and IL-2, we categorized each of the IFN-γ+, IL-17+ or DP cells into 4 subsets consisting of TNF-α+ IL-2+ cells, TNF-α+ or IL-2+ cells and TNF-α- IL-2- cells (Fig. 3.2). About 50% of Tc17 cells in the blood of normal macaques co-produced TNF-α and IL-2 demonstrating that a significant proportion of these cells are polyfunctional (Fig. 3.2B). Interestingly, the proportion of these cells was significantly higher in the Tc17 subset than the Tc1 subset ($p<0.0001$). Similarly, the proportion of polyfunctional cells was significantly higher in the DP CD8+ subset than in the Tc1 subset ($p=0.0082$). Similar to Tc17 cells, about 45% of Th17 cells also co-produced TNF-α and IL-2 (Fig. 3.2C). However, the proportion of these cells in the Th17 cells was not significantly different from Th1 cells. Interestingly, nearly 60% of the DP CD4+ subset consisted of polyfunctional cells and was significantly higher than Th17 and Th1 subset. Similar results were also observed in the gut mucosal tissue (data not shown).

Tc17 cells have more regulatory and less cytotoxic phenotype than Tc1 cells

We next characterized the Th17 and Tc17 cells for expression of β7 and CCR6 (gut homing potential), CTLA-4 (co-inhibitory receptor) and granzyme B (cytotoxic potential) (Fig. 3.3). α4β7 but not αEβ7 promotes migration to gut. However, previous study has shown that the majority of β7+ T cells in the blood are α4β7+ (168) and thus the anti-β7 antibody that we used here should primarily mark α4β7+ cells. Consistent with their predominance in the small intestine, about 25-65% of Tc17 and Th17 cells expressed gut homing markers β7 and CCR6. Importantly, both Tc17 and Th17 cells

expressed higher levels of these gut-homing markers than Tc1 and Th1 cells, respectively.

Interestingly, a significant proportion of Tc17 and Th17 cells exhibited an inhibitory phenotype and did not possess cytolytic potential (Fig 3.3B). CTLA-4 is an inhibitory receptor that is constitutively expressed on regulatory T cells, and on some activated CD4+ T cells. It is known to deliver negative signals that inhibit expansion of T cells. CD8+ T cells typically do not express CTLA-4. Interestingly, about 90% of Th17 and 50% of Tc17 cells expressed CTLA-4 post activation. These do not represent Tregs as none of these cells co-stained for FOXP3 (data not shown). Importantly, these levels were much higher compared to CTLA-4 expression on Th1/Tc1 cells. Only about 50% of the Th1 and 6% of Tc1 cells expressed CTLA-4. A small fraction of Tc17 cells expressed granzyme B suggesting that only a minority of these cells may have killing potential. Only about 10% of Tc17 cells were positive for intracellular granzyme B and this was 6-fold lower than Granzyme B expression by Tc1 cells. We could not measure expression of perforin, another molecule required for killing potential, because perforin is rapidly released from the cells following stimulation and thus is hard to stain for on stimulated cells.

The DP CD4+ and CD8+ T cells also expressed high levels of β7, CCR6 and CTLA-4 (Fig. 3.3). In general these were higher than the respective Th1/Tc1 cells and similar to respective Th17/Tc17 cells. Similarly, the expression of granzyme B by DP CD8+ T cells was lower than Tc1 cells and similar to Tc17 cells. These results suggest that DP T cells share more of a Th17/Tc17 lineage rather than Th1/Tc1 lineage.

Tc17 cells but not Tc1 cells are depleted during the end stage SIV infection

IL-17 has been shown to be important in controlling extracellular bacteria at mucosal surfaces and for enterocyte homeostasis. Consistently, our results demonstrate that Tc17 and Th17 cells are predominant in the small intestine (Fig 3.1B), one of the primary sites of viral replication. So, it is important to study the dynamics of Tc17/Th17 cells during the course of SIV infection to better understand the relative roles of these cells. We quantified the frequencies of Tc17, Tc1 and DP CD8+ cells in the blood (Fig. 3.4A) and colorectal mucosa (Fig. 3.4B) of 12 rhesus macaques prior to SIV infection, and at 2 and 16 weeks post infection. The frequencies of these cells were also quantified in a group of macaques that were chronically infected (>20 weeks) with SIV and were euthanized due to simian AIDS (Fig. 3.4C). We calculated the ratio of Tc1 over Tc17 cells to understand the relative proportions of these cell subsets at different time points and in various tissues studied (Fig. 3.4).

In blood, a transient reduction in the frequencies of Tc17 cells was observed at 2 weeks post SIV infection that recovered partially by 16 weeks (Fig. 3.4A). However, the frequency of these cells in animals with AIDS was significantly lower compared to normal (SIV-) animals demonstrating a loss of these cells during the end stage of disease. A similar pattern was also observed in the colorectal tissue (Fig. 3.4B) and for the frequency of DP CD8+ cells following SIV infection. In contrast, the frequencies of Tc1 cells showed a trend towards transient increase at 2 weeks post SIV infection and were similar to pre infection levels at 16 weeks post infection as well as end stage disease. These results demonstrate a preferential loss of IL-17 producing (Tc17 and DP) CD8+ T cells during the end stage AIDS. This preferential loss resulted in an altered balance

between Tc1 and Tc17 cells in blood, LN and large intestine. Prior to infection, there were about 45 Tc1 cells for every 1 Tc17 cells in blood and rectum whereas at end stage infection there were about 180 Tc1 cells for every 1 Tc17 cells. The difference in ratio was even greater in the LN, with 16 Tc1 cells for every 1 Tc17 prior to infection, and 500 Tc1 for each Tc17 cell at end stage disease.

We also performed temporal evaluation of Th17, Th1 and DP CD4+ T cells following infection and at end stage disease (Fig 3.5). For these analyses we calculated the absolute number rather than percentages because CD4+ T cells are infected and killed by the virus. Consistent with previous reports (63-65, 168) we observed a profound depletion of Th17 cells both in blood (Fig 3.5A) and colorectal tissue (Fig 3.5B) as early as 2 weeks post infection that sustained until the end stage disease (Fig 3.5C). A similar pattern was also observed for DP CD4+ T cells. The ratios of Th1:Th17 cells also indicated a preferential depletion of Th17 cells following infection.

SIV infection induces up-regulation of lymph node homing and gut homing markers on Th17 and Tc17 cells

SIV infection induced changes in the expression levels of cell surface receptors in Th1, Th17, Tc17, DP CD4+ and CD8+ cells (Fig 3.6). There was up-regulation of gut homing markers β7 on Th17 cells, and CCR6 on Th17, Tc17, Th1 and CD8+ DP cells. Th1 and CD4+ DP cells in SIV-infected macaques had significantly reduced expression levels of CTLA-4. Similarly, there was a trend for reduced expression of CTLA-4 in Th17 cells. SIV infection did not induce changes in the expression of pro-apoptotic

marker caspase-3 or cytotoxic molecule granzyme B. This indicates increased ability of Th17 and Tc17 to migrate into intestinal tissues, but with possible loss of inhibitory function.

ART increases Tc17 and Th17 cells in colorectal mucosa

We next studied the influence of ART on restoration of Tc17 and Th17 cells both in blood and colorectal mucosa in a cohort of 8 rhesus macaques that received therapy for a period of 8 weeks (Fig 3.7). This treatment was sufficient to cause a 2-3 fold log reduction in the viral load. Following ART, the frequency of both Tc17 and Tc1 cells increased by 2-8 fold in the colorectal tissue but not in blood (Fig 3.7A). However, the ratio of Tc1:Tc17 cells did not change demonstrating similar effects of ART on both of these subsets. A different pattern was observed for Th17 and Th1 cells (Fig 3.7B). The absolute number of Th17 and Th1 cells increased in blood, but incolorectal mucosa, significant increases were observed only in the Th1 subset.. The increases were higher for Th1 than Th17 resulting in a higher ratio of Th1:Th17 cells in both compartments. No significant changes were observed for DP CD8+ in either compartment, however there was significant increase in DP CD4+ T cells in blood. These results demonstrate that ART increases the frequency of Tc17 and Tc1 cells proportionally and the number of Th17 and Th1 cells disproportionally in the colorectal mucosa. We also tested the potential of Th17 and Tc17 cells to co-produce TNF-α or IL-2 and both subsets of cells were capable of secreting TNF-α and IL-2 at levels similar to prior to infection (data not shown). Thus, ART treatment was able to restore the magnitude and quality of response in colorectal mucosa.

Tc17 and Th17 cells are not depleted in SIV-infected sooty mangabeys

Previous studies demonstrated a preferential depletion of Th17 cells in pathogenic (rhesus macaques) but not in non-pathogenic (sooty mangabeys) SIV infections. However, no information is available on the status of Tc17 cells in SIV-infected sooty mangabeys. We determined the frequency of Tc17 and Th17 cells in the blood and colorectal tissue of SIV- and SIV+ sooty mangabeys to determine the effect of SIV-infection on these cells (Fig 3.8). The frequency of Tc17 cells and the absolute number of Th17 cells in the blood and colorectal tissue of SIV- and SIV+ sooty mangabeys were not significantly different demonstrating that these cells are not depleted following SIV infection. Furthermore, the frequency of TNF-α or IL-2 co-producing Tc17/Th17 cells was also not significantly different (data not shown).

Discussion

Chronic HIV/SIV infections are associated with impaired gut permeability and microbial translocation/control. IL-17 plays a critical role in regulating the permeability of gut epithelium and control of microbial and fungal infections. Previous studies have shown a rapid and preferential depletion of IL-17 producing CD4+ T cells (Th17) at the gut mucosa following pathogenic SIV infection of rhesus macaques and suggested a role for these cells in faster disease progression. However, it is increasingly becoming clear that IL-17 can also be produced by a subset of CD8+ T cells (Tc17) and it is important study the status of these cells during chronic HIV/SIV infection because these cells most likely are not killed by the virus. Our study, for the first time characterizing the anatomical distribution, phenotype and functional quality of Tc17 cells in normal and SIV-infected macaques demonstrates that, in contrast to Th17 cells, the magnitude and functional quality of Tc17 cells is not significantly altered during the acute phase of infection. Importantly, our results demonstrate that Tc17 cells indeed are depleted during the end stage disease. In addition, they demonstrate that Tc17 cells are not depleted in the blood and colorectal tissue of non-pathogenic SIV infection of sooty mangabeys. These results suggest that Tc17 cells may compensate for the loss of Th17 cells during the acute and early chronic phases of pathogenic SIV infection and may play a role in controlling disease progression.

Phenotypic characterization of Tc17 and Th17 cells revealed that these cells express high levels of the co-inhibitory marker CTLA-4, a finding that has not been appreciated before. This is particularly interesting for Tc17 cells because CD8+ T cells do not normally express high levels of CTLA-4 as has been observed for Tc1 cells in our

study. CTLA-4 binds to CD80 and CD86 that are normally expressed on antigen presenting cells such as dendritic cells and diminishes their T cell activation potential. The role of CTLA-4 expressed on Th17/Tc17 cells is not clear but perhaps is important to regulate the excessive Th1/Tc1 response (hyperimmune activation) in the gut. It is well established that hyperimmune activation plays a critical role in faster disease progression during pathogenic SIV infection and thus it is possible that loss of Th17/Tc17 cells contribute to enhanced hyperimmune activation during pathogenic SIV infection.

Unexpectedly, we found that Tc17 cells are similar to Th17 cells for many immune parameters that we studied here. Both subsets were predominant in the small intestine, consisted of high proportions of TNF-α and IL-2 co-producing cells, and expressed markers associated with gut homing potential. Furthermore, Tc17 cells despite being CD8+, expressed very little Granzyme B. This suggests that the function of Tc17 cells could be more of a helper rather than cytotoxicity. If this is true, it is not clear about the specific role of Tc17 cells. This requires further characterization of these cells with respect to production of other cytokines such as IL-22, cytotoxicity, suppressive potential and antigen specificity.

ART therapy was able to replenish Tc17 in colorectal mucosa and Th17 cells in the blood. This, along with low viral burden, may contribute for the partial restoration of gastrointestinal mucosa and immune function, as well as the alleviation of symptoms caused by pulmonary and gastrointestinal infection such as diarrhea due to common bacterial infections (Salmonella, Listeria, Shigella, and others). However, ART restored Th1 cells preferentially than Th17 cells resulting in an altered balance between the two subsets promoting a more pro-inflammatory response. The reasons for this preferential

restoration of Th1 cells are not clear. Perhaps longer duration of ART could further restore Th17 cells, and re-establish the relative proportion of these cells to what was seen prior to infection. Also, the levels of IL-21 have been shown to be decreased in HIV-infected individuals and that ART is only partially capable of restoring production of this cytokine (241). IL-21 is secreted by Th17 cells and drives IL-17 secretion in an autocrine manner. So, it is possible that combining ART with IL-21 therapy may preferentially restore Th17 cells over Th1 cells and thus enhance the function of gut mucosa. In conclusion, our results suggest an important role of Tc17 cells in regulating hyperimmune activation and disease progression during pathogenic SIV infection and therapeutic approaches that enhance the magnitude and function of these cells may have benefit in disease outcome.

Figure legends

Figure 3.1. Distribution of T cell subsets in normal rhesus macaques. (A) Gating pattern for Th1, Tc1, Th17, Tc17, DP CD4+ and CD8+ T cells in peripheral blood (PBMC) and jejunum. T cells were gated on lymphocyte gates, followed by exclusion of dead cells and gating on CD3+ T cells. CD3+CD4+CD8+- cells were identified as CD4+ T cells and CD3+CD4+-CD8+ cells were identified as CD8+ T cells. IL-17-secreting CD4+ or CD8+ T cells were identified as Th17 or Tc17 cells respectively. IFN-γ-secreting CD4+ or CD8+ T cells were identified as Th1 or Tc1 cells respectively. IL-17+IFN-γ+ CD4+ or CD8+ T cells were identified as DP CD4+ or CD8+ T cells, respectively. (B) Summary of the frequency of Th1, Tc1, Th17, TC17, DP CD4+ and CD8+ T cells and the ratio between Th1:Th17 and Tc1:Tc17 in various tissues. Error bars represent means ± sem; n=5.

Figure 3.2. Polyfunctionality of T cell subsets. (A) Representative flow plots depicting TNF-α or IL-2 secretion by Th1, Tc1, Th17, Tc17, DP CD4+ or CD8+ T cells. (B). Cytokine co-expression profiles by the different T cell subsets.

Figure 3.3. Characterization of T cell subsets. (A) Representative flow plots for expression of β7, CCR6, CTLA-4 and granzyme B by Th1, Th17, Tc1, Tc17 and DP CD4+ and CD8+ T cells. (B) Summary of expression levels of CTLA-4, β7, CCR6 and granzyme B for 4 rhesus macaques. Black bars represent Th1 or Tc1 cells, open bars represent Th17 or Tc17 cells, and gray bars represent DP CD4+ or CD8+ T cells.

*Indicates a p value of less than 0.05. ** Indicates a p value of less than 0.005. ***Indicates a p value of less than 0.0005.

Figure 3.4. Kinetics of CD8+ T cell subsets after pathogenic SIV infection. (A) Kinetics of the frequencies of Tc17, Tc1, CD8+ DP cells and ratio between Tc1:Tc17 cells in blood and (B) colorectal mucosa. (C) Frequencies of Tc17, Tc1 and CD8+ DP cells and ratios of Tc1:Tc17 cells in PBMC, axillary lymph node, duodenum and rectum. Error bars represent means ± sem. 12 rhesus macaques were followed post SIV infection. 5 normal uninfected and 4 rhesus macaques with clinical AIDS were euthanized for tissue analysis. *Indicates a p value of less than 0.05.

Figure 3.5. Kinetics of CD4+ T cell subsets after pathogenic SIV infection. (A) Kinetics of absolute numbers of Th17, Th1, CD4+ DP per 100,000 lymphocytes and ratio between Th1:Th17 cells in blood and (B) colorectal mucosa. (C) Absolute numbers of Th17, Th1 and CD4+ DP cells per 100,000 lymphocytes and ratios of Th1:Th17 cells in PBMC, axillary lymph node, duodenum and rectum of uninfected and SIV-infected rhesus macaques. Error bars represent means ± sem. 12 rhesus macaques were followed post SIV infection. 5 normal uninfected and 4 rhesus macaques with clinical AIDS were euthanized for tissue analysis. *Indicates a p value of less than 0.05. ** Indicates a p value of less than 0.005.

Figure 3.6. Characterization of T cell subsets post SIV infection. Summary of expression levels of β7, CCR6, CTLA-4, caspase-3 and granzyme B on (A) Th17, Th1

and CD4+ DP T cells and (B) Tc17, Tc1 and CD8+ DP T cells. Open bars represent data from 5 SIV-negative macaques, and black bars represent data from 4 macaques with clinical AIDS. *Indicates a p value of less than 0.05. ** Indicates a p value of less than 0.005. ***Indicates a p value of less than 0.0005.

Figure 3.7. Effect of ART on T cell subsets. (A) Frequencies of Tc17, Tc1 and CD8+ DP T cells before and after ART. Right panel summarizes ratios of Tc1:Tc17 cells before and after ART. (B) Number of Th17, Th1 and CD4+ DP T cells per 100,000 lymphocytes before and after ART. Right most panel summarizes the ratio of Th1:Th17 cells before and after ART. Data from colorectal mucosa and peripheral blood is shown. Each symbol represents an individual macaque. *Indicates a p value of less than 0.05. ***Indicates a p value of less than 0.0005.

Figure 3.8. Comparison of T cell subset levels in Sooty mangabeys. Number of Th17, Th1 and CD4+ DP T cells per 100,000 lymphocytes and frequencies of Tc17, Tc1 and CD8+ DP T cells in uninfected and infected sooties. (n=8 per group).

Figure 3.1

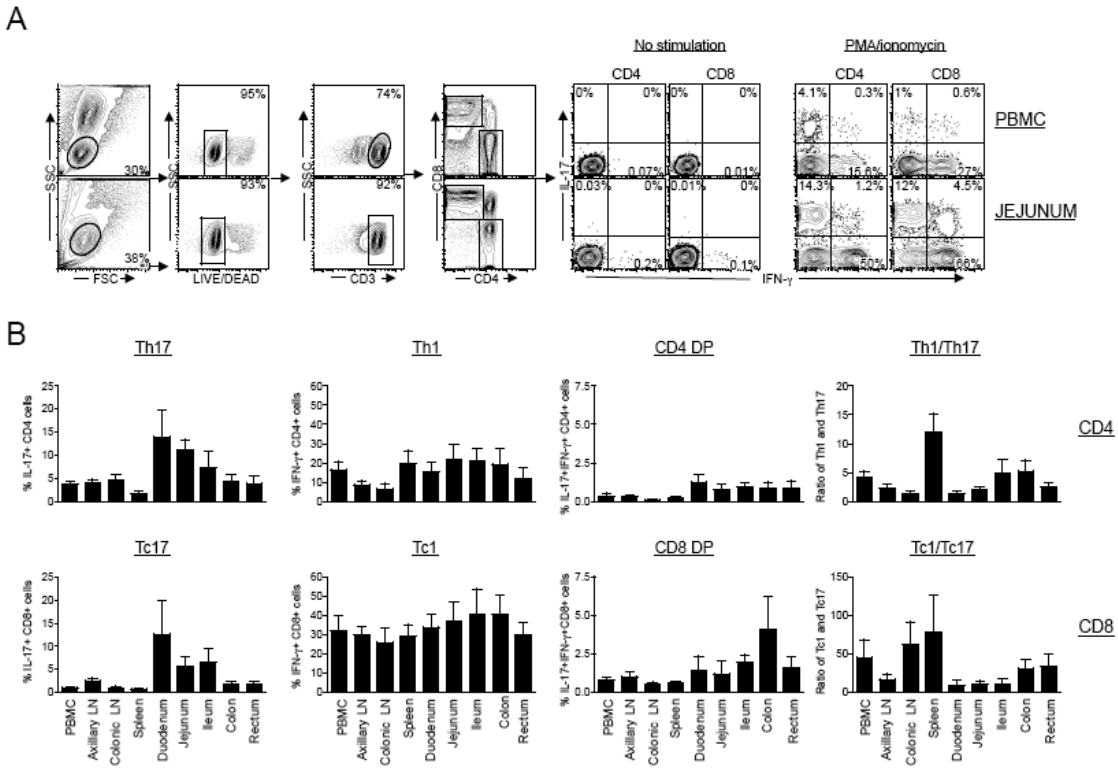

A
No stimulation
PMA/ionomycin
CD4
CD8
PBMC
JEJUNUM
SSC
FSC
LIVE/DEAD
CD3
CD4
IL-17
IFN-γ
B
Th17
Th1
CD4 DP
Th1/Th17
Tc17
Tc1
CD8 DP
Tc1/Tc17
CD4
CD8
PBMC
Axillary LN
Colonic LN
Spleen
Duodenum
Jejunum
Ileum
Colon
Rectum

Figure 3.2

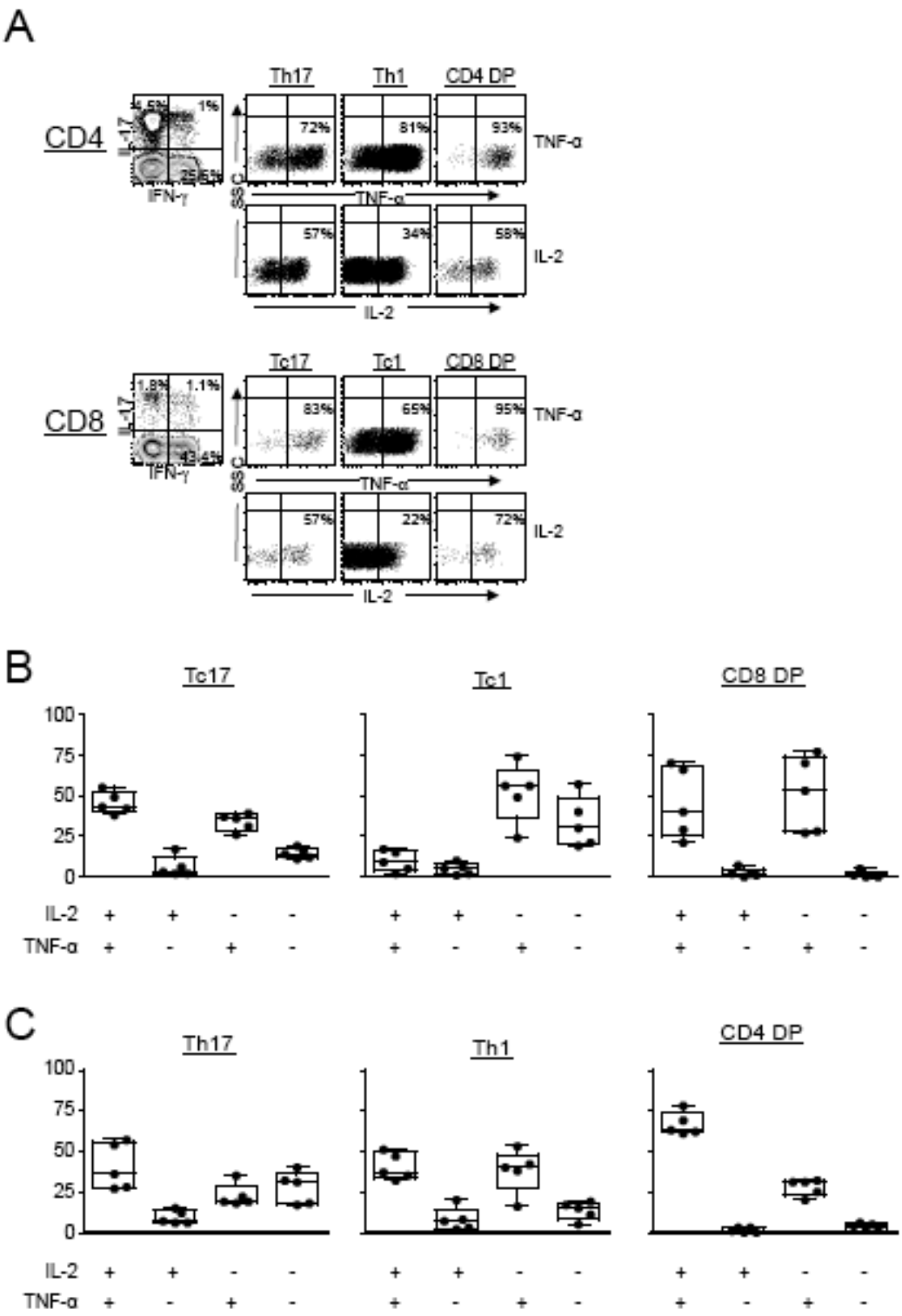

A
Th17
Th1
CD4 DP
CD4
1%
72%
81%
93%
TNF-α
IFN-γ
57%
34%
58%
IL-2
Tc17
Tc1
CD8 DP
CD8
1.8%
1.1%
83%
65%
95%
43.4%
22%
72%
B
Tc17
Tc1
CD8 DP
100
75
50
25
0
IL-2 + + - - + + - - + + - -
TNF-α + - + - + - + - + - + -
C
Th17
Th1
CD4 DP
100
75
50
25
0
IL-2 + + - - + + - - + + - -
TNF-α + - + - + - + - + - + -

Figure 3.3

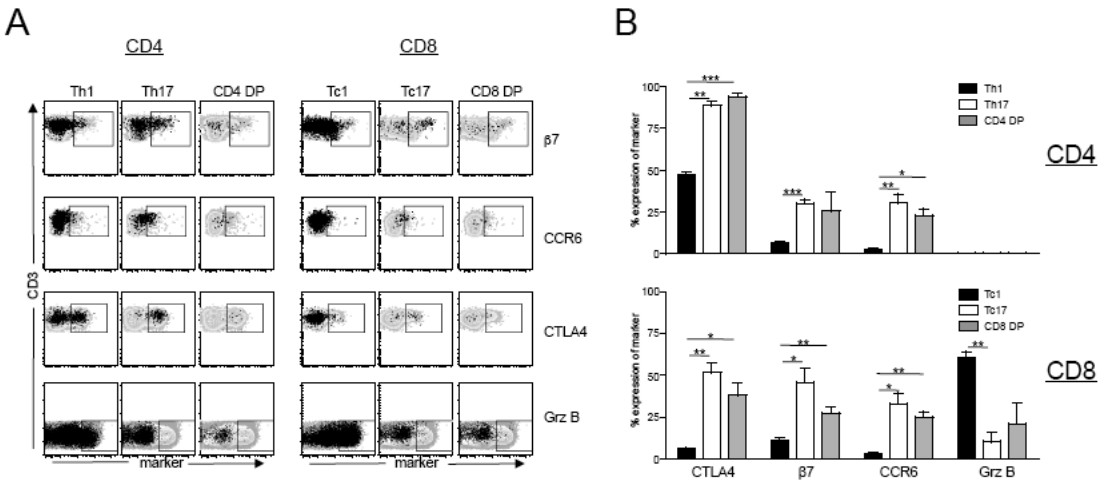

A
CD4
CD8
Th1
Th17
CD4 DP
Tc1
Tc17
CD8 DP
β7
CCR6
CTLA4
Grz B
CD3
marker
marker
B
Th1
Th17
CD4 DP
CD4
% expression of marker
Tc1
Tc17
CD8 DP
CD8
CTLA4
β7
CCR6
Grz B

Figure 3.4

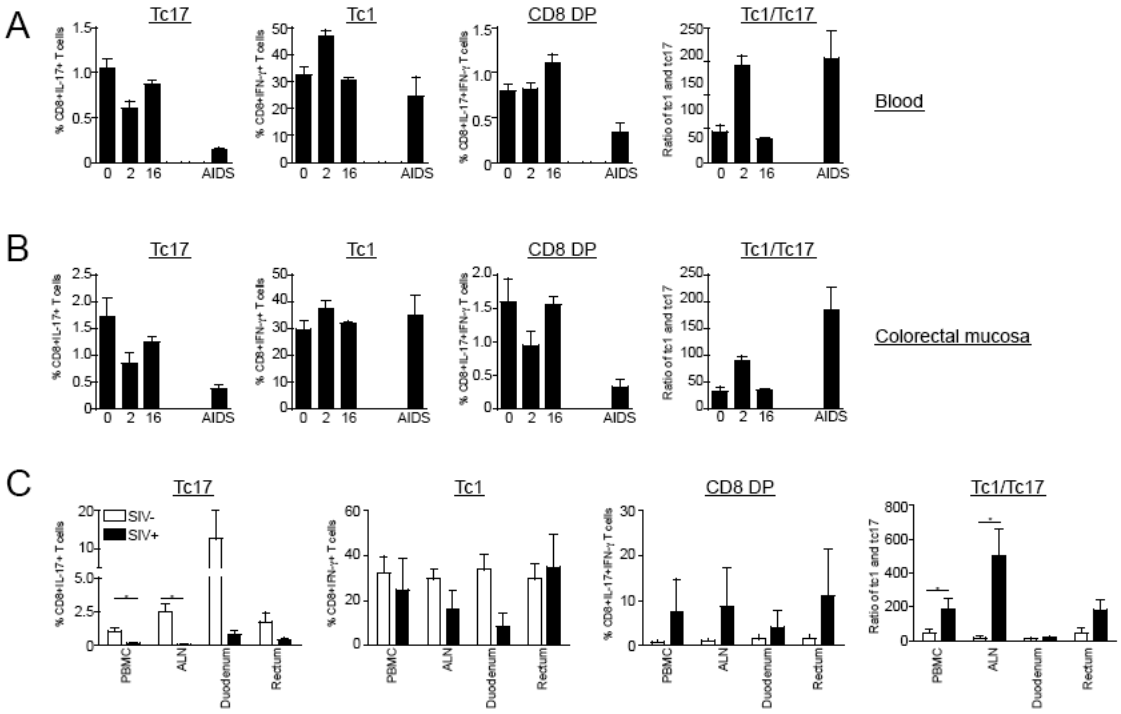

A
Tc17
Tc1
CD8 DP
Tc1/Tc17
% CD8+IL-17+ T cells
% CD8+IFN-γ+ T cells
% CD8+IL-17+IFN-γ T cells
Ratio of tc1 and tc17
0 2 16 AIDS
Blood
B
Colorectal mucosa
C
SIV-
SIV+
PBMC
ALN
Duodenum
Rectum

Figure 3.5

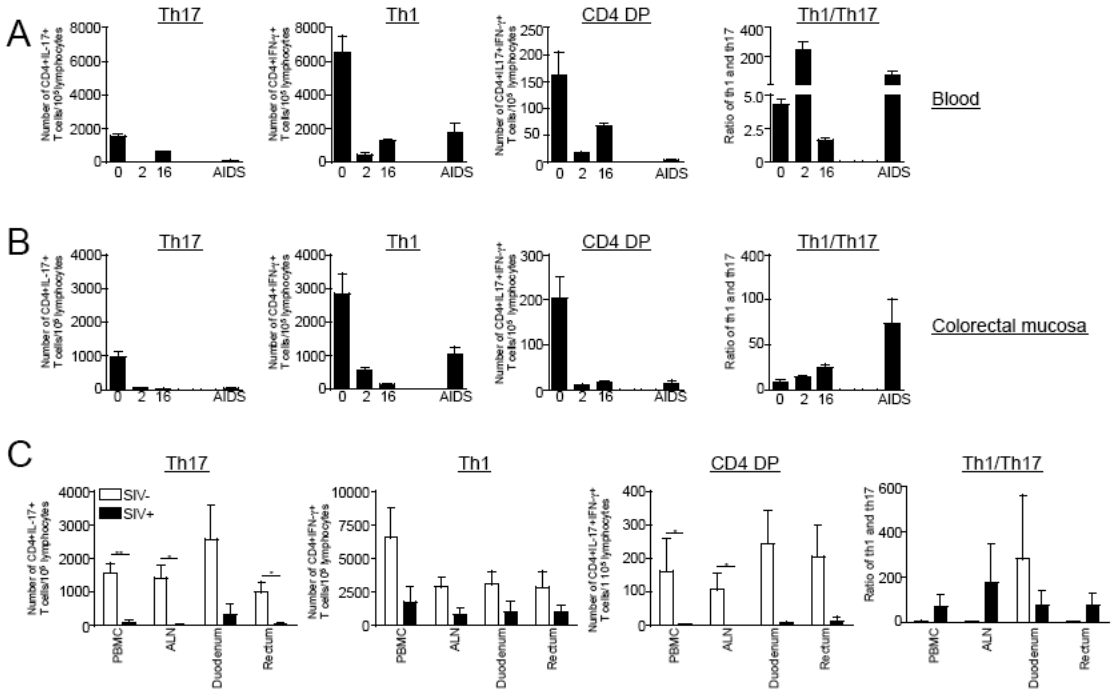

A
Th17
Th1
CD4 DP
Th1/Th17
Blood
B
Colorectal mucosa
C
SIV-
SIV+
PBMC
ALN
Duodenum
Rectum
0
2
16
AIDS

Figure 3.6

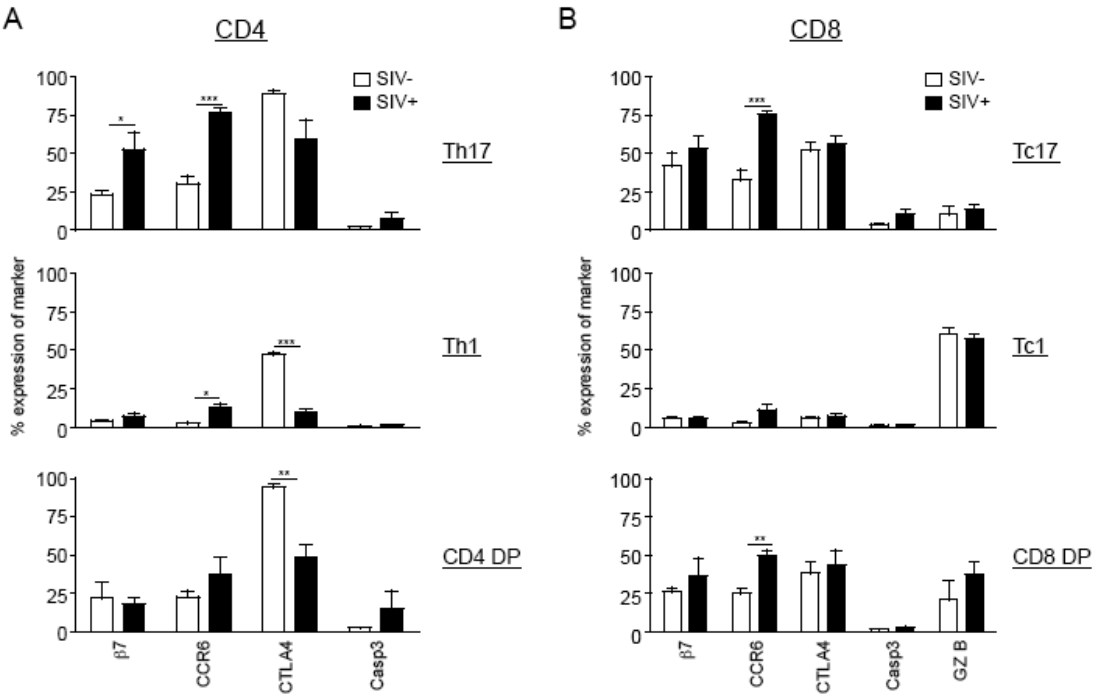

A
CD4
B
CD8
SIV-
SIV+
Th17
Th1
CD4 DP
Tc17
Tc1
CD8 DP
% expression of marker
100
75
50
25
0
β7
CCR6
CTLA4
Casp3
GZ B

Figure 3.7

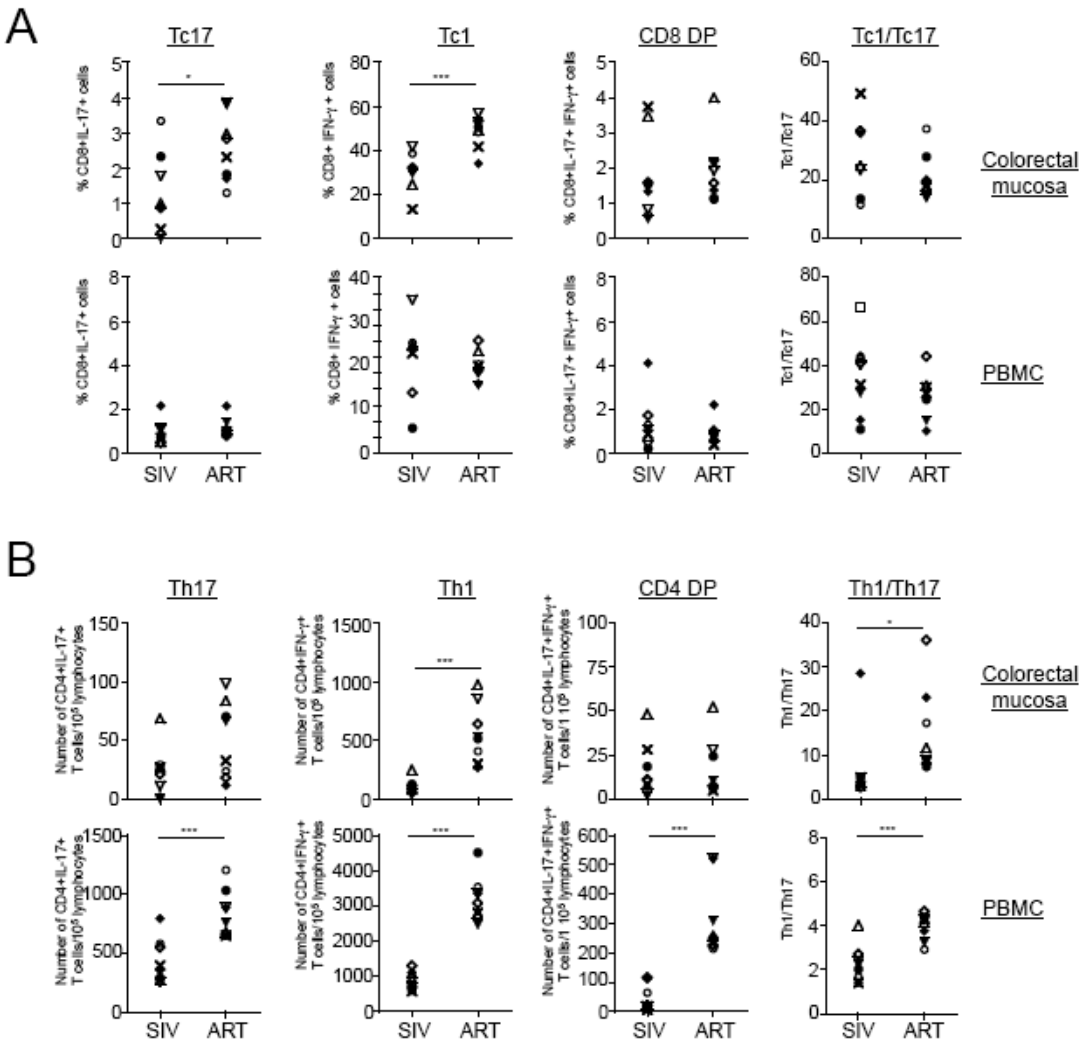
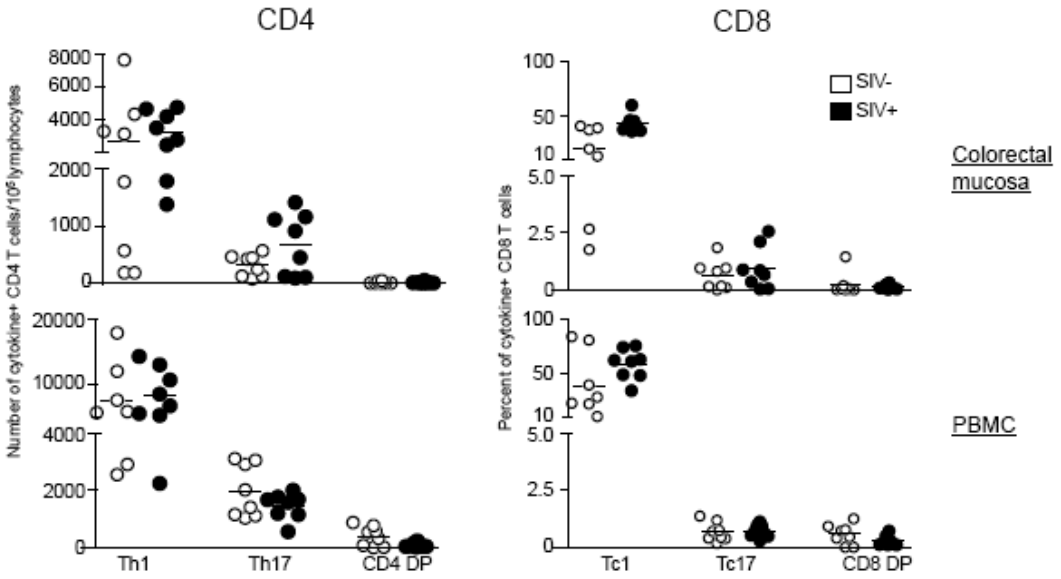

A
Tc17
Tc1
CD8 DP
Tc1/Tc17
% CD8+IL-17+ cells
% CD8+ IFN-γ+ cells
% CD8+IL-17+ IFN-γ+ cells
Tc1/Tc17
Colorectal mucosa
PBMC
SIV
ART
B
Th17
Th1
CD4 DP
Th1/Th17
Number of CD4+IL-17+ T cells/10^5 lymphocytes
Number of CD4+IFN-γ+ T cells/10^5 lymphocytes
Number of CD4+IL-17+IFN-γ+ T cells/1 10^5 lymphocytes
Th1/Th17
Colorectal mucosa
PBMC
SIV
ART

Figure 3.8


CD4
CD8
SIV-
SIV+
Number of cytokine+ CD4 T cells/10^6 lymphocytes
Percent of cytokine+ CD8 T cells
Colorectal mucosa
PBMC
Th1
Th17
CD4 DP
Tc1
Tc17
CD8 DP

CHAPTER 4

Discussion

The HIV epidemic has reached such great levels, that if left unchecked the number of people dying from AIDS shall continue to increase. The arduous efforts to generate a preventative vaccine for HIV have so far not yielded a final product that is able to generate sterilizing immunity. One of the main difficulties in generating a vaccine is the ability to elicit broadly neutralizing antibodies to HIV-1. These are considered to be important for optimal protection because they are associated with protection against most viral infections and they provide protection against immunodeficiency viral infections in non-human primates (242-245). This virus is highly mutable due to error-prone reverse transcription of the viral genome and thus the HIV isolates are highly variable. Current HIV vaccine development strategies are focusing on generation of virus-specific T cell response that have been shown to be effective in controlling viral replication in non-human primates (9, 246-248). However, it is necessary to better understand the pathogenesis of this disease by taking into account both viral and host factors. Managing this epidemic may include generating therapies that not only target control of viral replication, but also enhance the function of the host immune system.

Summary of results

Here, we have studied non-viral specific T cells during the course of SIV infection, and how they may influence progression to disease. Various studies have demonstrated the depletion of CD4 regulatory T cells and Th17 cells during the acute phase of SIV infection (62-64, 119, 130, 168), thus we focused on CD8 regulatory T cells and IL-17- secreting CD8 T cells (Tc17). The main findings in our study are:

1) Expansion of FOXP3+CD25+CD8+ T cells with suppressive potential predominantly in the colorectal tissues during the acute phase of SIV infection, and maintenance of these cells in the chronic phase of infection;

2) Loss of Tc17 cells that occurs only during end-stages of disease;

3) Treatment with anti-retroviral drugs increases the levels of Th1, Th17, Tc17 and CD4 regulatory T cells in colorectal mucosa. ART also reduces the levels of CD8 regulatory T cells in colorectal mucosa.

4) These events (expansion of CD8 regulatory T cells, and depletion of Th17, Tc17 cells and CD4 regulatory T cells) is specific to pathogenic SIV-infection, as it is not observed in non-pathogenic infection of sooty mangabeys.

Proposed role for Tc17 and CD8 regulatory T cells during pathogenic SIV infection

We propose a model in which CD8+ regulatory cells and Tc17 cells located in the gastrointestinal tract have critical roles in the immunopathogenesis of disease. Our data suggests that these two cell subsets have opposite functions in defining the outcome of HIV/SIV infections. Whereas CD8+ regulatory T cells help the virus by constraining

anti-viral immunity, Tc17 cells delay onset of disease by contributing to the maintenance of intestinal epithelial barrier. Below is the schematic of events we believe to occur in the gastrointestinal tract during the course of HIV/SIV infections.

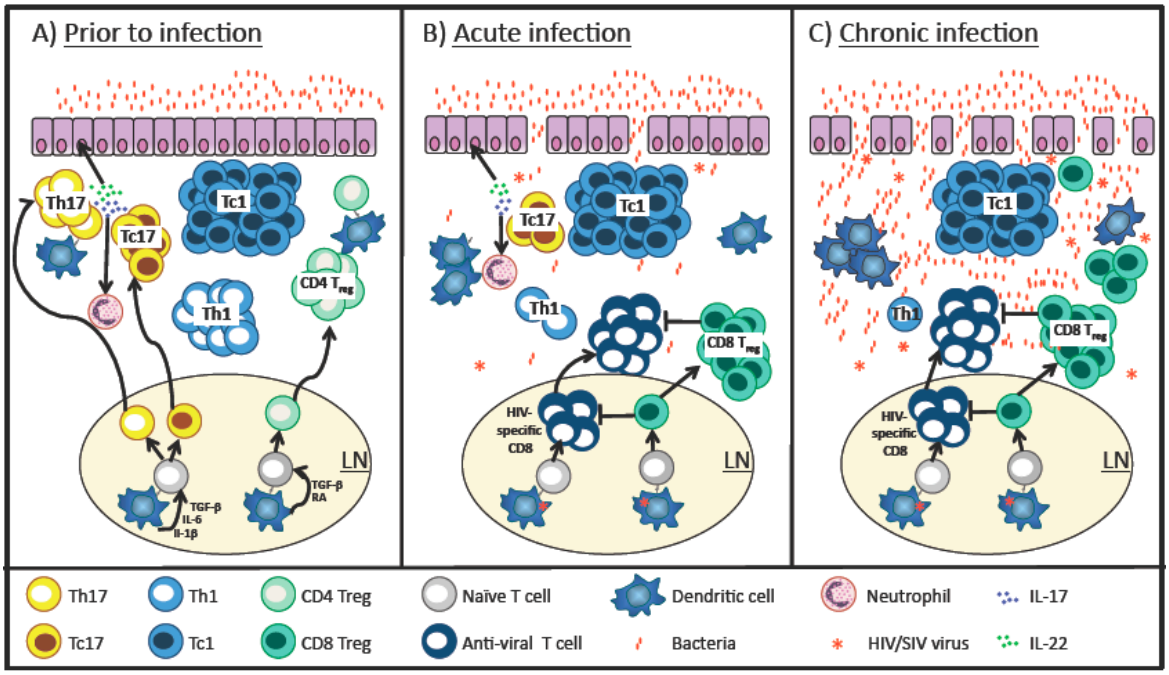


Figure 4.1: Proposed model of events during the SIV infection. A) Cell subsets present at the GI tract prior to SIV. B) Depletion of Th17 and CD4 Tregs during acute phase of infection; generation of anti-viral CD8 T cell response; expansion of CD8 Tregs; partial damage to epithelial barrier. C) Depletion of Tc17 cells; damage to gastrointestinal tract that leads to microbial translocation.

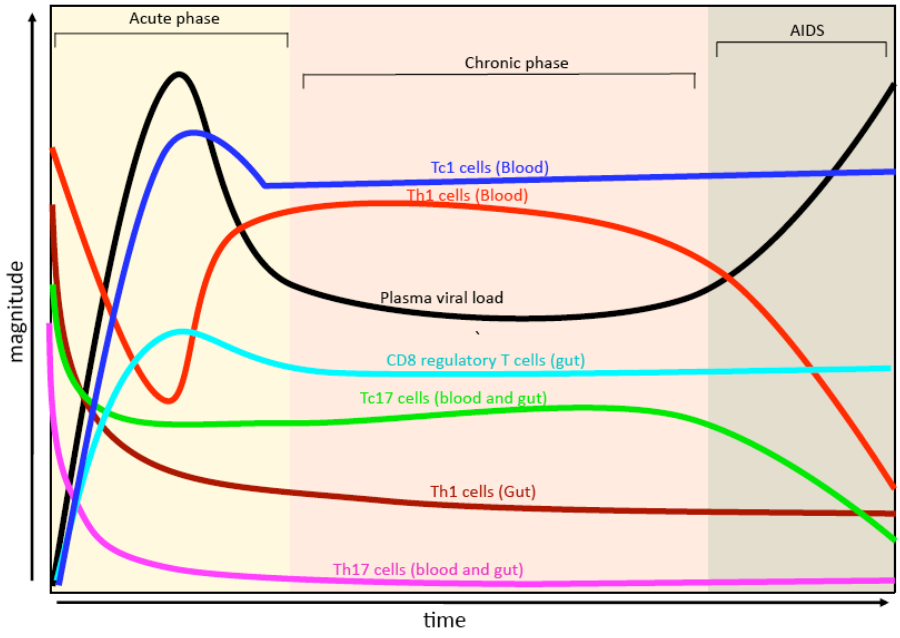


Figure 4.2: Timeline of events during the course of SIV infection.

The gastrointestinal tract is a complex system responsible for maintaining tolerance to the many microbes present in the lumen and also for generating immune responses that will clear any pathogens that breach the gut barrier. In normal, SIV-negative macaques, this microenvironment is rich in Th1 cells, Tc1 cells, CD4 regulatory T cells, Th17, and Tc17 cells. Th1 and Tc1 cells located in the gastrointestinal tract provide protective immunity against various viruses and other pathogens that may infect cells at this site. Th17 and Tc17 cells secrete the cytokines IL-17 and IL-22, which have important functions in pro-inflammatory responses and maintenance of the intestinal epithelial barrier (151). IL-17 can induce granulopoietic factors and promotes neutrophil chemotaxis (249-252). IL-22 induces pro-inflammatory responses (cytokine and chemokine secretion) and drives the secretion of anti-microbial peptides and proliferation of epithelial cells (253-255). Regulatory T cells are anti-inflammatory and generate a tolerizing response to bacteria that are present in the lumen. These cells also control for exacerbated immune responses that can lead to development of immunopathogenesis and autoimmunity.

Tc17 cells may compensate for the loss of Th17 cells during acute phase of infection

Once infection with HIV/SIV is established, various events occur that culminate in the development of symptoms and onset of disease. First, during the acute phase of infection, there is a transient depletion of Th1 cells in the blood. However, there is massive depletion of Th1 cells and Th17 cells in the gut mucosal tissues that occurs early following infection (10, 15, 62-65, 168). The majority of CD4+ T cells located in intestinal tissues are effector cells expressing high levels of co-receptor CCR5 that can be

rapidly infected and killed by HIV/SIV viral infection. Th17 cells in peripheral blood are also depleted very early following pathogenic SIV infection. The loss of Th17 cells implies in the loss of IL-17 and IL-22 secretion in the gut. IL-22 is important for the proliferation of enterocytes and its loss may compromise the integrity of the gut epithelium. CD8+ T cells are not likely to be infected or killed by HIV/SIV infections. Thus, we propose that the damage to intestinal mucosa may be partially contained due to the presence of Tc17 cells that actively secrete IL-17 and IL-22. CD4+ regulatory T cells also express viral co-receptor CCR5, and are depleted early following SIV infection. The depletion of CD4+ regulatory T cells may result in inability to maintain tolerance to microbes that translocate across the gut barrier. The presence of microbes induces the secretion of proinflammatory cytokines that recruit antigen-presenting cells (APCs) to the site of infection. APCs can present viral antigens and prime anti-viral specific Tc1 cells. These cells, however, are generated in the absence of helper T cells, and are only partially able to control viral replication. The consistent presence of bacterial load may contribute to the high levels of immune activation observed in pathogenic SIV infections.

CD8+ regulatory T cells expand rapidly in colorectal mucosa after pathogenic SIV infection and suppress anti-viral immune response

Prior to SIV infection, CD8+ regulatory T cells are present at very low levels in the blood and colorectal mucosal tissues (106, 140, 256). We demonstrate the rapid expansion of CD8+ regulatory T cells early following pathogenic SIV infection in both blood and gut mucosal tissues. We postulate that these cells may be expanding due to high levels of chronic hyper-immune activation observed in SIV-infection of non-natural

hosts, since their expansion is not observed in sooty mangabeys despite the presence of high viral loads. Our data provides evidence that CD8+ regulatory T cells are capable of suppressing the proliferation of SIV-specific T cell responses *in vitro* and *in vivo*. These cells are maintained at high levels in gut mucosal tissue during the chronic phase of infection, and continue to limit the anti-viral responses. The high levels of CD8+ regulatory T cells correlate directly with high levels of viremia in SIV+ rhesus macaques. Thus the expansion of CD8+ regulatory T cells with anti-viral suppressive capacity may be deleterious to the host and induce faster progression to disease.

Tc17 cells are depleted during end-stage disease

Although an initial loss in Tc17 cells is not observed early following pathogenic SIV infection, these cells are depleted in the gut during the end-stages of disease. We propose that Tc17 cell depletion abrogates the protection conferred to epithelial cell barrier by IL-17 and IL-22 secretion. Thus, the absence of these cytokines alters enterocyte homeostasis and leads to increase in the permeability of the gut, allowing for microbe translocation to occur. As CD8+ regulatory T cells are still present at high levels, these cells continue to restrict the anti-viral immune response generated by the host. Thus, the end-stage disease in macaques infected with pathogenic SIV is characterized by immune dysregulation, including, but not limited to the depletion of Th1, CD4+ regulatory T cells, Th17 and Tc17 and presence of high levels of CD8+regulatory T cells at the intestinal mucosal surfaces.

ART restores Th1, Th17, Tc17 and CD4+ regulatory T cells and reduces levels of CD8+regulatory T cells

Anti-retroviral therapies (ART) for SIV/HIV infections are known for the ability to lower viral load to below detection levels. ART has been shown to restore Th1 cells in the blood however, the restoration of these cells in intestinal mucosal surfaces occurs with delay and is incomplete (170-172). Our data shows that ART was able to replenish Tc17 cells and CD4+ regulatory T cells and reduce levels of CD8+ regulatory T cells in the colorectal mucosa. These events, in conjunction with reduced viremia may contribute for the partial restoration of gastrointestinal mucosa and immune function, as well as the alleviation of symptoms caused by pulmonary and gastrointestinal infection such as diarrhea due to common bacterial infections. However, ART preferentially restored Th1 over Th17 cells at the colorectal mucosa resulting in an altered balance between these two subsets that promotes a more pro-inflammatory response. It is not clear why ART preferential restores Th1 cells. It is possible that longer duration of ART could restore Th17 cells at the colorectal mucosa, and re-establish the ratios between these subsets of these cells to those observed before establishment of HIV/SIV infection. However, in HIV-infected individuals the levels of IL-21 have been shown to be decreased and ART is only partially capable of restoring secretion of this cytokine (241). IL-21 is secreted by Th17 cells and drives IL-17 secretion in an autocrine manner. Thus the low levels of IL-21 post ART may also contribute to the preferential replenishment of Th1 over Th17 cells. It's possible that treatment with anti-retroviral drugs and IL-21 therapy may restore the levels of Th17 cells and enhance gut mucosa function.

Immune dysfunction is specific to pathogenic SIV infection

SIV can establish infection in natural and non-natural hosts. Although CD4+ T cells appear to be the main cell type infected by this virus in both hosts, progression to disease is rarely observed in the natural host. The key difference between these two models of infection is the low levels of immune activation observed after SIV infection of natural host (22, 24-26). Consistent with this, we did not observe expansion of CD8 regulatory T cells, or depletion of Th17/Tc17 cells in sooty mangabeys chronically infected with SIV. It is possible that lower levels of CCR5 expression in CD4+ T cells of sooties may be associated with less trafficking to tissues, and thus these monkey may have less reservoirs for the virus. The lack of microbial translocation observed in sooties may be a direct result of the presence of Th17 and Tc17 cells at the colorectal mucosa, which allows for maintenance of gut permeability and morphology of the gastrointestinal tract. CD8+ regulatory T cells with suppressive capacity may not expand in the presence of low levels of immune activation, such as those observed in non-pathogenic SIV infections. Thus limiting the levels of immune activation may partially control for immune dysregulation in the gastrointestinal tract of rhesus macaques.

Conclusions and future directions

Our results have brought focus on two previously unexplored players in the context of HIV/SIV infections: CD8 regulatory T cells and Tc17 cells. We show that these cell subsets have important roles in regulating progression to disease. While CD8+

regulatory T cells help the virus by constraining anti-viral immunity, Tc17 cells delay onset of disease by contributing to the maintenance of intestinal epithelial barrier.

Studies conducted in large animal systems are not without their limitations. There are many questions that arise due to the findings reported here. Although ART reduced levels of CD8+ regulatory T cells, we did not address whether ART had an effect upon the ability of these cells to suppress anti-viral immunity. The expression of CTLA-4 and CD39 by CD8+ regulatory T cells are indicative of their mechanism of action. It would appropriate to conduct experiments that verify the exact mechanism of action, such as using blocking antibodies and confirming the loss of suppressive capacity of CD8+ regulatory T cells. It would be interesting to address whether the suppression of proliferation of SIV-specific T cells is the result of direct interaction of CD8+ regulatory T cells and antigen-specific cells or whether these cells act on dendritic cells which in turn shut down anti-viral immune response. The suppressive capacity of CD8+ regulatory T cells in HIV-infected individuals is yet to be confirmed.

The reasons for depletion of Tc17 cells during end-stage disease remain unclear in this report. It is possible that the depletion of these cells is due to higher activation levels, however further experiments are needed for confirmation. Although we propose that Tc17 cells compensate for the loss of Th17 cell loss during the acute phase of infection by partially maintaining the function of the epithelial barrier, immuno-histological studies would further elucidate the nature of events occurring at the gastrointestinal tract. In our proposed model, we speculate that there is greater infiltration of microbes during chronic phase than the acute phase of infection. We are yet to provide evidence for this. We demonstrate that Th17 and Tc17 cells express CTLA-4 and lack killing potential thus

suggest that these cell may have more of a regulatory role. It would be interesting to conduct studies that would corroborate the regulatory function of these cells. It is of utmost importance to ascertain that the findings reported here also occur in HIV-infected individuals.

References

1. Gottlieb MS, Schroff R, Schanker HM, Weisman JD, Fan PT, Wolf RA, Saxon A. 1981. Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. *N Engl J Med* 305: 1425-31
2. Masur H, Michelis MA, Greene JB, Onorato I, Stouwe RA, Holzman RS, Wormser G, Brettman L, Lange M, Murray HW, Cunningham-Rundles S. 1981. An outbreak of community-acquired Pneumocystis carinii pneumonia: initial manifestation of cellular immune dysfunction. *N Engl J Med* 305: 1431-8
3. Siegal FP, Lopez C, Hammer GS, Brown AE, Kornfeld SJ, Gold J, Hassett J, Hirschman SZ, Cunningham-Rundles C, Adelsberg BR, et al. 1981. Severe acquired immunodeficiency in male homosexuals, manifested by chronic perianal ulcerative herpes simplex lesions. *N Engl J Med* 305: 1439-44
4. Shattock RJ, Moore JP. 2003. Inhibiting sexual transmission of HIV-1 infection. *Nat Rev Microbiol* 1: 25-34
5. Lifson JD, Feinberg MB, Reyes GR, Rabin L, Banapour B, Chakrabarti S, Moss B, Wong-Staal F, Steimer KS, Engleman EG. 1986. Induction of CD4-dependent cell fusion by the HTLV-III/LAV envelope glycoprotein. *Nature* 323: 725-8
6. Regoes RR, Wodarz D, Nowak MA. 1998. Virus dynamics: the effect of target cell limitation and immune responses on virus evolution. *J Theor Biol* 191: 451-62
7. Stafford MA, Corey L, Cao Y, Daar ES, Ho DD, Perelson AS. 2000. Modeling plasma virus concentration during primary HIV infection. *J Theor Biol* 203: 285-301
8. Koup RA, Safrit JT, Cao Y, Andrews CA, McLeod G, Borkowsky W, Farthing C, Ho DD. 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J Virol* 68: 4650-5
9. Amara RR, Ibegbu C, Villinger F, Montefiori DC, Sharma S, Nigam P, Xu Y, McClure HM, Robinson HL. 2005. Studies using a viral challenge and CD8 T cell depletions on the roles of cellular and humoral immunity in the control of an SHIV-89.6P challenge in DNA/MVA-vaccinated macaques. *Virology* 343: 246-55
10. Mattapallil JJ, Douek DC, Hill B, Nishimura Y, Martin M, Roederer M. 2005. Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. *Nature* 434: 1093-7
11. Li Q, Duan L, Estes JD, Ma ZM, Rourke T, Wang Y, Reilly C, Carlis J, Miller CJ, Haase AT. 2005. Peak SIV replication in resting memory CD4+ T cells depletes gut lamina propria CD4+ T cells. *Nature* 434: 1148-52
12. Mehandru S, Poles MA, Tenner-Racz K, Horowitz A, Hurley A, Hogan C, Boden D, Racz P, Markowitz M. 2004. Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract. *J Exp Med* 200: 761-70
13. Mehandru S, Poles MA, Tenner-Racz K, Manuelli V, Jean-Pierre P, Lopez P, Shet A, Low A, Mohri H, Boden D, Racz P, Markowitz M. 2007. Mechanisms of

gastrointestinal CD4+ T-cell depletion during acute and early human immunodeficiency virus type 1 infection. *J Virol* 81: 599-612

14. Brenchley JM, Schacker TW, Ruff LE, Price DA, Taylor JH, Beilman GJ, Nguyen PL, Khoruts A, Larson M, Haase AT, Douek DC. 2004. CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *J Exp Med* 200: 749-59
15. Veazey RS, DeMaria M, Chalifoux LV, Shvetz DE, Pauley DR, Knight HL, Rosenzweig M, Johnson RP, Desrosiers RC, Lackner AA. 1998. Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. *Science* 280: 427-31
16. Sodora DL, Silvestri G. 2008. Immune activation and AIDS pathogenesis. *AIDS* 22: 439-46
17. Kovacs JA, Lempicki RA, Sidorov IA, Adelsberger JW, Herpin B, Metcalf JA, Sereti I, Polis MA, Davey RT, Tavel J, Falloon J, Stevens R, Lambert L, Dewar R, Schwartzentruber DJ, Anver MR, Baseler MW, Masur H, Dimitrov DS, Lane HC. 2001. Identification of dynamically distinct subpopulations of T lymphocytes that are differentially affected by HIV. *J Exp Med* 194: 1731-41
18. Hazenberg MD, Otto SA, van Benthem BH, Roos MT, Coutinho RA, Lange JM, Hamann D, Prins M, Miedema F. 2003. Persistent immune activation in HIV-1 infection is associated with progression to AIDS. *AIDS* 17: 1881-8
19. Sousa AE, Carneiro J, Meier-Schellersheim M, Grossman Z, Victorino RM. 2002. CD4 T cell depletion is linked directly to immune activation in the pathogenesis of HIV-1 and HIV-2 but only indirectly to the viral load. *J Immunol* 169: 3400-6
20. Talal AH, Irwin CE, Dieterich DT, Yee H, Zhang L. 2001. Effect of HIV-1 infection on lymphocyte proliferation in gut-associated lymphoid tissue. *J Acquir Immune Defic Syndr* 26: 208-17
21. Herbein G, Khan KA. 2008. Is HIV infection a TNF receptor signalling-driven disease? *Trends Immunol* 29: 61-7
22. Gordon SN, Klatt NR, Bosinger SE, Brenchley JM, Milush JM, Engram JC, Dunham RM, Paiardini M, Klucking S, Danesh A, Strobert EA, Apetrei C, Pandrea IV, Kelvin D, Douek DC, Staprans SI, Sodora DL, Silvestri G. 2007. Severe depletion of mucosal CD4+ T cells in AIDS-free simian immunodeficiency virus-infected sooty mangabeys. *J Immunol* 179: 3026-34
23. Muthukumar A, Zhou D, Paiardini M, Barry AP, Cole KS, McClure HM, Staprans SI, Silvestri G, Sodora DL. 2005. Timely triggering of homeostatic mechanisms involved in the regulation of T-cell levels in SIVsm-infected sooty mangabeys. *Blood* 106: 3839-45
24. Silvestri G. 2008. AIDS pathogenesis: a tale of two monkeys. *J Med Primatol* 37 Suppl 2: 6-12
25. Chakrabarti LA, Lewin SR, Zhang L, Gettie A, Luckay A, Martin LN, Skulsky E, Ho DD, Cheng-Mayer C, Marx PA. 2000. Normal T-cell turnover in sooty mangabeys harboring active simian immunodeficiency virus infection. *J Virol* 74: 1209-23
26. Kaur A, Di Mascio M, Barabasz A, Rosenzweig M, McClure HM, Perelson AS, Ribeiro RM, Johnson RP. 2008. Dynamics of T- and B-lymphocyte turnover in a natural host of simian immunodeficiency virus. *J Virol* 82: 1084-93

27. Onanga R, Kornfeld C, Pandrea I, Estaquier J, Souquiere S, Rouquet P, Mavoungou VP, Bourry O, M'Boup S, Barre-Sinoussi F, Simon F, Apetrei C, Roques P, Muller-Trutwin MC. 2002. High levels of viral replication contrast with only transient changes in CD4(+) and CD8(+) cell numbers during the early phase of experimental infection with simian immunodeficiency virus SIVmnd-1 in Mandrillus sphinx. *J Virol* 76: 10256-63
28. Brenchley JM, Price DA, Schacker TW, Asher TE, Silvestri G, Rao S, Kazzaz Z, Bornstein E, Lambotte O, Altmann D, Blazar BR, Rodriguez B, Teixeira-Johnson L, Landay A, Martin JN, Hecht FM, Picker LJ, Lederman MM, Deeks SG, Douek DC. 2006. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat Med* 12: 1365-71
29. Pandrea I, Apetrei C, Gordon S, Barbercheck J, Dufour J, Bohm R, Sumpter B, Roques P, Marx PA, Hirsch VM, Kaur A, Lackner AA, Veazey RS, Silvestri G. 2007. Paucity of CD4+CCR5+ T cells is a typical feature of natural SIV hosts. *Blood* 109: 1069-76
30. Cheroutre H. 2004. Starting at the beginning: new perspectives on the biology of mucosal T cells. *Annu Rev Immunol* 22: 217-46
31. Heise C, Miller CJ, Lackner A, Dandekar S. 1994. Primary acute simian immunodeficiency virus infection of intestinal lymphoid tissue is associated with gastrointestinal dysfunction. *J Infect Dis* 169: 1116-20
32. Heise C, Vogel P, Miller CJ, Halsted CH, Dandekar S. 1993. Simian immunodeficiency virus infection of the gastrointestinal tract of rhesus macaques. Functional, pathological, and morphological changes. *Am J Pathol* 142: 1759-71
33. Batman PA, Miller AR, Forster SM, Harris JR, Pinching AJ, Griffin GE. 1989. Jejunal enteropathy associated with human immunodeficiency virus infection: quantitative histology. *J Clin Pathol* 42: 275-81
34. Ullrich R, Zeitz M, Heise W, L'Age M, Hoffken G, Riecken EO. 1989. Small intestinal structure and function in patients infected with human immunodeficiency virus (HIV): evidence for HIV-induced enteropathy. *Ann Intern Med* 111: 15-21
35. Cummins AG, LaBrooy JT, Stanley DP, Rowland R, Shearman DJ. 1990. Quantitative histological study of enteropathy associated with HIV infection. *Gut* 31: 317-21
36. MacDonald TT, Spencer J. 1992. Cell-mediated immune injury in the intestine. *Gastroenterol Clin North Am* 21: 367-86
37. Clayburgh DR, Shen L, Turner JR. 2004. A porous defense: the leaky epithelial barrier in intestinal disease. *Lab Invest* 84: 282-91
38. George MD, Wehkamp J, Kays RJ, Leutenegger CM, Sabir S, Grishina I, Dandekar S, Bevins CL. 2008. In vivo gene expression profiling of human intestinal epithelial cells: analysis by laser microdissection of formalin fixed tissues. *BMC Genomics* 9: 209
39. Sankaran S, George MD, Reay E, Guadalupe M, Flamm J, Prindiville T, Dandekar S. 2008. Rapid onset of intestinal epithelial barrier dysfunction in primary human immunodeficiency virus infection is driven by an imbalance between immune response and mucosal repair and regeneration. *J Virol* 82: 538-45

40. Sankaran S, Guadalupe M, Reay E, George MD, Flamm J, Prindiville T, Dandekar S. 2005. Gut mucosal T cell responses and gene expression correlate with protection against disease in long-term HIV-1-infected nonprogressors. *Proc Natl Acad Sci U S A* 102: 9860-5
41. Abel K, Rocke DM, Chohan B, Fritts L, Miller CJ. 2005. Temporal and anatomic relationship between virus replication and cytokine gene expression after vaginal simian immunodeficiency virus infection. *J Virol* 79: 12164-72
42. Reka S, Garro ML, Kotler DP. 1994. Variation in the expression of human immunodeficiency virus RNA and cytokine mRNA in rectal mucosa during the progression of infection. *Lymphokine Cytokine Res* 13: 391-8
43. Olsson J, Poles M, Spetz AL, Elliott J, Hultin L, Giorgi J, Andersson J, Anton P. 2000. Human immunodeficiency virus type 1 infection is associated with significant mucosal inflammation characterized by increased expression of CCR5, CXCR4, and beta-chemokines. *J Infect Dis* 182: 1625-35
44. McGowan I, Elliott J, Fuerst M, Taing P, Boscardin J, Poles M, Anton P. 2004. Increased HIV-1 mucosal replication is associated with generalized mucosal cytokine activation. *J Acquir Immune Defic Syndr* 37: 1228-36
45. Brenchley JM, Price DA, Douek DC. 2006. HIV disease: fallout from a mucosal catastrophe? *Nat Immunol* 7: 235-9
46. Walker CM, Moody DJ, Stites DP, Levy JA. 1986. CD8+ lymphocytes can control HIV infection in vitro by suppressing virus replication. *Science* 234: 1563-6
47. Rinaldo C, Huang XL, Fan ZF, Ding M, Beltz L, Logar A, Panicali D, Mazzara G, Liebmann J, Cottrill M, et al. 1995. High levels of anti-human immunodeficiency virus type 1 (HIV-1) memory cytotoxic T-lymphocyte activity and low viral load are associated with lack of disease in HIV-1-infected long-term nonprogressors. *J Virol* 69: 5838-42
48. Musey L, Hughes J, Schacker T, Shea T, Corey L, McElrath MJ. 1997. Cytotoxic-T-cell responses, viral load, and disease progression in early human immunodeficiency virus type 1 infection. *N Engl J Med* 337: 1267-74
49. Ogg GS, Jin X, Bonhoeffer S, Dunbar PR, Nowak MA, Monard S, Segal JP, Cao Y, Rowland-Jones SL, Cerundolo V, Hurley A, Markowitz M, Ho DD, Nixon DF, McMichael AJ. 1998. Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. *Science* 279: 2103-6
50. Schmitz JE, Kuroda MJ, Santra S, Sasseville VG, Simon MA, Lifton MA, Racz P, Tenner-Racz K, Dalesandro M, Scallon BJ, Ghrayeb J, Forman MA, Montefiori DC, Rieber EP, Letvin NL, Reimann KA. 1999. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science* 283: 857-60
51. Schnittman SM, Lane HC, Higgins SE, Folks T, Fauci AS. 1986. Direct polyclonal activation of human B lymphocytes by the acquired immune deficiency syndrome virus. *Science* 233: 1084-6
52. Moir S, Malaspina A, Li Y, Chun TW, Lowe T, Adelsberger J, Baseler M, Ehler LA, Liu S, Davey RT, Jr., Mican JA, Fauci AS. 2000. B cells of HIV-1-infected patients bind virions through CD21-complement interactions and transmit infectious virus to activated T cells. *J Exp Med* 192: 637-46

53. Kacani L, Prodinger WM, Sprinzl GM, Schwendinger MG, Spruth M, Stoiber H, Dopper S, Steinhuber S, Steindl F, Dierich MP. 2000. Detachment of human immunodeficiency virus type 1 from germinal centers by blocking complement receptor type 2. *J Virol* 74: 7997-8002
54. Malaspina A, Moir S, Nickle DC, Donoghue ET, Ogwaro KM, Ehler LA, Liu S, Mican JA, Dybul M, Chun TW, Mullins JI, Fauci AS. 2002. Human immunodeficiency virus type 1 bound to B cells: relationship to virus replicating in CD4+ T cells and circulating in plasma. *J Virol* 76: 8855-63
55. Moir S, Fauci AS. 2008. Pathogenic mechanisms of B-lymphocyte dysfunction in HIV disease. *J Allergy Clin Immunol* 122: 12-9; quiz 20-1
56. Ehrhardt GR, Hsu JT, Gartland L, Leu CM, Zhang S, Davis RS, Cooper MD. 2005. Expression of the immunoregulatory molecule FcRH4 defines a distinctive tissue-based population of memory B cells. *J Exp Med* 202: 783-91
57. Ehrhardt GR, Hijikata A, Kitamura H, Ohara O, Wang JY, Cooper MD. 2008. Discriminating gene expression profiles of memory B cell subpopulations. *J Exp Med* 205: 1807-17
58. Kao CY, Chen Y, Thai P, Wachi S, Huang F, Kim C, Harper RW, Wu R. 2004. IL-17 markedly up-regulates beta-defensin-2 expression in human airway epithelium via JAK and NF-kappaB signaling pathways. *J Immunol* 173: 3482-91
59. Liang SC, Tan XY, Luxenberg DP, Karim R, Dunussi-Joannopoulos K, Collins M, Fouser LA. 2006. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med* 203: 2271-9
60. Kolls JK, Linden A. 2004. Interleukin-17 family members and inflammation. *Immunity* 21: 467-76
61. Balagopal A, Philp FH, Astemborski J, Block TM, Mehta A, Long R, Kirk GD, Mehta SH, Cox AL, Thomas DL, Ray SC. 2008. Human immunodeficiency virus-related microbial translocation and progression of hepatitis C. *Gastroenterology* 135: 226-33
62. Brenchley JM, Paiardini M, Knox KS, Asher AI, Cervasi B, Asher TE, Scheinberg P, Price DA, Hage CA, Kholi LM, Khoruts A, Frank I, Else J, Schacker T, Silvestri G, Douek DC. 2008. Differential Th17 CD4 T-cell depletion in pathogenic and nonpathogenic lentiviral infections. *Blood* 112: 2826-35
63. Cecchinato V, Trindade CJ, Laurence A, Heraud JM, Brenchley JM, Ferrari MG, Zaffiri L, Tryniszewska E, Tsai WP, Vaccari M, Parks RW, Venzon D, Douek DC, O'Shea JJ, Franchini G. 2008. Altered balance between Th17 and Th1 cells at mucosal sites predicts AIDS progression in simian immunodeficiency virus-infected macaques. *Mucosal Immunol* 1: 279-88
64. Favre D, Lederer S, Kanwar B, Ma ZM, Proll S, Kasakow Z, Mold J, Swainson L, Barbour JD, Baskin CR, Palermo R, Pandrea I, Miller CJ, Katze MG, McCune JM. 2009. Critical loss of the balance between Th17 and T regulatory cell populations in pathogenic SIV infection. *PLoS Pathog* 5: e1000295
65. Raffatellu M, Santos RL, Verhoeven DE, George MD, Wilson RP, Winter SE, Godinez I, Sankaran S, Paixao TA, Gordon MA, Kolls JK, Dandekar S, Baumler AJ. 2008. Simian immunodeficiency virus-induced mucosal interleukin-17 deficiency promotes Salmonella dissemination from the gut. *Nat Med* 14: 421-8

66. Shellito JE. 2004. Failure of host defenses in human immunodeficiency virus. *Semin Respir Crit Care Med* 25: 73-84
67. Ellis M, Gupta S, Galant S, Hakim S, VandeVen C, Toy C, Cairo MS. 1988. Impaired neutrophil function in patients with AIDS or AIDS-related complex: a comprehensive evaluation. *J Infect Dis* 158: 1268-76
68. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 136: 2348-57
69. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, Weaver CT. 2005. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 6: 1123-32
70. Yao Z, Fanslow WC, Seldin MF, Rousseau AM, Painter SL, Comeau MR, Cohen JI, Spriggs MK. 1995. Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor. *Immunity* 3: 811-21
71. Luzza F, Parrello T, Monteleone G, Sebkova L, Romano M, Zarrilli R, Imeneo M, Pallone F. 2000. Up-regulation of IL-17 is associated with bioactive IL-8 expression in Helicobacter pylori-infected human gastric mucosa. *J Immunol* 165: 5332-7
72. Aggarwal S, Ghilardi N, Xie MH, de Sauvage FJ, Gurney AL. 2003. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *J Biol Chem* 278: 1910-4
73. Bluestone JA, Abbas AK. 2003. Natural versus adaptive regulatory T cells. *Nat Rev Immunol* 3: 253-7
74. Fontenot JD, Rudensky AY. 2005. A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. *Nat Immunol* 6: 331-7
75. Sakaguchi S. 2000. Regulatory T cells: key controllers of immunologic self-tolerance. *Cell* 101: 455-8
76. Ivanov, II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, Cua DJ, Littman DR. 2006. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 126: 1121-33
77. Yang XO, Pappu BP, Nurieva R, Akimzhanov A, Kang HS, Chung Y, Ma L, Shah B, Panopoulos AD, Schluns KS, Watowich SS, Tian Q, Jetten AM, Dong C. 2008. T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. *Immunity* 28: 29-39
78. Chen Z, Laurence A, O'Shea JJ. 2007. Signal transduction pathways and transcriptional regulation in the control of Th17 differentiation. *Semin Immunol* 19: 400-8
79. de Beaucoudrey L, Puel A, Filipe-Santos O, Cobat A, Ghandil P, Chrabieh M, Feinberg J, von Bernuth H, Samarina A, Janniere L, Fieschi C, Stephan JL, Boileau C, Lyonnet S, Jondeau G, Cormier-Daire V, Le Merrer M, Hoarau C, Lebranchu Y, Lortholary O, Chandesris MO, Tron F, Gambineri E, Bianchi L, Rodriguez-Gallego C, Zitnik SE, Vasconcelos J, Guedes M, Vitor AB, Marodi L, Chapel H, Reid B, Roifman C, Nadal D, Reichenbach J, Caragol I, Garty BZ, Dogu F, Camcioglu Y, Gulle S, Sanal O, Fischer A, Abel L, Stockinger B, Picard

C, Casanova JL. 2008. Mutations in STAT3 and IL12RB1 impair the development of human IL-17-producing T cells. *J Exp Med* 205: 1543-50
80. Nurieva R, Yang XO, Martinez G, Zhang Y, Panopoulos AD, Ma L, Schluns K, Tian Q, Watowich SS, Jetten AM, Dong C. 2007. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* 448: 480-3
81. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, Weiner HL, Kuchroo VK. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441: 235-8
82. Happel KI, Dubin PJ, Zheng M, Ghilardi N, Lockhart C, Quinton LJ, Odden AR, Shellito JE, Bagby GJ, Nelson S, Kolls JK. 2005. Divergent roles of IL-23 and IL-12 in host defense against Klebsiella pneumoniae. *J Exp Med* 202: 761-9
83. Khader SA, Bell GK, Pearl JE, Fountain JJ, Rangel-Moreno J, Cilley GE, Shen F, Eaton SM, Gaffen SL, Swain SL, Locksley RM, Haynes L, Randall TD, Cooper AM. 2007. IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during Mycobacterium tuberculosis challenge. *Nat Immunol* 8: 369-77
84. Volpe E, Servant N, Zollinger R, Bogiatzi SI, Hupe P, Barillot E, Soumelis V. 2008. A critical function for transforming growth factor-beta, interleukin 23 and proinflammatory cytokines in driving and modulating human T(H)-17 responses. *Nat Immunol* 9: 650-7
85. Fragale A, Gabriele L, Stellacci E, Borghi P, Perrotti E, Ilari R, Lanciotti A, Remoli AL, Venditti M, Belardelli F, Battistini A. 2008. IFN regulatory factor-1 negatively regulates CD4+ CD25+ regulatory T cell differentiation by repressing Foxp3 expression. *J Immunol* 181: 1673-82
86. Takaki H, Ichiyama K, Koga K, Chinen T, Takaesu G, Sugiyama Y, Kato S, Yoshimura A, Kobayashi T. 2008. STAT6 Inhibits TGF-beta1-mediated Foxp3 induction through direct binding to the Foxp3 promoter, which is reverted by retinoic acid receptor. *J Biol Chem* 283: 14955-62
87. Xiao S, Jin H, Korn T, Liu SM, Oukka M, Lim B, Kuchroo VK. 2008. Retinoic acid increases Foxp3+ regulatory T cells and inhibits development of Th17 cells by enhancing TGF-beta-driven Smad3 signaling and inhibiting IL-6 and IL-23 receptor expression. *J Immunol* 181: 2277-84
88. Kang SG, Lim HW, Andrisani OM, Broxmeyer HE, Kim CH. 2007. Vitamin A metabolites induce gut-homing FoxP3+ regulatory T cells. *J Immunol* 179: 3724-33
89. Mucida D, Park Y, Kim G, Turovskaya O, Scott I, Kronenberg M, Cheroutre H. 2007. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science* 317: 256-60
90. Khayrullina T, Yen JH, Jing H, Ganea D. 2008. In vitro differentiation of dendritic cells in the presence of prostaglandin E2 alters the IL-12/IL-23 balance and promotes differentiation of Th17 cells. *J Immunol* 181: 721-35
91. Woolard MD, Hensley LL, Kawula TH, Frelinger JA. 2008. Respiratory Francisella tularensis live vaccine strain infection induces Th17 cells and prostaglandin E2, which inhibits generation of gamma interferon-positive T cells. *Infect Immun* 76: 2651-9

92. Chizzolini C, Chicheportiche R, Alvarez M, de Rham C, Roux-Lombard P, Ferrari-Lacraz S, Dayer JM. 2008. Prostaglandin E2 synergistically with interleukin-23 favors human Th17 expansion. *Blood* 112: 3696-703
93. Napolitani G, Acosta-Rodriguez EV, Lanzavecchia A, Sallusto F. 2009. Prostaglandin E2 enhances Th17 responses via modulation of IL-17 and IFN-gamma production by memory CD4+ T cells. *Eur J Immunol* 39: 1301-12
94. Gershon RK, Kondo K. 1970. Cell interactions in the induction of tolerance: the role of thymic lymphocytes. *Immunology* 18: 723-37
95. Gershon RK, Kondo K. 1971. Infectious immunological tolerance. *Immunology* 21: 903-14
96. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155: 1151-64
97. Hori S, Nomura T, Sakaguchi S. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299: 1057-61
98. Khattri R, Cox T, Yasayko SA, Ramsdell F. 2003. An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat Immunol* 4: 337-42
99. Fontenot JD, Gavin MA, Rudensky AY. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 4: 330-6
100. Mills KH. 2004. Regulatory T cells: friend or foe in immunity to infection? *Nat Rev Immunol* 4: 841-55
101. Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J, Enk AH. 2001. Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. *J Exp Med* 193: 1285-94
102. Wu K, Bi Y, Sun K, Wang C. 2007. IL-10-producing type 1 regulatory T cells and allergy. *Cell Mol Immunol* 4: 269-75
103. Weiner HL. 2001. Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells. *Immunol Rev* 182: 207-14
104. Siegmund K, Ruckert B, Ouaked N, Burgler S, Speiser A, Akdis CA, Schmidt-Weber CB. 2009. Unique phenotype of human tonsillar and in vitro-induced FOXP3+CD8+ T cells. *J Immunol* 182: 2124-30
105. Manavalan JS, Kim-Schulze S, Scotto L, Naiyer AJ, Vlad G, Colombo PC, Marboe C, Mancini D, Cortesini R, Suciu-Foca N. 2004. Alloantigen specific CD8+CD28- FOXP3+ T suppressor cells induce ILT3+ ILT4+ tolerogenic endothelial cells, inhibiting alloreactivity. *Int Immunol* 16: 1055-68
106. Lim A, French MA, Price P. 2009. CD4+ and CD8+ T cells expressing FoxP3 in HIV-infected patients are phenotypically distinct and influenced by disease severity and antiretroviral therapy. *J Acquir Immune Defic Syndr* 51: 248-57
107. Kaestner KH, Knochel W, Martinez DE. 2000. Unified nomenclature for the winged helix/forkhead transcription factors. *Genes Dev* 14: 142-6
108. Bettelli E, Dastrange M, Oukka M. 2005. Foxp3 interacts with nuclear factor of activated T cells and NF-kappa B to repress cytokine gene expression and effector functions of T helper cells. *Proc Natl Acad Sci U S A* 102: 5138-43

109. Lankford S, Petty C, LaVoy A, Reckling S, Tompkins W, Dean GA. 2008. Cloning of feline FOXP3 and detection of expression in CD4+CD25+ regulatory T cells. *Vet Immunol Immunopathol* 122: 159-66
110. Du J, Huang C, Zhou B, Ziegler SF. 2008. Isoform-specific inhibition of ROR alpha-mediated transcriptional activation by human FOXP3. *J Immunol* 180: 4785-92
111. Ichiyama K, Yoshida H, Wakabayashi Y, Chinen T, Saeki K, Nakaya M, Takaesu G, Hori S, Yoshimura A, Kobayashi T. 2008. Foxp3 inhibits RORgammat-mediated IL-17A mRNA transcription through direct interaction with RORgammat. *J Biol Chem* 283: 17003-8
112. Mailer RK, Falk K, Rotzschke O. 2009. Absence of leucine zipper in the natural FOXP3Delta2Delta7 isoform does not affect dimerization but abrogates suppressive capacity. *PLoS One* 4: e6104
113. Kim HP, Leonard WJ. 2007. CREB/ATF-dependent T cell receptor-induced FoxP3 gene expression: a role for DNA methylation. *J Exp Med* 204: 1543-51
114. Janson PC, Winerdal ME, Marits P, Thorn M, Ohlsson R, Winqvist O. 2008. FOXP3 promoter demethylation reveals the committed Treg population in humans. *PLoS One* 3: e1612
115. Li B, Greene MI. 2007. FOXP3 actively represses transcription by recruiting the HAT/HDAC complex. *Cell Cycle* 6: 1432-6
116. Tao R, de Zoeten EF, Ozkaynak E, Chen C, Wang L, Porrett PM, Li B, Turka LA, Olson EN, Greene MI, Wells AD, Hancock WW. 2007. Deacetylase inhibition promotes the generation and function of regulatory T cells. *Nat Med* 13: 1299-307
117. Lucas JL, Mirshahpanah P, Haas-Stapleton E, Asadullah K, Zollner TM, Numerof RP. 2009. Induction of Foxp3+ regulatory T cells with histone deacetylase inhibitors. *Cell Immunol* 257: 97-104
118. Pereira LE, Villinger F, Onlamoon N, Bryan P, Cardona A, Pattanapanysat K, Mori K, Hagen S, Picker L, Ansari AA. 2007. Simian immunodeficiency virus (SIV) infection influences the level and function of regulatory T cells in SIV-infected rhesus macaques but not SIV-infected sooty mangabeys. *J Virol* 81: 4445-56
119. Chase AJ, Sedaghat AR, German JR, Gama L, Zink MC, Clements JE, Siliciano RF. 2007. Severe depletion of CD4+ CD25+ regulatory T cells from the intestinal lamina propria but not peripheral blood or lymph nodes during acute simian immunodeficiency virus infection. *J Virol* 81: 12748-57
120. Jiao Y, Fu J, Xing S, Fu B, Zhang Z, Shi M, Wang X, Zhang J, Jin L, Kang F, Wu H, Wang FS. 2009. The decrease of regulatory T cells correlates with excessive activation and apoptosis of CD8+ T cells in HIV-1-infected typical progressors, but not in long-term non-progressors. *Immunology* 128: e366-75
121. Andersson J, Boasso A, Nilsson J, Zhang R, Shire NJ, Lindback S, Shearer GM, Chougnet CA. 2005. The prevalence of regulatory T cells in lymphoid tissue is correlated with viral load in HIV-infected patients. *J Immunol* 174: 3143-7
122. Nilsson J, Boasso A, Velilla PA, Zhang R, Vaccari M, Franchini G, Shearer GM, Andersson J, Chougnet C. 2006. HIV-1-driven regulatory T-cell accumulation in lymphoid tissues is associated with disease progression in HIV/AIDS. *Blood* 108: 3808-17

123. Chase AJ, Yang HC, Zhang H, Blankson JN, Siliciano RF. 2008. Preservation of FoxP3+ regulatory T cells in the peripheral blood of human immunodeficiency virus type 1-infected elite suppressors correlates with low CD4+ T-cell activation. *J Virol* 82: 8307-15
124. Card CM, McLaren PJ, Wachihi C, Kimani J, Plummer FA, Fowke KR. 2009. Decreased immune activation in resistance to HIV-1 infection is associated with an elevated frequency of CD4(+)CD25(+)FOXP3(+) regulatory T cells. *J Infect Dis* 199: 1318-22
125. Boasso A, Vaccari M, Hryniewicz A, Fuchs D, Nacsa J, Cecchinato V, Andersson J, Franchini G, Shearer GM, Chougnet C. 2007. Regulatory T-cell markers, indoleamine 2,3-dioxygenase, and virus levels in spleen and gut during progressive simian immunodeficiency virus infection. *J Virol* 81: 11593-603
126. Epple HJ, Loddenkemper C, Kunkel D, Troger H, Maul J, Moos V, Berg E, Ullrich R, Schulzke JD, Stein H, Duchmann R, Zeitz M, Schneider T. 2006. Mucosal but not peripheral FOXP3+ regulatory T cells are highly increased in untreated HIV infection and normalize after suppressive HAART. *Blood* 108: 3072-8
127. Bi X, Suzuki Y, Gatanaga H, Oka S. 2009. High frequency and proliferation of CD4+ FOXP3+ Treg in HIV-1-infected patients with low CD4 counts. *Eur J Immunol* 39: 301-9
128. Montes M, Lewis DE, Sanchez C, de Castilla DL, Graviss EA, Seas C, Gotuzzo E, White AC, Jr. 2006. Foxp3+ regulatory T cells in antiretroviral-naive HIV patients. *AIDS* 20: 1669-71
129. Allers K, Loddenkemper C, Hofmann J, Unbehaun A, Kunkel D, Moos V, Kaup FJ, Stahl-Hennig C, Sauermann U, Epple HJ, Schneider T. 2010. Gut mucosal FOXP3+ regulatory CD4+ T cells and Nonregulatory CD4+ T cells are differentially affected by simian immunodeficiency virus infection in rhesus macaques. *J Virol* 84: 3259-69
130. Baker CA, Clark R, Ventura F, Jones NG, Guzman D, Bangsberg DR, Cao H. 2007. Peripheral CD4 loss of regulatory T cells is associated with persistent viraemia in chronic HIV infection. *Clin Exp Immunol* 147: 533-9
131. Antons AK, Wang R, Oswald-Richter K, Tseng M, Arendt CW, Kalams SA, Unutmaz D. 2008. Naive precursors of human regulatory T cells require FoxP3 for suppression and are susceptible to HIV infection. *J Immunol* 180: 764-73
132. Kinter AL, Hennessey M, Bell A, Kern S, Lin Y, Daucher M, Planta M, McGlaughlin M, Jackson R, Ziegler SF, Fauci AS. 2004. CD25(+)CD4(+) regulatory T cells from the peripheral blood of asymptomatic HIV-infected individuals regulate CD4(+) and CD8(+) HIV-specific T cell immune responses in vitro and are associated with favorable clinical markers of disease status. *J Exp Med* 200: 331-43
133. Kinter A, McNally J, Riggin L, Jackson R, Roby G, Fauci AS. 2007. Suppression of HIV-specific T cell activity by lymph node CD25+ regulatory T cells from HIV-infected individuals. *Proc Natl Acad Sci U S A* 104: 3390-5
134. Kinter AL, Horak R, Sion M, Riggin L, McNally J, Lin Y, Jackson R, O'Shea A, Roby G, Kovacs C, Connors M, Migueles SA, Fauci AS. 2007. CD25+ regulatory T cells isolated from HIV-infected individuals suppress the cytolytic and nonlytic

antiviral activity of HIV-specific CD8+ T cells in vitro. *AIDS Res Hum Retroviruses* 23: 438-50

135. Aandahl EM, Michaelsson J, Moretto WJ, Hecht FM, Nixon DF. 2004. Human CD4+ CD25+ regulatory T cells control T-cell responses to human immunodeficiency virus and cytomegalovirus antigens. *J Virol* 78: 2454-9
136. Ji J, Cloyd MW. 2009. HIV-1 binding to CD4 on CD4+CD25+ regulatory T cells enhances their suppressive function and induces them to home to, and accumulate in, peripheral and mucosal lymphoid tissues: an additional mechanism of immunosuppression. *Int Immunol* 21: 283-94
137. Elrefaei M, Barugahare B, Ssali F, Mugyenyi P, Cao H. 2006. HIV-specific IL-10-positive CD8+ T cells are increased in advanced disease and are associated with decreased HIV-specific cytolysis. *J Immunol* 176: 1274-80
138. Torheim EA, Ndhlovu LC, Pettersen FO, Larsen TL, Jha AR, Torgersen KM, Kvale D, Nixon DF, Tasken K, Aandahl EM. 2009. Interleukin-10-secreting T cells define a suppressive subset within the HIV-1-specific T-cell population. *Eur J Immunol* 39: 1280-7
139. Elrefaei M, Ventura FL, Baker CA, Clark R, Bangsberg DR, Cao H. 2007. HIV-specific IL-10-positive CD8+ T cells suppress cytolysis and IL-2 production by CD8+ T cells. *J Immunol* 178: 3265-71
140. Karlsson I, Malleret B, Brochard P, Delache B, Calvo J, Le Grand R, Vaslin B. 2007. FoxP3+ CD25+ CD8+ T-cell induction during primary simian immunodeficiency virus infection in cynomolgus macaques correlates with low CD4+ T-cell activation and high viral load. *J Virol* 81: 13444-55
141. Cao W, Jamieson BD, Hultin LE, Hultin PM, Detels R. 2009. Regulatory T cell expansion and immune activation during untreated HIV type 1 infection are associated with disease progression. *AIDS Res Hum Retroviruses* 25: 183-91
142. Ye P, Rodriguez FH, Kanaly S, Stocking KL, Schurr J, Schwarzenberger P, Oliver P, Huang W, Zhang P, Zhang J, Shellito JE, Bagby GJ, Nelson S, Charrier K, Peschon JJ, Kolls JK. 2001. Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. *J Exp Med* 194: 519-27
143. Nakae S, Komiyama Y, Nambu A, Sudo K, Iwase M, Homma I, Sekikawa K, Asano M, Iwakura Y. 2002. Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses. *Immunity* 17: 375-87
144. Grimbacher B, Holland SM, Gallin JI, Greenberg F, Hill SC, Malech HL, Miller JA, O'Connell AC, Puck JM. 1999. Hyper-IgE syndrome with recurrent infections--an autosomal dominant multisystem disorder. *N Engl J Med* 340: 692-702
145. Ma CS, Chew GY, Simpson N, Priyadarshi A, Wong M, Grimbacher B, Fulcher DA, Tangye SG, Cook MC. 2008. Deficiency of Th17 cells in hyper IgE syndrome due to mutations in STAT3. *J Exp Med* 205: 1551-7
146. Yu JJ, Gaffen SL. 2008. Interleukin-17: a novel inflammatory cytokine that bridges innate and adaptive immunity. *Front Biosci* 13: 170-7
147. Rouvier E, Luciani MF, Mattei MG, Denizot F, Golstein P. 1993. CTLA-8, cloned from an activated T cell, bearing AU-rich messenger RNA instability

sequences, and homologous to a herpesvirus saimiri gene. *J Immunol* 150: 5445-56
148. Yang XO, Chang SH, Park H, Nurieva R, Shah B, Acero L, Wang YH, Schluns KS, Broaddus RR, Zhu Z, Dong C. 2008. Regulation of inflammatory responses by IL-17F. *J Exp Med* 205: 1063-75
149. Ishigame H, Kakuta S, Nagai T, Kadoki M, Nambu A, Komiyama Y, Fujikado N, Tanahashi Y, Akitsu A, Kotaki H, Sudo K, Nakae S, Sasakawa C, Iwakura Y. 2009. Differential roles of interleukin-17A and -17F in host defense against mucoepithelial bacterial infection and allergic responses. *Immunity* 30: 108-19
150. Dong C. 2008. Regulation and pro-inflammatory function of interleukin-17 family cytokines. *Immunol Rev* 226: 80-6
151. Ouyang W, Kolls JK, Zheng Y. 2008. The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity* 28: 454-67
152. Ye P, Garvey PB, Zhang P, Nelson S, Bagby G, Summer WR, Schwarzenberger P, Shellito JE, Kolls JK. 2001. Interleukin-17 and lung host defense against Klebsiella pneumoniae infection. *Am J Respir Cell Mol Biol* 25: 335-40
153. Aujla SJ, Chan YR, Zheng M, Fei M, Askew DJ, Pociask DA, Reinhart TA, McAllister F, Edeal J, Gaus K, Husain S, Kreindler JL, Dubin PJ, Pilewski JM, Myerburg MM, Mason CA, Iwakura Y, Kolls JK. 2008. IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. *Nat Med* 14: 275-81
154. Zheng Y, Valdez PA, Danilenko DM, Hu Y, Sa SM, Gong Q, Abbas AR, Modrusan Z, Ghilardi N, de Sauvage FJ, Ouyang W. 2008. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nat Med* 14: 282-9
155. Fedele G, Nasso M, Spensieri F, Palazzo R, Frasca L, Watanabe M, Ausiello CM. 2008. Lipopolysaccharides from Bordetella pertussis and Bordetella parapertussis differently modulate human dendritic cell functions resulting in divergent prevalence of Th17-polarized responses. *J Immunol* 181: 208-16
156. Siciliano NA, Skinner JA, Yuk MH. 2006. Bordetella bronchiseptica modulates macrophage phenotype leading to the inhibition of CD4+ T cell proliferation and the initiation of a Th17 immune response. *J Immunol* 177: 7131-8
157. Komiyama Y, Nakae S, Matsuki T, Nambu A, Ishigame H, Kakuta S, Sudo K, Iwakura Y. 2006. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J Immunol* 177: 566-73
158. Hofstetter HH, Ibrahim SM, Koczan D, Kruse N, Weishaupt A, Toyka KV, Gold R. 2005. Therapeutic efficacy of IL-17 neutralization in murine experimental autoimmune encephalomyelitis. *Cell Immunol* 237: 123-30
159. Tzartos JS, Friese MA, Craner MJ, Palace J, Newcombe J, Esiri MM, Fugger L. 2008. Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. *Am J Pathol* 172: 146-55
160. Bromley SK, Mempel TR, Luster AD. 2008. Orchestrating the orchestrators: chemokines in control of T cell traffic. *Nat Immunol* 9: 970-80
161. Acosta-Rodriguez EV, Rivino L, Geginat J, Jarrossay D, Gattorno M, Lanzavecchia A, Sallusto F, Napolitani G. 2007. Surface phenotype and antigenic

specificity of human interleukin 17-producing T helper memory cells. *Nat Immunol* 8: 639-46

162. Reboldi A, Coisne C, Baumjohann D, Benvenuto F, Bottinelli D, Lira S, Uccelli A, Lanzavecchia A, Engelhardt B, Sallusto F. 2009. C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE. *Nat Immunol* 10: 514-23
163. Croft M, Carter L, Swain SL, Dutton RW. 1994. Generation of polarized antigen-specific CD8 effector populations: reciprocal action of interleukin (IL)-4 and IL-12 in promoting type 2 versus type 1 cytokine profiles. *J Exp Med* 180: 1715-28
164. Hamada H, Garcia-Hernandez Mde L, Reome JB, Misra SK, Strutt TM, McKinstry KK, Cooper AM, Swain SL, Dutton RW. 2009. Tc17, a unique subset of CD8 T cells that can protect against lethal influenza challenge. *J Immunol* 182: 3469-81
165. Kondo T, Takata H, Matsuki F, Takiguchi M. 2009. Cutting edge: Phenotypic characterization and differentiation of human CD8+ T cells producing IL-17. *J Immunol* 182: 1794-8
166. Yue FY, Merchant A, Kovacs CM, Loutfy M, Persad D, Ostrowski MA. 2008. Virus-specific interleukin-17-producing CD4+ T cells are detectable in early human immunodeficiency virus type 1 infection. *J Virol* 82: 6767-71
167. Arthos J, Cicala C, Martinelli E, Macleod K, Van Ryk D, Wei D, Xiao Z, Veenstra TD, Conrad TP, Lempicki RA, McLaughlin S, Pascuccio M, Gopaul R, McNally J, Cruz CC, Censoplano N, Chung E, Reitano KN, Kottilil S, Goode DJ, Fauci AS. 2008. HIV-1 envelope protein binds to and signals through integrin alpha4beta7, the gut mucosal homing receptor for peripheral T cells. *Nat Immunol* 9: 301-9
168. Kader M, Wang X, Piatak M, Lifson J, Roederer M, Veazey R, Mattapallil JJ. 2009. Alpha4(+)beta7(hi)CD4(+) memory T cells harbor most Th-17 cells and are preferentially infected during acute SIV infection. *Mucosal Immunol* 2: 439-49
169. Kader M, Bixler S, Roederer M, Veazey R, Mattapallil JJ. 2009. CD4 T cell subsets in the mucosa are CD28+Ki-67-HLA-DR-CD69+ but show differential infection based on alpha4beta7 receptor expression during acute SIV infection. *J Med Primatol* 38 Suppl 1: 24-31
170. Guadalupe M, Reay E, Sankaran S, Prindiville T, Flamm J, McNeil A, Dandekar S. 2003. Severe CD4+ T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy. *J Virol* 77: 11708-17
171. Mehandru S, Poles MA, Tenner-Racz K, Jean-Pierre P, Manuelli V, Lopez P, Shet A, Low A, Mohri H, Boden D, Racz P, Markowitz M. 2006. Lack of mucosal immune reconstitution during prolonged treatment of acute and early HIV-1 infection. *PLoS Med* 3: e484
172. Guadalupe M, Sankaran S, George MD, Reay E, Verhoeven D, Shacklett BL, Flamm J, Wegelin J, Prindiville T, Dandekar S. 2006. Viral suppression and immune restoration in the gastrointestinal mucosa of human immunodeficiency virus type 1-infected patients initiating therapy during primary or chronic infection. *J Virol* 80: 8236-47

173. Kader M, Bixler S, Piatak M, Lifson J, Mattapallil JJ. 2009. Anti-retroviral therapy fails to restore the severe Th-17: Tc-17 imbalance observed in peripheral blood during simian immunodeficiency virus infection. *J Med Primatol* 38 Suppl 1: 32-8
174. Macal M, Sankaran S, Chun TW, Reay E, Flamm J, Prindiville TJ, Dandekar S. 2008. Effective CD4+ T-cell restoration in gut-associated lymphoid tissue of HIV-infected patients is associated with enhanced Th17 cells and polyfunctional HIV-specific T-cell responses. *Mucosal Immunol* 1: 475-88
175. Ivanov, II, Frutos Rde L, Manel N, Yoshinaga K, Rifkin DB, Sartor RB, Finlay BB, Littman DR. 2008. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell Host Microbe* 4: 337-49
176. Mucida D, Kutchukhidze N, Erazo A, Russo M, Lafaille JJ, Curotto de Lafaille MA. 2005. Oral tolerance in the absence of naturally occurring Tregs. *J Clin Invest* 115: 1923-33
177. Curotto de Lafaille MA, Kutchukhidze N, Shen S, Ding Y, Yee H, Lafaille JJ. 2008. Adaptive Foxp3+ regulatory T cell-dependent and -independent control of allergic inflammation. *Immunity* 29: 114-26
178. Gu Y, Yang J, Ouyang X, Liu W, Li H, Bromberg J, Chen SH, Mayer L, Unkeless JC, Xiong H. 2008. Interleukin 10 suppresses Th17 cytokines secreted by macrophages and T cells. *Eur J Immunol* 38: 1807-13
179. Heo YJ, Joo YB, Oh HJ, Park MK, Heo YM, Cho ML, Kwok SK, Ju JH, Park KS, Cho SG, Park SH, Kim HY, Min JK. 2010. IL-10 suppresses Th17 cells and promotes regulatory T cells in the CD4+ T cell population of rheumatoid arthritis patients. *Immunol Lett* 127: 150-6
180. Mjosberg J, Berg G, Jenmalm MC, Ernerudh J. 2010. FOXP3+ regulatory T cells and T helper 1, T helper 2, and T helper 17 cells in human early pregnancy decidua. *Biol Reprod* 82: 698-705
181. Borsellino G, Kleinewietfeld M, Di Mitri D, Sternjak A, Diamantini A, Giometto R, Hopner S, Centonze D, Bernardi G, Dell'Acqua ML, Rossini PM, Battistini L, Rotzschke O, Falk K. 2007. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood* 110: 1225-32
182. Deaglio S, Dwyer KM, Gao W, Friedman D, Usheva A, Erat A, Chen JF, Enjyoji K, Linden J, Oukka M, Kuchroo VK, Strom TB, Robson SC. 2007. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med* 204: 1257-65
183. Fletcher JM, Lonergan R, Costelloe L, Kinsella K, Moran B, O'Farrelly C, Tubridy N, Mills KH. 2009. CD39+Foxp3+ regulatory T Cells suppress pathogenic Th17 cells and are impaired in multiple sclerosis. *J Immunol* 183: 7602-10
184. Giorgi JV, Hultin LE, McKeating JA, Johnson TD, Owens B, Jacobson LP, Shih R, Lewis J, Wiley DJ, Phair JP, Wolinsky SM, Detels R. 1999. Shorter survival in advanced human immunodeficiency virus type 1 infection is more closely associated with T lymphocyte activation than with plasma virus burden or virus chemokine coreceptor usage. *J Infect Dis* 179: 859-70

185. Deeks SG, Kitchen CM, Liu L, Guo H, Gascon R, Narvaez AB, Hunt P, Martin JN, Kahn JO, Levy J, McGrath MS, Hecht FM. 2004. Immune activation set point during early HIV infection predicts subsequent CD4+ T-cell changes independent of viral load. *Blood* 104: 942-7
186. Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA, Wilkinson JE, Galas D, Ziegler SF, Ramsdell F. 2001. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet* 27: 68-73
187. Chatila TA, Blaeser F, Ho N, Lederman HM, Voulgaropoulos C, Helms C, Bowcock AM. 2000. JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic disregulation syndrome. *J Clin Invest* 106: R75-81
188. Wildin RS, Ramsdell F, Peake J, Faravelli F, Casanova JL, Buist N, Levy-Lahad E, Mazzella M, Goulet O, Perroni L, Bricarelli FD, Byrne G, McEuen M, Proll S, Appleby M, Brunkow ME. 2001. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet* 27: 18-20
189. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, Kelly TE, Saulsbury FT, Chance PF, Ochs HD. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet* 27: 20-1
190. Read S, Malmstrom V, Powrie F. 2000. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J Exp Med* 192: 295-302
191. Ramsdell F. 2003. Foxp3 and natural regulatory T cells: key to a cell lineage? *Immunity* 19: 165-8
192. Sakaguchi S. 2003. The origin of FOXP3-expressing CD4+ regulatory T cells: thymus or periphery. *J Clin Invest* 112: 1310-2
193. Thornton AM, Shevach EM. 2000. Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. *J Immunol* 164: 183-90
194. Stephens LA, Mottet C, Mason D, Powrie F. 2001. Human CD4(+)CD25(+) thymocytes and peripheral T cells have immune suppressive activity in vitro. *Eur J Immunol* 31: 1247-54
195. Levings MK, Sangregorio R, Roncarolo MG. 2001. Human cd25(+)cd4(+) t regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. *J Exp Med* 193: 1295-302
196. Wu K, Bi YT, Sun K, Wang CZ. 2007. IL-10-Producing Type 1 Regulatory T Cells and Allergy. *Cell Mol Immunol* 4: 269-75
197. Morgan ME, van Bilsen JHM, Bakker AM, Heemskerk B, Schilham MW, Hartgers FC, Elferink BG, van der Zanden L, de Vries RRP, Huizinga TWJ, Ottenhoff THM, Toes REM. 2005. Expression of FOXP3 mRNA is not confined to CD4+CD25+ T regulatory cells in humans. *Human Immunology* 66: 13-20
198. Wang J, Ioan-Facsinay A, van der Voort EI, Huizinga TW, Toes RE. 2007. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. *Eur J Immunol* 37: 129-38
199. Allan SE, Crome SQ, Crellin NK, Passerini L, Steiner TS, Bacchetta R, Roncarolo MG, Levings MK. 2007. Activation-induced FOXP3 in human T

effector cells does not suppress proliferation or cytokine production. *Int. Immunol.* 19: 345-54
200. Tran DQ, Ramsey H, Shevach EM. 2007. Induction of FOXP3 expression in naive human CD4+FOXP3 T cells by T-cell receptor stimulation is transforming growth factor-{beta} dependent but does not confer a regulatory phenotype. *Blood* 110: 2983-90
201. Pillai V, Ortega SB, Wang CK, Karandikar NJ. 2007. Transient regulatory T-cells: a state attained by all activated human T-cells. *Clin Immunol* 123: 18-29
202. Lim A, French MA, Price P. 2009. CD4+ and CD8+ T Cells Expressing FoxP3 in HIV-Infected Patients Are Phenotypically Distinct and Influenced by Disease Severity and Antiretroviral Therapy. *J Acquir Immune Defic Syndr*
203. Ahmadzadeh M, Antony PA, Rosenberg SA. 2007. IL-2 and IL-15 each mediate de novo induction of FOXP3 expression in human tumor antigen-specific CD8 T cells. *J Immunother* 30: 294-302
204. Popmihajlov Z, Smith KA. 2008. Negative feedback regulation of T cells via interleukin-2 and FOXP3 reciprocity. *PLoS ONE* 3: e1581
205. Billerbeck E, Nakamoto N, Seigel B, Blum HE, Chang KM, Thimme R. 2009. Determinants of in vitro expansion of different human virus-specific FoxP3+ regulatory CD8+ T cells in chronic hepatitis C virus infection. *J Gen Virol* 90: 1692-701
206. Billerbeck E, Blum HE, Thimme R. 2007. Parallel expansion of human virus-specific FoxP3- effector memory and de novo-generated FoxP3+ regulatory CD8+ T cells upon antigen recognition in vitro. *J Immunol* 179: 1039-48
207. Chaput N, Louafi S, Bardier A, Charlotte F, Vaillant JC, Menegaux F, Rosenzwajg M, Lemoine F, Klatzmann D, Taieb J. 2009. Identification of CD8+CD25+Foxp3+ suppressive T cells in colorectal cancer tissue. *Gut* 58: 520-9
208. Kinter AL, Hennessey M, Bell A, Kern S, Lin Y, Daucher M, Planta M, McGlaughlin M, Jackson R, Ziegler SF, Fauci AS. 2004. CD25+CD4+ Regulatory T Cells from the Peripheral Blood of Asymptomatic HIV-infected Individuals Regulate CD4+ and CD8+ HIV-specific T Cell Immune Responses In Vitro and Are Associated with Favorable Clinical Markers of Disease Status. *J. Exp. Med.* 200: 331-43
209. Oswald-Richter K, Grill SM, Shariat N, Leelawong M, Sundrud MS, Haas DW, Unutmaz D. 2004. HIV infection of naturally occurring and genetically reprogrammed human regulatory T-cells. *PLoS Biol* 2: E198
210. Chase AJ, Sedaghat AR, German JR, Gama L, Zink MC, Clements JE, Siliciano RF. 2007. Severe Depletion of CD4+CD25+ Regulatory T Cells from the Intestinal Lamina Propria but not Peripheral Blood or Lymph Nodes During Acute SIV Infection. *J Virol* 81: 12748-57
211. Estes JD, Wietgrefe S, Schacker T, Southern P, Beilman G, Reilly C, Milush JM, Lifson JD, Sodora DL, Carlis JV, Haase AT. 2007. Simian immunodeficiency virus-induced lymphatic tissue fibrosis is mediated by transforming growth factor beta 1-positive regulatory T cells and begins in early infection. *J Infect Dis* 195: 551-61

212. Estes JD, Li Q, Reynolds MR, Wietgrefe S, Duan L, Schacker T, Picker LJ, Watkins DI, Lifson JD, Reilly C, Carlis J, Haase AT. 2006. Premature induction of an immunosuppressive regulatory T cell response during acute simian immunodeficiency virus infection. *J Infect Dis* 193: 703-12
213. Kannanganat S, Kapogiannis BG, Ibegbu C, Chennareddi L, Goepfert P, Robinson HL, Lennox J, Amara RR. 2007. Human immunodeficiency virus type 1 controllers but not noncontrollers maintain CD4 T cells coexpressing three cytokines. *J Virol* 81: 12071-6
214. Amara RR, Smith JM, Staprans SI, Montefiori DC, Villinger F, Altman JD, O'Neil SP, Kozyr NL, Xu Y, Wyatt LS, Earl PL, Herndon JG, McNicholl JM, McClure HM, Moss B, Robinson HL. 2002. Critical role for Env as well as Gag-Pol in control of a simian-human immunodeficiency virus 89.6P challenge by a DNA prime/recombinant modified vaccinia virus Ankara vaccine. *J Virol* 76: 6138-46
215. Allen TM, Vogel TU, Fuller DH, Mothe BR, Steffen S, Boyson JE, Shipley T, Fuller J, Hanke T, Sette A, Altman JD, Moss B, McMichael AJ, Watkins DI. 2000. Induction of AIDS virus-specific CTL activity in fresh, unstimulated peripheral blood lymphocytes from rhesus macaques vaccinated with a DNA prime/modified vaccinia virus Ankara boost regimen. *J Immunol* 164: 4968-78
216. Amara RR, Villinger F, Altman JD, Lydy SL, O'Neil SP, Staprans S, Montefiori DC, Xu Y, Herndon JG, Wyatt LS, Candido MA, Kozyr NL, Earl PL, Smith JM, Ma H-L, Grimm BD, Hulsey ML, Miller J, McClure HM, McNicholl JM, Moss B, Robinson HL. 2001. Control of a Mucosal Challenge and Prevention of AIDS by a Multiprotein DNA/MVA Vaccine. *Science* 292: 69-74
217. Robinson HL, Amara RR. 2005. T cell vaccines for microbial infections. *Nat Med* 11: S25-32
218. Parry RV, Chemnitz JM, Frauwirth KA, Lanfranco AR, Braunstein I, Kobayashi SV, Linsley PS, Thompson CB, Riley JL. 2005. CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. *Mol Cell Biol* 25: 9543-53
219. Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, Gottlieb PA, Kapranov P, Gingeras TR, Fazekas de St Groth B, Clayberger C, Soper DM, Ziegler SF, Bluestone JA. 2006. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med* 203: 1701-11
220. Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A, Solomon M, Selby W, Alexander SI, Nanan R, Kelleher A, Fazekas de St Groth B. 2006. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med* 203: 1693-700
221. Silvestri G, Paiardini M, Pandrea I, Lederman MM, Sodora DL. 2007. Understanding the benign nature of SIV infection in natural hosts. *J Clin Invest* 117: 3148-54
222. Karlsson I, Malleret B, Brochard P, Delache B, Calvo J, Le Grand R, Vaslin B. 2007. FoxP3+CD25+CD8+ T-cell induction during primary SIV infection in cynomolgus macaques correlates with low CD4+ T-cell activation and high viral load. *J Virol*
223. Takahashi T, Tagami T, Yamazaki S, Uede T, Shimizu J, Sakaguchi N, Mak TW, Sakaguchi S. 2000. Immunologic self-tolerance maintained by CD25(+)CD4(+)

regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med* 192: 303-10

224. Shimizu J, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S. 2002. Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. *Nat Immunol* 3: 135-42
225. Bopp T, Becker C, Klein M, Klein-Hessling S, Palmetshofer A, Serfling E, Heib V, Becker M, Kubach J, Schmitt S, Stoll S, Schild H, Staege MS, Stassen M, Jonuleit H, Schmitt E. 2007. Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression. *J Exp Med* 204: 1303-10
226. Vieira PL, Christensen JR, Minaee S, O'Neill EJ, Barrat FJ, Boonstra A, Barthlott T, Stockinger B, Wraith DC, O'Garra A. 2004. IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4+CD25+ regulatory T cells. *J Immunol* 172: 5986-93
227. Levings MK, Sangregorio R, Sartirana C, Moschin AL, Battaglia M, Orban PC, Roncarolo MG. 2002. Human CD25+CD4+ T suppressor cell clones produce transforming growth factor beta, but not interleukin 10, and are distinct from type 1 T regulatory cells. *J Exp Med* 196: 1335-46
228. Cao X, Cai SF, Fehniger TA, Song J, Collins LI, Piwnica-Worms DR, Ley TJ. 2007. Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. *Immunity* 27: 635-46
229. Curti A, Pandolfi S, Valzasina B, Aluigi M, Isidori A, Ferri E, Salvestrini V, Bonanno G, Rutella S, Durelli I, Horenstein AL, Fiore F, Massaia M, Colombo MP, Baccarani M, Lemoli RM. 2007. Modulation of tryptophan catabolism by human leukemic cells results in the conversion of CD25- into CD25+ T regulatory cells. *Blood* 109: 2871-7
230. Josefowicz SZ, Wilson CB, Rudensky AY. 2009. Cutting edge: TCR stimulation is sufficient for induction of Foxp3 expression in the absence of DNA methyltransferase 1. *J Immunol* 182: 6648-52
231. Silvestri G, Sodora DL, Koup RA, Paiardini M, O'Neil SP, McClure HM, Staprans SI, Feinberg MB. 2003. Nonpathogenic SIV infection of sooty mangabeys is characterized by limited bystander immunopathology despite chronic high-level viremia. *Immunity* 18: 441-52
232. Mandl JN, Barry AP, Vanderford TH, Kozyr N, Chavan R, Klucking S, Barrat FJ, Coffman RL, Staprans SI, Feinberg MB. 2008. Divergent TLR7 and TLR9 signaling and type I interferon production distinguish pathogenic and nonpathogenic AIDS virus infections. *Nat Med* 14: 1077-87
233. Stevceva L, Yoon V, Carville A, Pacheco B, Santosuosso M, Korioth-Schmitz B, Mansfield K, Poznansky MC. 2008. The efficacy of T cell-mediated immune responses is reduced by the envelope protein of the chimeric HIV-1/SIV-KB9 virus in vivo. *J Immunol* 181: 5510-21
234. Gougeon ML, Lecoeur H, Dulioust A, Enouf MG, Crouvoiser M, Goujard C, Debord T, Montagnier L. 1996. Programmed cell death in peripheral lymphocytes from HIV-infected persons: increased susceptibility to apoptosis of CD4 and CD8 T cells correlates with lymphocyte activation and with disease progression. *J Immunol* 156: 3509-20

235. Viollet L, Monceaux V, Petit F, Ho Tsong Fang R, Cumont MC, Hurtrel B, Estaquier J. 2006. Death of CD4+ T cells from lymph nodes during primary SIVmac251 infection predicts the rate of AIDS progression. *J Immunol* 177: 6685-94
236. Yen HR, Harris TJ, Wada S, Grosso JF, Getnet D, Goldberg MV, Liang KL, Bruno TC, Pyle KJ, Chan SL, Anders RA, Trimble CL, Adler AJ, Lin TY, Pardoll DM, Huang CT, Drake CG. 2009. Tc17 CD8 T cells: functional plasticity and subset diversity. *J Immunol* 183: 7161-8
237. Zhao Y, Balato A, Fishelevich R, Chapoval A, Mann DL, Gaspari AA. 2009. Th17/Tc17 infiltration and associated cytokine gene expression in elicitation phase of allergic contact dermatitis. *Br J Dermatol* 161: 1301-6
238. Huber M, Heink S, Grothe H, Guralnik A, Reinhard K, Elflein K, Hunig T, Mittrucker HW, Brustle A, Kamradt T, Lohoff M. 2009. A Th17-like developmental process leads to CD8(+) Tc17 cells with reduced cytotoxic activity. *Eur J Immunol* 39: 1716-25
239. Ciric B, El-behi M, Cabrera R, Zhang GX, Rostami A. 2009. IL-23 drives pathogenic IL-17-producing CD8+ T cells. *J Immunol* 182: 5296-305
240. Velu V, Kannanganat S, Ibegbu C, Chennareddi L, Villinger F, Freeman GJ, Ahmed R, Amara RR. 2007. Elevated expression levels of inhibitory receptor programmed death 1 on simian immunodeficiency virus-specific CD8 T cells during chronic infection but not after vaccination. *J Virol* 81: 5819-28
241. Iannello A, Boulassel MR, Samarani S, Debbeche O, Tremblay C, Toma E, Routy JP, Ahmad A. 2010. Dynamics and consequences of IL-21 production in HIV-infected individuals: a longitudinal and cross-sectional study. *J Immunol* 184: 114-26
242. Mascola JR, Lewis MG, Stiegler G, Harris D, VanCott TC, Hayes D, Louder MK, Brown CR, Sapan CV, Frankel SS, Lu Y, Robb ML, Katinger H, Birx DL. 1999. Protection of Macaques against pathogenic simian/human immunodeficiency virus 89.6PD by passive transfer of neutralizing antibodies. *J Virol* 73: 4009-18
243. Baba TW, Liska V, Hofmann-Lehmann R, Vlasak J, Xu W, Ayehunie S, Cavacini LA, Posner MR, Katinger H, Stiegler G, Bernacky BJ, Rizvi TA, Schmidt R, Hill LR, Keeling ME, Lu Y, Wright JE, Chou TC, Ruprecht RM. 2000. Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection. *Nat Med* 6: 200-6
244. Nishimura Y, Igarashi T, Haigwood NL, Sadjadpour R, Donau OK, Buckler C, Plishka RJ, Buckler-White A, Martin MA. 2003. Transfer of neutralizing IgG to macaques 6 h but not 24 h after SHIV infection confers sterilizing protection: implications for HIV-1 vaccine development. *Proc Natl Acad Sci U S A* 100: 15131-6
245. Parren PW, Marx PA, Hessell AJ, Luckay A, Harouse J, Cheng-Mayer C, Moore JP, Burton DR. 2001. Antibody protects macaques against vaginal challenge with a pathogenic R5 simian/human immunodeficiency virus at serum levels giving complete neutralization in vitro. *J Virol* 75: 8340-7
246. Amara RR, Villinger F, Altman JD, Lydy SL, O'Neil SP, Staprans SI, Montefiori DC, Xu Y, Herndon JG, Wyatt LS, Candido MA, Kozyr NL, Earl PL, Smith JM, Ma HL, Grimm BD, Hulsey ML, Miller J, McClure HM, McNicholl JM, Moss B,

Robinson HL. 2001. Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. *Science* 292: 69-74

247. Liu J, O'Brien KL, Lynch DM, Simmons NL, La Porte A, Riggs AM, Abbink P, Coffey RT, Grandpre LE, Seaman MS, Landucci G, Forthal DN, Montefiori DC, Carville A, Mansfield KG, Havenga MJ, Pau MG, Goudsmit J, Barouch DH. 2009. Immune control of an SIV challenge by a T-cell-based vaccine in rhesus monkeys. *Nature* 457: 87-91
248. Gaufin T, Ribeiro RM, Gautam R, Dufour J, Mandell D, Apetrei C, Pandrea I. 2010. Experimental depletion of CD8+ cells in acutely SIVagm-Infected African Green Monkeys results in increased viral replication. *Retrovirology* 7: 42
249. Fossiez F, Djossou O, Chomarat P, Flores-Romo L, Ait-Yahia S, Maat C, Pin JJ, Garrone P, Garcia E, Saeland S, Blanchard D, Gaillard C, Das Mahapatra B, Rouvier E, Golstein P, Banchereau J, Lebecque S. 1996. T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. *J Exp Med* 183: 2593-603
250. Laan M, Cui ZH, Hoshino H, Lotvall J, Sjostrand M, Gruenert DC, Skoogh BE, Linden A. 1999. Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. *J Immunol* 162: 2347-52
251. Kawaguchi M, Kokubu F, Kuga H, Matsukura S, Hoshino H, Ieki K, Imai T, Adachi M, Huang SK. 2001. Modulation of bronchial epithelial cells by IL-17. *J Allergy Clin Immunol* 108: 804-9
252. Jones CE, Chan K. 2002. Interleukin-17 stimulates the expression of interleukin-8, growth-related oncogene-alpha, and granulocyte-colony-stimulating factor by human airway epithelial cells. *Am J Respir Cell Mol Biol* 26: 748-53
253. Boniface K, Bernard FX, Garcia M, Gurney AL, Lecron JC, Morel F. 2005. IL-22 inhibits epidermal differentiation and induces proinflammatory gene expression and migration of human keratinocytes. *J Immunol* 174: 3695-702
254. Brand S, Beigel F, Olszak T, Zitzmann K, Eichhorst ST, Otte JM, Diepolder H, Marquardt A, Jagla W, Popp A, Leclair S, Herrmann K, Seiderer J, Ochsenkuhn T, Goke B, Auernhammer CJ, Dambacher J. 2006. IL-22 is increased in active Crohn's disease and promotes proinflammatory gene expression and intestinal epithelial cell migration. *Am J Physiol Gastrointest Liver Physiol* 290: G827-38
255. Wolk K, Kunz S, Witte E, Friedrich M, Asadullah K, Sabat R. 2004. IL-22 increases the innate immunity of tissues. *Immunity* 21: 241-54
256. Nigam P, Velu V, Kannanganat S, Chennareddi L, Kwa S, Siddiqui M, Amara RR. 2010. Expansion of FOXP3+ CD8 T cells with suppressive potential in colorectal mucosa following a pathogenic simian immunodeficiency virus infection correlates with diminished antiviral T cell response and viral control. *J Immunol* 184: 1690-701