INTRACELLULAR SIGNALING DEFECTS CONTRIBUTE TO TH17 DYSREGULATION DURING HIV INFECTION

by

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DEDICATION

For my brother – I wish you were here to enjoy this with me

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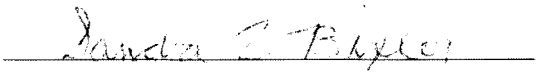
[Signature] 

Sandra L. Bixler

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ABSTRACT

Intracellular Signaling Defects Contribute to Th17 Dysregulation during HIV Infection:

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Thesis directed by: Dr. Joseph J. Mattapallil, Associate Professor, Department of Microbiology and Immunology

Human immunodeficiency virus (HIV) is characterized by profound loss of CD4+ T cells particularly in the gastrointestinal tract, chronic immune activation, and evidence of translocation of microbial products from the gut. Along with the loss of CD4+ T cells, IL-17-producing CD4+ T cells known as Th17 cells are also lost during HIV infection and fail to recover during the course of disease. Th17 cells are an important component of the mucosal immune system as they help maintain the intestinal epithelial barrier and protect against mucosal pathogens. The loss of Th17 cells likely contributes to increased translocation of microbial products and chronic immune activation during HIV infection. Why Th17 cells do not recover during infection is not known. We hypothesized that the failure of Th17 cells to recover during HIV infection is due to the dysregulation of the signaling pathways essential for the generation of Th17 responses. Using the simian immunodeficiency virus (SIV) model, we sought to determine if the Th17 differentiation and transcriptional pathways were altered during infection. Using flow cytometry and relative qRT-pCR, we found that Th17 responses were suppressed very early in acute

infection and persisted into chronic infection. The loss of Th17 cells correlated with increased expression of markers of microbial translocation and immune activation, suggesting that the loss of these cells during infection contributes to pathogenesis. There were no major changes in the expression of Th17-promoting cytokines or their receptors on CD4+ T cells during infection, indicating that the key factors required for differentiation of Th17 cells were not altered during infection. Surprisingly, there was no difference in the levels of phosphorylated STAT3 between infected and healthy animals, yet IL-17 expression remained suppressed suggesting that signaling pathways downstream of STAT3 activation were likely altered during infection. Interestingly, PIAS3, a negative regulator of STAT3 that prevents binding of p-STAT3 to the IL-17 promoter, was elevated during SIV infection. Overexpression of PIAS3 in an IL-17-producing cell line was associated with suppression of IL-17 responses. Our results suggest that elevated PIAS3 levels contribute to the suppression of Th17 cells during HIV and SIV infection.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: Introduction

HUMAN IMMUNODEFICIENCY VIRUS

Identification of HIV as the causative agent of AIDS

Human immunodeficiency virus (HIV) is the causative agent of acquired immunodeficiency syndrome (AIDS) that was first identified as a new syndrome in the early 1980s in the Morbidity and Mortality Weekly Report from the Centers for Disease Control and Prevention (1). The report outlined a cluster of *Pneumocystis carinii* pneumonia cases in Los Angeles, California in several homosexual young men (1). Similar reports of other rare infections and cancers in young homosexual men, as well as blood transfusion recipients and intravenous drug users, were reported in large urban centers across the U.S. (2) suggesting the presence of a previously unidentified immune deficiency (109; 206). All these cases had low CD4 T+ cell counts (109). In 1982, the CDC ascribed the term AIDS to the rash of cases observed across the country (214).

Simultaneously, several research groups began to search for the causative agent of the newly identified immunodeficiency syndrome. In the early 1980s, a novel retrovirus was separately isolated by several different groups from patients with lymphadenopathy and was found to be distinct from the previously identified human retrovirus human T lymphotropic virus (HTLV) (109; 206; 214). As such, the newly identified virus was designated as human immunodeficiency virus (HIV) in 1986 by the International Committee on Taxonomy of Viruses (109). Since then, two major strains of HIV have been identified: HIV-1 and HIV-2 (109; 214). HIV-1 is highly diverse and subdivided into numerous groups and subtypes based on sequence diversity (109). HIV-1 is the more

common type as it is distributed worldwide and is highly infective and pathogenic (109). Distribution of the less pathogenic HIV-2 is mainly restricted to West Africa (109; 214).

The origin of HIV is likely repeated independent cross-species transmission events from non-human primates into humans (117; 118; 237). As HIV-1 is most genetically similar to SIVcpz, it is therefore thought to have originated from chimpanzees (117; 185) while the origin of HIV-2 is thought to be sooty mangabeys based on homology to SIVsmm (118; 237).

Global burden of HIV and AIDS

Since its identification, HIV has risen to become one of the foremost global health threats. To date, over 60 million infections and 25 million deaths have been attributed to HIV (161). The most recent figures from 2012 indicate that over 35 million individuals are infected with HIV worldwide, with the majority of those infections centered in sub-Saharan Africa (161; 206).

As the fourth leading cause of death in the world, HIV is responsible for approximately 1.8 million deaths each year (161). Despite being a significant cause of mortality worldwide, recent data indicates a decline in HIV-related mortality that is associated with an increase in the number of individuals living with the infection (161). Better clinical care and the availability of therapeutic options likely contributed to the improved patient outcome and decline in mortality (161).

HIV structure and binding

HIV is a human retrovirus that belongs to the genus *Lentivrus* and family *Retroviridae* (109; 214). As with all other lentiviruses, infection with HIV is

characterized by a long period of clinical latency, which can last as long as a decade or more in some patients (45; 212; 219).

HIV is a spherical enveloped virus that is ~0.1 μM in size (109; 124). The lipid envelope is derived from the host cell membrane and is coated with the glycoproteins gp120 and gp41 (109; 214; 223; 360; 369). The gp120 glycoprotein binds to the primary receptor for the virus, CD4 (79; 198) whereas gp41 is the fusion protein that mediates fusion between the viral envelope and the cell membrane (109; 214). Receptor engagement by gp120 results in a series of conformational changes that expose the fusion protein gp41, which is inserted in the host cell membrane prior to virus-host cell fusion and entry (109; 116; 148; 214). As such, CD4+ helper T cells serve as the primary target cell for infection by the virus (109; 197; 214; 314). Macrophages and dendritic cells, which have been shown to express low levels of CD4, also serve as an additional reservoir for the virus (11; 74; 109; 123; 200; 214).

Binding of CD4 alone is not sufficient for viral entry; HIV additionally requires one of two co-receptors for entry: CCR5 and CXCR4 (12; 28; 30; 60; 85; 90; 107; 213). Most of the primary isolates of HIV use the CCR5 co-receptor whereas CXCR4 is used as the viruses evolve in the infected host, leading to co-receptor switching (109). Alternative receptors, such as DC-SIGN and GalC, have been shown to play a role in infection of additional cell types, such as dendritic cells (32; 33; 109; 121; 122; 145; 214). HIV viruses are generally designated as either R5 or macrophage-tropic viruses, or X4 or T cell-tropic viruses depending on their co-receptor usage (28; 29; 109; 214; 363).

HIV genome and replication

The HIV virion contains a conical capsid composed of p24, which encases the positive single-stranded RNA viral genome (43; 109; 214). Although the genome is only ~ 9.8 kb in size, it codes for a variety of structural, regulatory, and accessory proteins through RNA splicing and processing of polyproteins (214). Its three primary structural genes – gag, pol, and env – each encode a polyprotein that is further processed into the structural components (109; 214). The Gag polyprotein is cleaved into capsid (CA), matrix (MA), and nucleocapsid (NC) while Pol encodes the three viral reverse transcriptase (RT), protease (PR), and integrase (IN) enzymes (109; 214). The envelope protein gp160 is further cleaved into the gp120 surface and gp41 transmembrane proteins (109; 214).

HIV also encodes several regulatory and accessory proteins. The transactivator (Tat) interacts with the viral long terminal repeat (LTR) at the 5' end of the viral genome to promote viral transcription and replication (49; 109; 181; 187; 214). The RNA regulator Rev regulates viral mRNA expression by enabling unspliced and partially spliced viral mRNA transcripts to be exported from the nucleus and packaged into new virions (65; 86; 106; 109; 141; 214; 361). Four accessory proteins nef, vif, vpu, and vpr, are produced by the virus during replication, which serve as virulence factors and promote viral infectivity (109; 214). Nef promotes infectivity by downregulation of CD4 and major histocompatibility complex (MHC) expression on infected cells (16; 109; 119; 214; 228; 233; 311). Vif counteracts the antiviral defense mechanism APOBEC3G by preventing its incorporation into the virion (109; 214; 313; 357). Vpr aids in viral transactivation and replication by enabling HIV to replicate in non-dividing cells such as macrophages (7; 25; 68; 69; 109; 150; 214; 344). Finally, vpu functions in CD4

degradation, which allows for release of viral progeny from infected cells (109; 214; 260; 332).

Following viral entry and fusion with the host cell, the viral genome enters the cell along with reverse transcriptase, integrase, and other viral proteins (43; 109; 214). Like all other retroviruses, HIV uses the enzyme reverse transcriptase to convert its RNA genome into a dsDNA intermediate, which is subsequently integrated into the host genome by the viral integrase enzyme (109; 214). It is here that the virus may lie dormant for many years and from where its genus name "lentivirus" for slow originates (102). HIV reverse transcriptase lacks editing capabilities and is therefore highly error prone, which contributes to the extensive level of mutation that the virus undergoes (109).

Integrated viral DNA is transcribed by host cell RNA polymerase and is exported from the nucleus with the aid of Rev (106; 109; 214). Viral transcripts may remain unspliced as complete viral genomes, be partially spliced into Gag, Gag-Pol and Env polyproteins, or be fully spliced into transcripts for accessory proteins (47; 109; 214). Translation of viral proteins is mediated by host cell machinery, after which viral proteins and transcripts are transported to the plasma membrane where they assemble into new virions (109; 214). Upon release from the host cell, the HIV virion undergoes a maturation step where proteases cleave the capsid proteins leading to rearrangement of the capsid and rendering the virion infectious (109; 214; 284).

DYNAMICS OF CLINICAL HIV INFECTION

The course of HIV infection can be divided into three broad clinical stages: acute, chronic or latency, and AIDS (137; 206) (**Figure 1**). In humans, the acute stage of infection lasts for several weeks (206). Following transmission, the virus replicates

Figure 1. Changes in mucosal and blood $CD4^+$ T-cell numbers and in viremia level over the course of HIV infection. In the acute phase (left portion), mucosal T cells (purple) are rapidly lost, viremia (green) rapidly rises to a maximum, CD4+ cell counts (blue) decline sharply at first because of trapping in lymphoid tissues but then rise again to a moderately subnormal level. The immune system becomes highly activated (red). In the chronic phase, immune activation steadily increases. Mucosal CD4+ T-cell number remains low, CD4+ T-cell count slowly declines, and viremia rises. As overt AIDS is approached, changes seen in the chronic phase accelerate and the number of mucosal T cells becomes very small.

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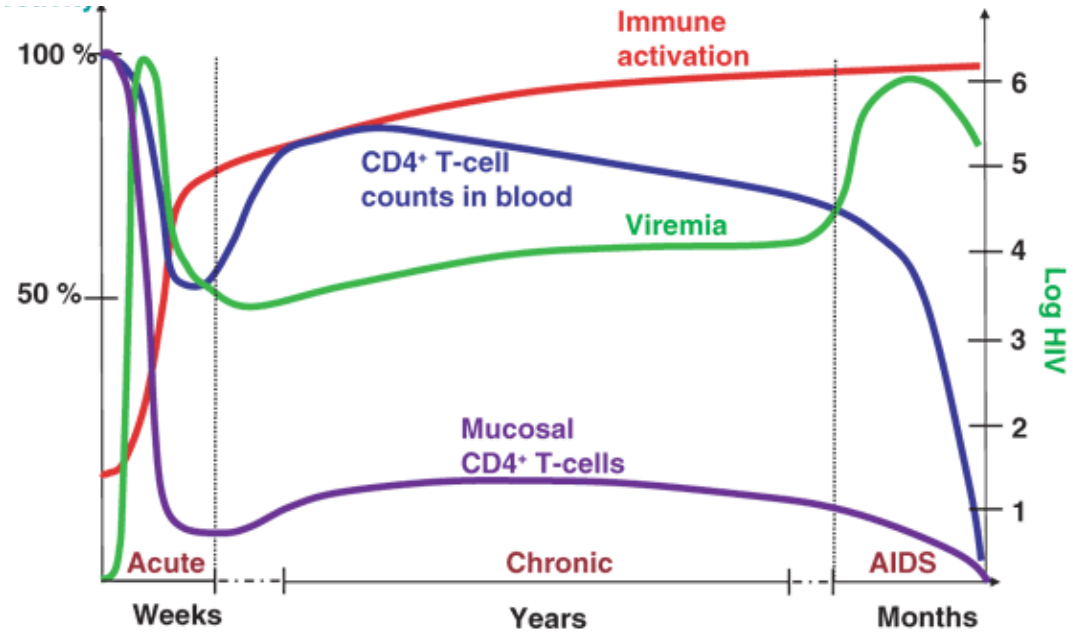

100 %
50 %
Immune
activation
CD4⁺ T-cell
counts in blood
Viremia
Mucosal
CD4⁺ T-cells
Log HIV
6
5
4
3
2
1
Acute
Chronic
AIDS
Weeks
Years
Months

locally in the mucosa, leading to massive infection and destruction of memory CD4+ T cells (206; 214; 251; 364). The virus then disseminates to draining lymph nodes and other lymphoid tissues, where it infects CD4+ target cells (42; 206; 241). Dissemination coincides with a rapid increase in viral loads, which peak at 1-2 weeks after infection (206; 222; 241; 285). Peak viremia also coincides with the onset of patient symptoms, which are often non-specific and flu-like in nature (71; 206; 266; 327).

Following the acute phase of infection, viral loads steadily decrease until they reach a stable level known as set point (162; 206; 241; 297; 305). Viral set point is a delicate balance between viral turnover and the immune response that keeps viral replication checked (37; 206; 214; 241). The viral set point level predicts disease progression, as higher set points are associated with faster progression to end-stage disease (206; 244). Stabilization of viral loads coincides with partial recovery of CD4+ T cells in peripheral blood, but not at mucosal sites (42; 206; 214; 238; 251; 336).

It is at this point that the chronic or latent stage of infection begins (206; 214). During chronic infection, the virus is maintained at set point but still replicates, albeit at lower levels (66; 162; 206; 214; 251; 282; 285; 347). Infection is largely contained in lymphoid tissues with persistent generalized lymphadenopathy and destruction of lymph node architecture (206; 214; 278; 279). Chronic infection is characterized by a progressive decline in CD4+ T cells, which continue to be infected and killed, as well as steady loss of immune control (206). Chronic infection can persist for years or even decades in some patients, depending on host genetic factors, viral set point, and therapy status (206).

When CD4+ T cell counts fall below 200 cells/μl in blood, the patient enters the third and final stage of infection known as AIDS (3; 206). Loss of immune control corresponds with a sharp increase in viremia (206). The clinical portrait in AIDS patients includes cachexia, fever, and diarrhea (3; 206). The acquisition of opportunistic infections is common as the immune response is compromised as a result of CD4+ T cell loss (206). Opportunistic infection can be viral, bacterial, fungal or parasitic in nature; common opportunistic pathogens seen in AIDS patients include CMV, *Mycobacterium tuberculosis*, and *Candida* spp, among others (3; 206). Secondary neoplasms such as Kaposi sarcoma and various lymphomas are common at this stage, as well as neurological disorders (3; 206; 214). In untreated patients, death is often the result of opportunistic infections (206).

TREATMENT OF HIV INFECTION

To date there is no sterilizing cure for HIV and no effective vaccine to prevent transmission of infection. Current patient care guidelines emphasize the use of antiretroviral therapy (ART) in order to keep HIV infection under control (92; 206; 214). There are six classes of antiretrovirals which target different steps in the viral life cycle (206). Reverse transcriptase inhibitors such as the nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) are nucleoside and nucleotide analogs that interfere with reverse transcription (83; 84; 206; 214). Protease inhibitors inhibit protease activity required for assembly of progeny virions (84; 193; 348; 352) whereas fusion inhibitors inhibit viral entry by preventing gp41-mediated fusion (206; 214; 239). Integrase inhibitors interfere with integration of

viral genome into host DNA (144; 206). Co-receptor antagonists bind to CCR5, preventing its interaction with gp120 and subsequent viral entry (88; 103; 206).

Current patient care guidelines recommend the use of several different classes of drugs in combination, known as highly active antiretroviral therapy (HAART) (92; 206). The use of HAART is encouraged to increase effectiveness of therapy and diminish the likelihood of drug resistance developing (92; 206). While antiretroviral therapy is not a sterilizing cure for infection, it reduces viral loads to nearly undetectable levels, leading to a corresponding recovery of CD4+ T cell counts in treated patients (23; 206). However, ART fails to lead to repopulation at mucosal sites (138; 242). HAART has the dual benefit of not only improving patient quality of life, but also reducing viral loads and the subsequent likelihood of transmission to uninfected persons (206).

SIMIAN IMMUNODEFICIENCY VIRUS AS AN ANIMAL MODEL

Limitation of HIV models

Studies involving HIV have been limited by the fact that viral infection and replication is restricted to humans, as the virus does not replicate in other animals (14; 120). Chimpanzees can be infected with HIV-1 in the laboratory setting but the infection is asymptomatic and does not resemble the immunodeficiency that characterizes HIV infection in humans (120). Although the virus does not replicate naturally in rodents (253), several transgenic and humanized mouse models have been developed (109; 151). Transgenic mice, rats, and rabbits expressing the human proteins required for viral entry have been developed, but the virus does not replicate efficiently in these models and disease is absent (44; 91; 186). To more effectively mimic the human immune system, humanized mouse models, in which severe combined immunodeficiency (SCID) mice

have been engrafted with human tissues, have been generated (151) and used to investigate the immune response to infection (109).

SIV as model for primate lentiviruses

The most extensively used animal model for studying HIV in the laboratory is the simian immunodeficiency virus (SIV) model in non-human primates. Simian immunodeficiency viruses encompass a group of lentiviruses that are closely related to HIV (102). SIV was first identified as the causative agent of an AIDS-like illness in rhesus macaques (81).

SIV infection is found to be naturally occurring in many African primate species, including African green monkeys and sooty mangabeys (39; 102; 151). SIV species are designated by their natural primate hosts, with common strains including SIVagm, SIVmac, and SIVsmm (102). Infection of these "natural" non-human primate hosts is generally non-pathogenic in nature with no resulting immunodeficiency (39; 102; 151). Interestingly though, infection of natural hosts with their respective SIVs does lead to high levels of viral replication and infection and loss of CD4+ T cells (39; 102). However, many of the other classic hallmarks of pathogenic infection, such as progressive depletion of CD4+ T cells and immune activation, are absent in these animals (39; 54; 102).

Unlike non-human primates of African origin, Asian origin non-human primates have not co-evolved with their own strains of SIV (39; 102; 151). Infection of these "non-natural" hosts, such as Asian- and Chinese-origin rhesus macaques and pig-tailed macaques, with certain SIV strains is highly pathogenic, ultimately resulting in an immunodeficiency syndrome which mimics the classic characteristics of HIV and AIDS

in humans (102). This includes progressive depletion of CD4+ T cells, progression to end-stage disease, and acquisition of opportunistic infections (39; 42; 102; 217; 238; 336). The SIV strains SIVagm, SIVmac, SIVsmm all cause disease in Asian macaques (102).

Despite the immunological similarities, the SIV model in rhesus macaques deviates from HIV infection in humans in several ways. One of the most obvious and advantageous to research efforts is the difference in disease time course. In humans, chronic infection can last for many years, with progression to end-stage disease and AIDS sometimes taking as long as decades. In non-human primates, the disease course is considerably shorter at 1-2 years, with faster disease progression (331). Additionally, viral set point in SIV-infected macaques tends to be higher than that in humans (331).

Along with SIVmac239 and E660, SIVmac251 is one of the most extensively used strains of SIV for laboratory models of infection in non-human primates. SIVmac251 is a highly pathogenic uncloned strain of SIV derived from an Indian-origin rhesus macaque that developed B-cell lymphoma following serial passage (81). Characteristics of SIVmac251 infection include CCR5 co-receptor usage and infection of memory CD4+ T cells (151). Following infection with SIVmac251, macaques progress to an AIDS-like illness within 1-2 years. SIVmac239 is closely related to SIVmac251, but is a molecular clone derived after serial passage (188). E660 is an uncloned SIV isolate from sooty mangabeys that was passaged in rhesus macaques (160).

The important similarities between SIVmac251 infection in Asian macaques and HIV infection in humans have made SIVmac251 one of the primary models for studying HIV infection and disease (151). Like HIV, SIVmac251 is heterogeneous mix of viral

variants; as such SIVmac251 has been commonly used as a challenge strain in testing potential vaccine candidates and new therapeutic options (151).

MUCOSAL PATHOGENESIS

Mucosal sites are the important sites of entry for the virus, as well as the sites where the most significant damage occurs. This is likely due to the fact that many of the CD4+ T cells at mucosal sites are activated memory T cells that express CCR5, making them ideal targets for viral infection (42; 337; 338). Important mucosal sites for HIV and SIV infection include the gastrointestinal tract, vaginal tract, and the rectal mucosa (102; 214). It is here that most CD4+ T cell in the body reside and massive CD4+ T cell depletion occurs during the acute stage of infection, with nearly all CD4+ T cells infected and lost from these sites (102; 238; 329; 336; 338).

While CD4+ T cells in other peripheral compartments repopulate during the later stages of infection and following HAART, CD4+ T cells at mucosal sites fail to repopulate and remain severely depleted throughout the course of disease (42; 138; 177; 242). It is not known why CD4+ T cells fail to repopulate at mucosal sites during infection and despite long-term HAART. It is possible that ongoing viral replication creates a constant state of lymphocyte activation at mucosal sites, creating new target cells throughout the course of infection (177). Alternatively, other studies have suggested that dysregulation of CD4+ T cell homing to mucosal sites may be responsible for the failure of CD4+ T cell recovery at these sites (240).

The loss of mucosal CD4+ T cells, particularly in the gastrointestinal tract, is accompanied by clinical signs and symptoms of mucosal dysfunction and enteropathy (80; 204). Chronic diarrhea and increased intestinal permeability are common clinical

findings in HIV-infected patients (182; 204; 303). While some cases of diarrhea have infectious etiology associated with opportunistic infections, in other instances no secondary infection can be identified (303). Other clinical symptoms associated with gastrointestinal dysfunction include malabsorption, dehydration, and weight loss (80; 155; 182; 204; 295; 312; 316). As HIV and SIV do not infect the structural components of the gastrointestinal tract, it is thought that the loss of CD4+ T cells at mucosal sites may lead to breakdown of the intestinal barrier (99). Additionally, diarrhea may be due to immune activation and pro-inflammatory cytokines such as IL-6, which is upregulated during infection (99).

IMMUNE ACTIVATION

One of the hallmarks of HIV infection is chronic, systemic, generalized activation of the immune system (89). Immune activation is characterized by increased turnover of cells (156), proliferation and activation (153; 156; 209), and increased production of pro-inflammatory cytokines (19; 330). As activated CD4+ T cells are the primary targets for viral infection, immune activation in the context of HIV infection likely increases the pool of potential target cells for the virus (339), which is continuously infected and depleted. Therefore, immune activation serves as a strong predictor of disease progression in HIV-infected patients (19; 127; 128; 152; 225; 304). In fact, studies have suggested that immune activation may be a more accurate predictor of disease progression than plasma viral loads (19).

Although immune activation is generally considered to be a chronic phenomenon, high levels of activation are also observed early in acute HIV and SIV infection (93; 184; 252; 263; 319). Increased activation can be found in both the innate and adaptive arms of

the immune response, as evidenced by increased T cell activation and production of pro-inflammatory cytokines and chemokines (93; 184; 252; 263; 319).

Furthermore, immune activation in HIV infection has been linked to increased morbidity and mortality. In addition to opportunistic infections, HIV-infected individuals are at a higher risk for other diseases including cardiovascular disease, hypertension, neurocognitive disorders, and osteoporosis (139; 143; 206; 273; 303).

Chronic immune activation likely contributes to the immune dysfunction and pathology observed during infection. The increased turnover of cells accompanied by continuous exposure to viral antigen can promote B and T cell exhaustion during infection (89). Release of pro-inflammatory cytokines such as IL-1β and IL-6 likely contributes to pathology and inflammation observed in infection (143; 303).

Interestingly, chronic immune activation is a hallmark of pathogenic SIV infection of non-natural hosts like rhesus macaques, while it is less apparent in non-pathogenic infection of natural hosts (100; 101; 130; 277; 315). SIV infection of natural hosts results in elevated levels of acute immune activation that do not persist into the chronic phase (100). As CD4+ T cell depletion is not sufficient for the development of AIDS, it has been hypothesized that immune activation may be one of the key factors that contributes to immunodeficiency (89).

Several potential mechanisms have been suggested to explain the high levels of immune activation observed during infection. Immune activation may be due at least in part to direct effects of viral infection and replication, particularly in acute infection. Several studies have shown that the virus is capable of activating the innate immune system (13; 243). Similarly, other studies have suggested that the high antigenic load

present in infection, both viral and concurrent infections, contributes to immune activation (19).

MICROBIAL TRANSLOCATION

Although several mechanisms have been proposed, there is an increasing body of evidence that one of the causes of immune activation may be microbial translocation during infection (41; 63; 89; 303). Microbial translocation refers to translocation of bacteria and their associated products from the lumen of the intestine, where they are typically confined in a healthy state, into systemic circulation. These microbial products, such as lipopolysaccharide (LPS), are pro-inflammatory in nature and capable of stimulating an immune response through toll-like receptor activation (303). In support of a link between microbial translocation and immune activation in HIV infection, studies have shown that increased levels of microbial translocation in infection correlate with increased production of pro-inflammatory cytokines, activation of CD8+ T cells, and enhanced cell proliferation (51; 63; 78; 268; 303).

Markers of microbial translocation

Evidence of microbial translocation is provided by a variety of markers of microbial products that can be detected in the plasma, including soluble CD14 (sCD14), LPS, lipopolysaccharide binding protein (LBP), and endotoxin core antibody (EndoCAb). Some of these markers are bacterial structural and genetic components, such as LPS and bacterial 16S rDNA. LPS constitutes a major structural element of the cell wall of Gram negative bacteria and is known to stimulate the immune system via signaling through toll-like receptor 4 (TLR4) (303). Historically, LPS has been used as a measure of intestinal permeability and translocation in other diseases and conditions, including graft

versus host disease (GVHD), inflammatory bowel disease (IBD), and gastrointestinal surgery (50; 70; 306). However, elevated levels of LPS have not been observed in all studies involving chronic HIV infection (63; 211; 291). Similarly, increased levels of bacterial 16S rDNA have also been detected in HIV infection (173; 205).

Other markers of microbial translocation such as LBP and sCD14 are released in response to LPS. Soluble CD14 is secreted by CD14+ monocytes and macrophages and binds to LPS (157; 208; 303) while LBP is secreted by the gastrointestinal and hepatic epithelial cells in response to LPS (41). Finally, antibodies against the LPS core protein are produced in response to LPS, bind to LPS, and remove it from circulation. EndoCAb titers decrease as LPS is bound and eliminated. Therefore, while elevated levels of LPS, sCD14, and LBP are all indicative of microbial translocation, decreased expression of EndoCAb can alternatively be used as a measure of translocation (41; 46; 304). As LBP, sCD14, and EndoCAb all bind to LPS, this may explain the discrepancies in LPS levels in HIV infection (303).

Microbial translocation in HIV/SIV

A seminal paper by Brenchley *et al* provided strong evidence of microbial translocation in HIV infection as indicated by increased levels of LBP and sCD14 (41). Increased levels of microbial translocation were linked to immune activation in both the innate and adaptive compartments of the immune system (41). Like immune activation, microbial translocation appears to be a function of pathogenic infections, as SIV infection of natural hosts such as sooty mangabeys does not result in increased levels of plasma LPS or sCD14 (41; 303) and these animals maintain an intact mucosal epithelium with little evidence of immune activation (101).

Microbial translocation during HIV infection is likely the result of the breakdown of the intestinal barrier (**Figure 2**) (101; 303). In healthy individuals, an intact mucosal barrier restricts microbial products from entering systemic circulation. Immunohistochemistry studies in SIV-infected macaques have shown evidence for breaks in the mucosal barrier in the intestine during infection (101). More importantly, bacterial products such as LPS are co-localized with these mucosal breaches (101). Decreased tight junction formation and disruption of enterocytes by apoptotic mechanisms have been hypothesized to result in increased permeability and damage to the mucosal barrier during infection (20; 97; 101; 258; 303).

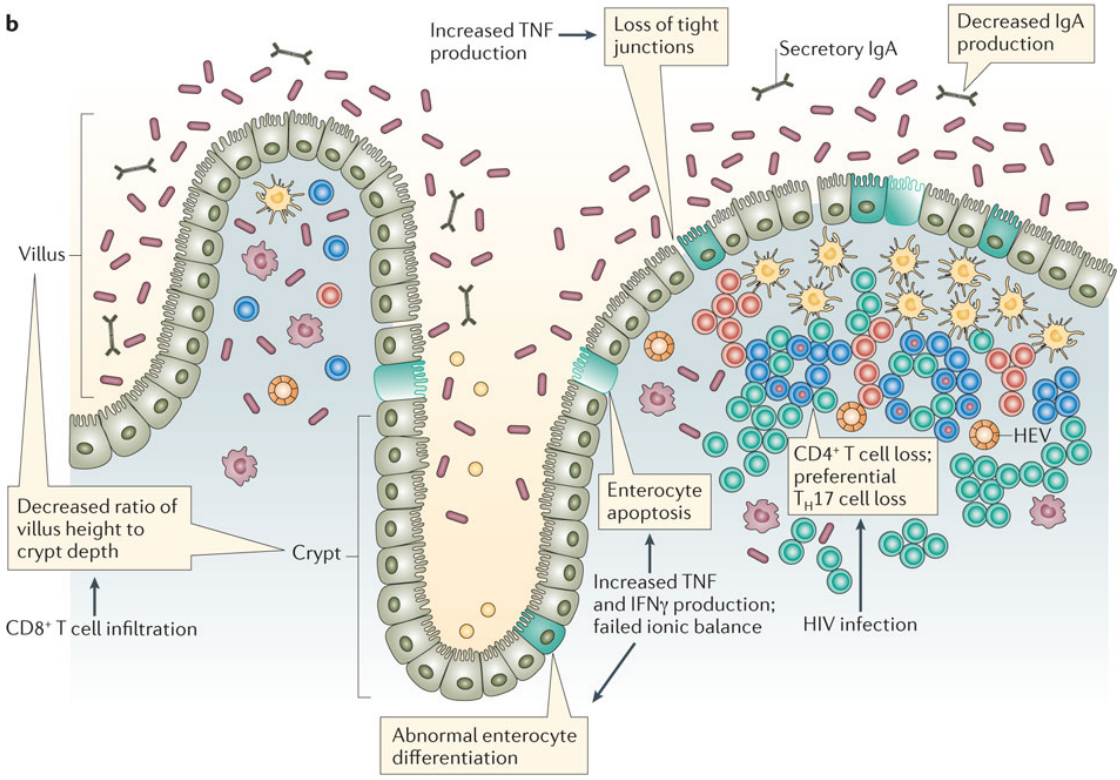
The clinical relevance of immune activation and microbial translocation is evident in several human studies in which elevated levels of microbial translocation were linked to disease progression (15; 234; 268). In a study which examined various biomarkers from 379 HIV-infected patients, sCD14 was positively correlated with viral loads and negatively correlated with CD4 counts, suggesting that viral infection and subsequent loss of CD4+ T cells in fact drives microbial translocation (234). Increased expression of sCD14 negatively correlated with CD4 counts and along with increased levels of LPS, correlated with faster clinical progression of HIV-infected patients (234).

T-HELPER-17 CELLS

Characterization of Th17 cells

T-helper-17 (Th17) cells are a recently identified subset of CD4+ T helper cells that play a distinct role in the immune response from their Th1 and Th2 counterparts (146; 281; 298; 321). They were identified as a novel subset of T helper cells based on their generation under anti-Th1 and –Th2 polarizing conditions (146; 281; 298; 321).

Figure 2. The intestinal epithelium in an HIV-infected individual. The villus height in the intestine of HIV-infected individual decreases with an increase in crypt depth, and this has been associated with CD8+ T cell infiltration. The decreased villus height/crypt depth ratio may be the result of abnormal enterocyte differentiation and enterocyte apoptosis, which may be caused by failure of the cells to maintain ionic balance and by increased production of interferon-γ (IFNγ) and tumour necrosis factor (TNF). Increased TNF production may also lead to the destruction of the tight junctions. B cell dysfunction may contribute to decreased luminal IgA concentrations, and HIV infection of CD4+ T cells is likely to drive the loss of CD4+ T cells, particularly TH17 cells, from the GALT. Lower IgA levels and TH17 cell loss may allow bacterial overgrowth, which may also contribute to increased microbial translocation. The continued presence of the high endothelial venules (HEVs) suggests there is not an anatomical abnormality preventing T cells from trafficking into the GALT.


b
Increased TNF production
Loss of tight junctions
Secretory IgA
Decreased IgA production
Villus
Decreased ratio of villus height to crypt depth
Crypt
CD8+ T cell infiltration
Enterocyte apoptosis
Increased TNF and IFNγ production; failed ionic balance
Abnormal enterocyte differentiation
CD4+ T cell loss; preferential TH17 cell loss
HIV infection
HEV
Nature Reviews | Microbiology

Th17 cells are CD4+ T cells that express the memory marker CD95 (5; 131), as well as CD26, CD161 and interleukin-4-inducible gene (18; 26; 73; 199; 230; 265). Th17 cells also express a variety of chemokine receptors such as CCR4, CCR6, CCR9, and the mucosal homing receptor α4β7, which likely promote the homing of Th17 cells to mucosal sites (5; 18; 178; 240).

Th17 cells were initially identified as a pro-inflammatory subset in the context of experimental autoimmune encephalitis (EAE), a multiple sclerosis model in mice (31; 210). Th1 cells were initially hypothesized to be the primary effector cells that mediated EAE symptoms and myelin degeneration; surprisingly however, EAE severity increased in response to ablation of Th1 cells (108). Subsequent experiments identified an IL-17-producing subset of CD4+ T cells that appeared to be the primary effector cell in EAE (108; 132; 362).

Role of Th17 cells in the immune response

Th17 cells preferentially produce pro-inflammatory cytokines, including IL-17, IL-22, and IL-21 (146; 218; 281; 298; 321). The IL-17 family has a number of isoforms such as IL-17A or IL-17, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F (115; 254). However, only IL-17 and IL-17F are known to be secreted by Th17 cells (115; 254). Although IL-17 and IL-17F share approximately 50% homology and likely share overlapping functions, IL-17 is considered to be the prototypic cytokine of the IL-17 family and demonstrates more potent activity than IL-17F (115; 254).

Through their release of IL-17 which serves as a chemoattractant, Th17 cells promote the recruitment of neutrophils to sites of infection (207; 249). IL-17, along with

IL-21 and IL-22, also promotes the release of antimicrobial peptides such as defensins from epithelial cells, particularly in mucosal settings (22; 180; 218).

The crucial role for Th17 cells in the control of extracellular pathogens is evident in hyper-IgE syndrome or Job's Syndrome which is characterized by high circulating levels of IgE and a paucity of Th17 cells (133; 247). Clinically, Job's Syndrome is distinguished by severe recurrent bacterial and fungal infections of the mucosa and skin (133; 247).

Th17 cells are enriched at mucosal sites (21; 40; 170) where they are thought to play an important role in preservation of the mucosal epithelial barrier at such sites as the gastrointestinal tract. Studies have shown that IL-17 upregulates expression of proteins claudin-1, claudin-2, and zona occludens-1, thereby promoting tight junction formation between epithelial cells at mucosal sites (75; 192). The Th17-associated product IL-22 also enhances the mucosal barrier by promoting enterocyte homeostasis (286).

Th17 cells likely play a dual role in the immune response as a result of their pro-inflammatory activity. The critical roles they play in the immune response to bacterial and fungal pathogens, as well as in mucosal immunity, clearly indicate a protective role for Th17 cells in the host defense (256). However, their pro-inflammatory nature has proven to be detrimental in the setting of many autoimmune and inflammatory diseases, such as asthma and allergy (271), psoriasis (215; 227), and inflammatory bowel disease (110; 201). It is likely that the tenor of a Th17 response may be dependent on careful regulation and balance with other components of the immune system, such as regulatory T cells.

TH17 CELLS IN HIV AND SIV INFECTION

Th17 dynamics in infection

Th17 cells, like other CD4+ T cells, are depleted during HIV and SIV infection (40; 52; 104; 175; 178; 245; 288; 302). The rapid loss of Th17 cells, particularly in the gastrointestinal tract, begins during the acute stage of infection and persists into chronic infection (40; 52; 95; 104; 175; 178). Interestingly, Th17 cells were not depleted at other mucosal sites such as the lungs (40). Studies have shown an increase in IL-17 responses during the early phase of SIV infection (48; 176). As CD4+ T cells are depleted at this time, innate immune cells such as natural killer cells and γδ T cells (48; 353) are likely the source of IL-17 at these time points.

Unlike other CD4+ T cells that repopulate the periphery following acute infection, Th17 cells specifically remain depleted throughout the entire course of HIV and SIV infection and therapy (40; 52; 104; 175). Importantly, other CD4+ T cells such as regulatory T cells and Th1 cells are present during the chronic phase of infection, suggesting that the loss of IL-17-producing CD4+ T cells is selective (40; 104).

Th17 cells, microbial translocation and immune activation in infection

Due to their role in maintaining immunity and structural integrity at the mucosal barrier, the loss of Th17 cells in HIV and SIV infection has been linked to mucosal damage, microbial translocation, and immune activation (52; 101; 104; 195; 229; 290). Work by Raffatellu *et al* (290) provided key evidence for the role of Th17 loss in driving microbial translocation and immune activation. This study demonstrated that the loss of IL-17 responses in the gastrointestinal tract during SIV infection promoted translocation of *Salmonella* from the intestine into systemic circulation (290).

Interestingly, the loss of Th17 cells appears to be a feature of pathogenic infections of non-human primates as Th17 cells are preserved in SIV infection of natural hosts (40). SIV infection of natural non-human primate hosts does not result in mucosal barrier damage (101) or high levels of chronic immune activation that accompany pathogenic infections (40; 303). Maintenance of the Th17 subset in SIV infection of natural hosts suggests that the loss of this subset is a critical component of pathogenesis.

Role of Th17 cells in the immune response to HIV and SIV infection

It is unclear what role, if any, Th17 cells play in the antiviral immune response to HIV infection. Virus-specific Th17 cells are detectable during the early stages of infection (359) but do not persist into the later stages of infection (40; 359). In other viral infections such as hepatitis C (55), virus-specific Th17 cells are thought to play a detrimental role through their contributions to immunopathology and inflammation (236). However, the primary cytokine product of Th17 cells, IL-17, has been associated with clearance of virus and protection from viral challenge in rotavirus and influenza models (142; 317).

Effect on therapeutic interventions on Th17 cells in infection

The effect of antiretroviral therapy on Th17 dynamics during HIV and SIV infection remains controversial. While several studies have found a lack of Th17 repopulation in HAART-treated patients (95; 131), other studies reported that ART might lead to only partial or incomplete repopulation of Th17 cells during infection (62; 112; 154; 229; 240). Incomplete restoration may be due to an overall failure of CD4+ T cell repopulation, which occurs in approximately 20% of HIV patients undergoing therapy (112). Other studies have implicated a role for mucosal reconstitution and/or homing in

Th17 repopulation. Macal *et al* suggested that Th17 repopulation may be dependent on achieving a sufficient level of total CD4+ repopulation in mucosal tissues (229). Defective homing likely plays a role as Mavinger *et al* reported that the homing of a subset of CCR9+β7+CD4+ T cells to the gastrointestinal tract, of which Th17 cells are known to be a part, are defective (240).

The development of pro- and pre-biotic regimens in recent years may improve Th17 repopulation in the context of anti-retroviral therapy. While HAART alone was not successful in achieving Th17 repopulation, concurrent treatment with prebiotics/probiotics led to repopulation of Th17 cells at mucosal sites, in addition to improved overall CD4+ repopulation and mucosal immunity (77; 194). The repopulation of Th17 cells was accompanied by increased numbers of IL-23-producing cells (194), which may promote maintenance and development of Th17 cells.

However, there is debate about the effect of prebiotic/probiotic therapy on immune activation and microbial translocation. One study found the use of prebiotics/probiotics in HIV infection to result in lower levels of microbial translocation and inflammation (129) while another found no discernible effect of combined probiotic treatment on immune activation or microbial translocation in the context of HIV infection (310).

DIFFERENTIATION AND DEVELOPMENT OF TH17 CELLS

The development of the Th17 helper subset requires various cytokines and signaling effectors. As Th17 cells are memory CD4+ T cells (5; 131), differentiation of a naïve CD4+ T cell into a Th17 cell is first initiated by recognition of antigen by the T cell receptor in the presence of the appropriate co-stimulatory signals (159; 322). The specific

cytokine milieu present directs the T cell differentiation pathway towards a specific lineage i.e. Th1, Th2, etc.

Role of TLRs in Th17 differentiation

Studies conducted using mice have recognized four cytokines as being critical for the skewing of T cells towards a Th17 phenotype: interleukin (IL)-6, IL-21, IL-23, and TGF-β. The production of several of these Th17-promoting cytokines results from activation of toll-like receptors (TLRs) on antigen-presenting cells (APCs) by pathogen-associated molecular patterns (PAMPs) (159; 322). The particular PAMPs present and TLRs that are activated are critical in determining the nature of the cytokine response. Activation of a wide variety of TLRs, including TLR1/2, TLR3, TLR4, TLR7/8, and TLR9, have all been shown to play a role in Th17 development (27; 159; 183; 296; 322; 340).

While TLRs have classically been considered in the context of antigen-presenting cells, there is increasing evidence that T cells themselves express their own TLRs. Th17 cells have been shown to express TLR2, with TLR2 signaling leading to enhanced expression of IL-17 and other Th17 products (296).

TLR engagement by pathogen-associated motifs results in recruitment of adaptor proteins, leading to activation of the signaling cascade and culminating in cytokine production. The adaptor protein MyD88, which is utilized by all TLRs with the exception of TLR3, has been shown to be important in promoting Th17 cells (111). In addition to TLRs, other signaling molecules expressed on APCs, such as dectin-1 and DC-SIGN, can also lead to enhanced Th17 induction (5; 134-136; 333).

Differentiation of Th17 cells

Differentiation of a Th17 cell can be divided into 3 stages: initial differentiation, amplification, and maintenance (**Figure 3**). Studies conducted in mice and humans have suggested that there may be some species differences in the differentiation requirements for Th17 cells. Based on the results of differentiation studies in mice, the initial stage of Th17 differentiation requires two cytokines, IL-6 and TGF-β (31; 232; 341). Human cells, however, appear to have less stringent cytokine requirements as IL-1β appears to be a key cytokine, and pairing it with any other of the four is sufficient to induce a Th17 response (4; 231; 351).

Interleukin-6 and development of Th17 cells

IL-6 was initially identified as a critical factor for Th17 development in a series of mouse studies in which IL-6 signaling was inhibited (264; 322; 341). Through either IL-6 antibody treatment (322; 341) or expression of a mutant version of the IL-6R component gp130 (264), Th17 development failed to occur in the absence of IL-6 signaling. In Th17 differentiation, IL-6 signaling leads to activation of the Th17-transcription factors STAT3 and RORγt, resulting in transcription of IL-17 and other Th17-associated genes (264; 355; 367). Two of these genes, IL-21 and IL-23R, help promote Th17 development in the subsequent amplification and maintenance steps of the differentiation pathway (367).

TGF-β and development of Th17 cells

The requirement for TGF-β in Th17 differentiation is interesting, considering its typical role as an anti-inflammatory cytokine important for T regulatory cells and immunosuppression. The key appears to lie in the concentration of TGF-β present, as high levels of TGF-β skew cells away from a Th17 phenotype and towards regulatory T

Figure 3. Th17 differentiation pathway. TH17-cell differentiation is initiated by the activation of STAT3, which induces the expression of IL-21 and cooperates with transforming growth factor-β (TGFβ) signalling to induce the expression of retinoic-acid-receptor-related orphan receptor-γt (RORγt), IL-17 and IL-23R, and STAT3 activation is attenuated by IL-2-induced STAT5. IL-21 and IL-23 drive the production of IL-17 and IL-22 and TH17-cell differentiation. These pathways also induce epigenetic remodelling at genes that encode lineage-restricted transcription factors and cytokines to facilitate heritable patterns of gene expression and lineage commitment. IFNGR, IFNγ receptor; NK, natural killer.

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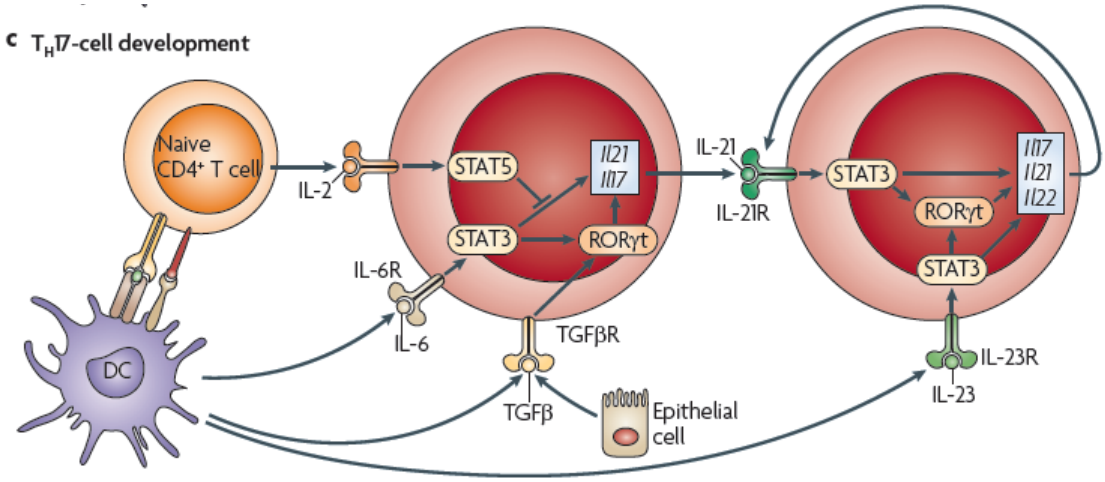
c T$_H$17-cell development
Naive CD4+ T cell
IL-2
STAT5
Il21
Il17
STAT3
RORγt
IL-21
IL-21R
STAT3
Il17
Il21
Il22
RORγt
STAT3
IL-6R
IL-6
TGFβR
TGFβ
Epithelial cell
DC
IL-23R
IL-23

cell development through induction of the T regulatory (Treg)-transcription factor FoxP3 (172; 368). Conversely, low concentrations of TGF-β combined with IL-6 or other Th17-promoting cytokines induces Th17 development through activation of the Th17-lineage specific transcription factor RORγt (172; 368). TGF-β may also promote Th17 development through inhibition of SOCS3, a negative regulator of Th17 cells (289). Knockout of TGF-β, either through genetic manipulation in mice (216; 232) or treatment with anti-TGF-β antibodies, resulted in decreased Th17 differentiation and IL-17 production (289).

IL-21 and development of Th17 cells

Following initial differentiation of a naïve T cell into a Th17 cell, the second stage of development is amplification of the Th17 phenotype. Amplification is facilitated by the autocrine common gamma chain cytokine IL-21, which is produced by Th17 cells following initial differentiation (172; 235; 269). Through a positive feedback mechanism, IL-21 signals through the heterodimeric complex of IL-21R and common gamma chain subunits expressed on Th17 cells, stimulating additional expression of IL-17 (235; 269; 346; 367). In addition to enhancing IL-17 production, IL-21 promotes the Th17 phenotype by inhibiting components of other differentiation pathways such as FoxP3 and IFNγ, thereby skewing away from a Treg and Th1 response respectively, and towards a Th17 response (163; 235; 269; 346). IL-21 also induces upregulation of IL-23R for later stages of differentiation (367).

The role of IL-21 in Th17 development has been elucidated in mouse studies in which a lack of IL-21 signaling, either through IL-21-deficiency or IL-21R deficiency, resulted in a failure of Th17 cells to develop, even after stimulation with IL-6 (235; 269;

346). However, recent studies have demonstrated that IL-21 may be dispensable for Th17 differentiation, as IL-6 signaling appeared to be sufficient to induce Th17 differentiation in IL-21 and IL-21R knockout mice (72; 318).

IL-23 and development of Th17 cells

The pro-inflammatory cytokine IL-23 is responsible for the final stage of Th17 differentiation, long-term maintenance. IL-23 is a heterodimeric cytokine composed of the IL-12p40 and IL-23p19 subunits (165) and is produced by antigen-presenting cells such as macrophages and dendritic cells in response to specific PAMPs (5; 125; 333).

While IL-23 was not required for initial polarization of Th17 cells, studies found that it was required for later stages of development including survival and maintenance (6; 87; 146; 172; 210; 232; 281; 325). Expression of IL-23R is limited to activated memory T cells (280), which provides additional evidence that IL-23 is dispensable for early differentiation steps (355; 367). In fact, initial differentiation of Th17 cells leads to upregulated expression of IL-23R (355). In the absence of IL-23 signaling, Th17 cells failed to continuously produce IL-17 in long-term culture experiments (325). Studies have shown that IL-23R is expressed in lamina propria T cells only in the presence of the Th17-promoting cytokine IL-6 (171), while other studies have indicated that IL-23R was induced by RORγt and IL-21 (355; 367). The exact mechanism by which IL-23 promotes differentiation of Th17 cells is unknown. However, it has been hypothesized that IL-23 may either provide proliferative signals or promote epigenetic modifications that provide permanence to the phenotype (87; 172).

Alternative differentiation pathways may exist for Th17 cell development. Studies have suggested that some plasticity in the Th17 induction pathway may exist. For

example, one study found that IL-21 could be substituted for IL-6 in Th17 differentiation experiments as IL-21 and TGF-β appeared to be sufficient to induce IL-17 in mice lacking IL-6 (203). Another study found that TGF-β and IL-23 were sufficient to induce IL-17 expression in the absence of IL-6 (367).

Transcriptional pathways in Th17 cells

Signaling by Th17-promoting cytokines leads to activation of transcriptional pathways that ultimately result in production of IL-17. The central pathway to Th17 induction involves the transcription factor signal transducer and activator of transcription-3 (STAT3) (158; 270). Binding of Th17-promoting cytokines, such as IL-6, to their cognate receptors initiates the Janus kinase (JAK)/STAT signaling cascade (**Figure 4**). Following ligand-receptor interaction, receptors dimerize which bring JAKs, associated with the intracellular domains of the receptors, into close proximity. JAKs then perform a series of three phosphorylation events, the first being auto-phosphorylation. Following auto-phosphorylation, JAKs phosphorylate sites on the intracellular domains of the receptors, providing a docking site for STAT molecules. Once docked, STATs are phosphorylated by JAKs. Phosphorylated STAT molecules subsequently dimerize, translocate to the nucleus, and bind to the promoter regions of target genes, inducing their transcription.

STAT3

During Th17 differentiation, IL-6, IL-21, and IL-23 have all been shown to induce activation of the transcription factor STAT3 (57; 172; 346). The kinases JAK1 and Tyk2 have been shown to phosphorylate STAT3 and be important for IL-17 induction (140; 248). In addition to IL-17, activation of STAT3 induces transcription of a

Figure 4. Signal transduction from the ligand binding to STAT activation.

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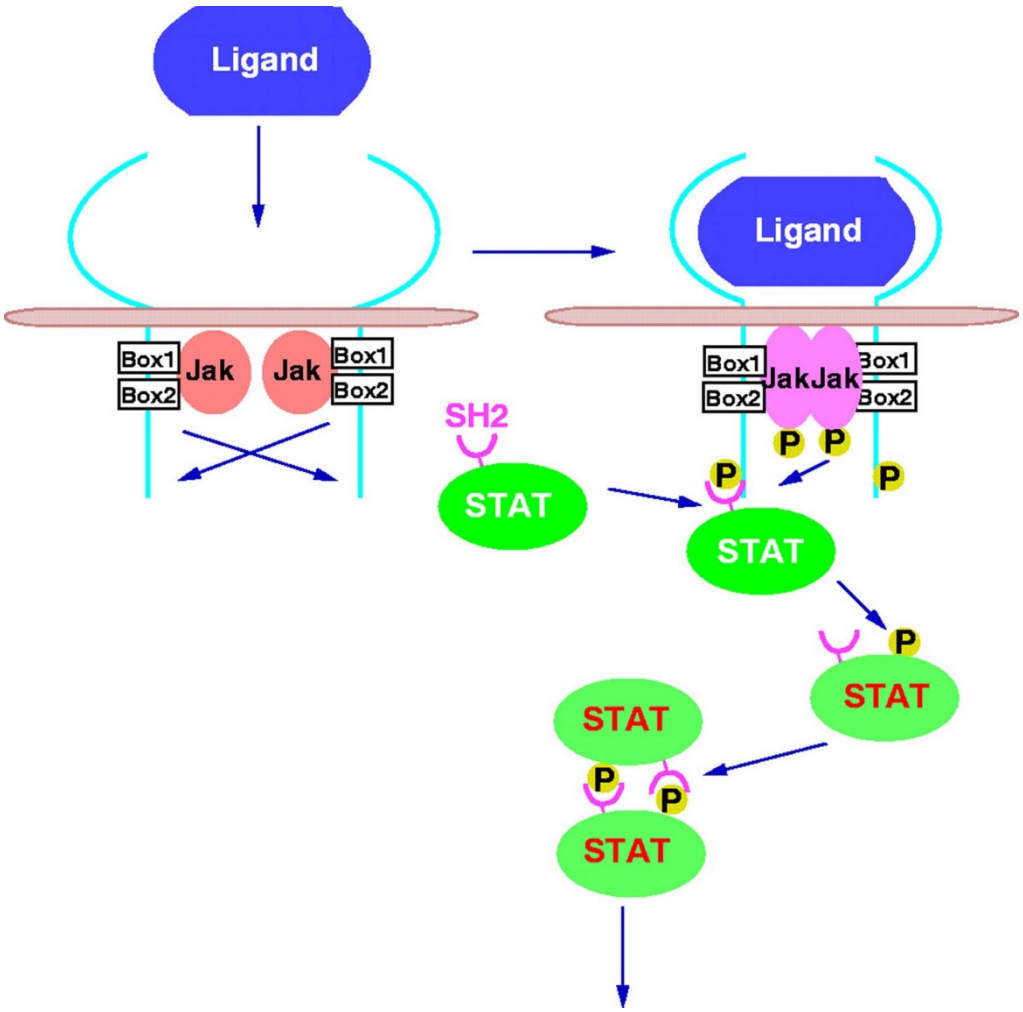
Ligand
Box1
Box2
Jak
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Box1
Box2
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Box1
Box2
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Box2
P
P
P
P
SH2
STAT
STAT
P
STAT
STAT
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P
STAT

number of additional Th17-associated genes and products, including IL-21, IL-22, and IL-23R (269; 355; 367). STAT3 binds directly to the IL-21 and IL-17 promoters to activate transcription (56; 346).

The importance of STAT3 as a critical signaling mediator in the Th17 induction pathway is evident from both human and mouse studies. Th17 development was inhibited in mice lacking STAT3 (147; 355). Patients with Job's syndrome (Hyper IgE syndrome) suffer from a myriad of immunological deficiencies, among them a lack of IL-17 production (133; 247). Studies have shown this is due to a mutation in STAT3 rendering it non-functional (82; 247). As a result of this, IL-17 responses are significantly impaired in these patients, and they suffer from serious recurrent bacterial and fungal infections which they are unable to control (133; 247).

STAT3 also induces expression of the Th17-lineage specific transcription factor RORγt (172; 269; 355; 356; 367). RORγt binds to ROR-dependent enhancer elements in the conserved non-coding sequence (CNS)-2 upstream of the IL-17A promoter (356). Following induction by Th17-promoting cytokines such as TGF-β, IL-6, and IL-21 (171; 269; 367), RORγt also binds directly to the IL-17 promoter to induce transcription of IL-17 (169). RORγt overexpression has been additionally shown to upregulate other Th17-associated genes such as IL-22 and IL-23R (171). The importance RORγt in Th17 development has been demonstrated in studies in which RORγt knockout resulted in reduced IL-17 production *in vitro* and reduced numbers of Th17 cells in the lamina propria of mice (171; 269; 367).

Regulation of the STAT3 pathway

Several proteins namely, suppressor of cytokine signaling-3 (SOCS3), protein tyrosine phosphatase (SHP2), and protein inhibitor of activated STAT3-3 (PIAS3), are known to negatively regulate the STAT3 signaling pathway (**Figure 5**). Each of these regulatory proteins targets a different step in the STAT3 activation pathway that ultimately prevents STAT3-mediated induction of downstream target genes. Expression of these regulatory proteins likely serves as a feedback mechanism to help temper the degree of STAT3 activation.

SOCS3

The negative regulator SOCS3 prevents STAT3 activation by acting at one of the earliest steps in the activation pathway: phosphorylation. SOCS3 binds to JAK and prevents phosphorylation and activation of STAT3 (94). Expression of SOCS3 is induced by several of the same cytokines that promote Th17 development, namely IL-6, IL-21, and IL-23 (56; 289). However, one of the important roles of TGF-β in Th17 development appears to be inhibition of SOCS3, thereby enabling activation of the STAT3 pathway (289).

The role of SOCS3 in Th17 differentiation is apparent from studies using conditional knockdown of SOCS3 in targeted T cell populations that resulted in expansion of Th17 cells (56). Similarly, siRNA knockdown of SOCS3 resulted in increased expression of Th17-associated transcription factors and ultimately enhanced Th17 development (289). Conversely, Th17 differentiation was impaired under conditions in which SOCS3 was overexpressed (326).

Figure 5. Regulation of the STAT3 signal transduction pathway. SOCS proteins inhibit or terminate JAK/STAT signals by binding to tyrosine-phosphorylated JAKs and/or cytokine receptors and targeting them for degradation. The STAT3signal can also be attenuated by PIAS3, a member of the protein inhibitors of activated STATs (PIAS) family of proteins. PIAS3 binds selectively to activated STAT3 dimers and blocks their ability to activate gene transcription.

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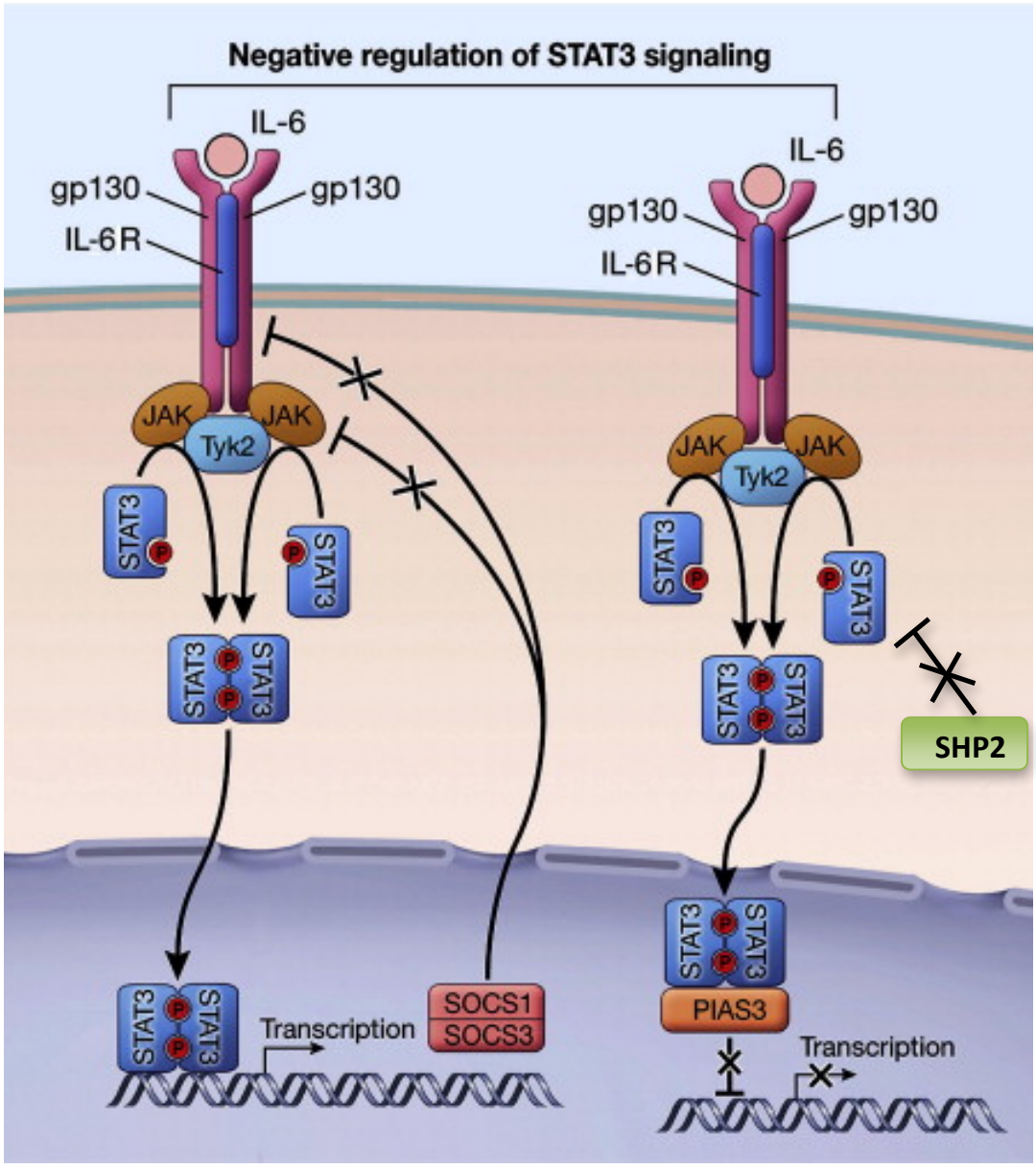

Negative regulation of STAT3 signaling
IL-6
gp130
gp130
IL-6R
JAK
JAK
Tyk2
STAT3
STAT3
STAT3
STAT3
Transcription
SOCS1
SOCS3
IL-6
gp130
gp130
IL-6R
JAK
JAK
Tyk2
STAT3
STAT3
STAT3
STAT3
SHP2
STAT3
STAT3
PIAS3
Transcription

SHP2

The protein tyrosine phosphatase SHP2 also prevents activation of STAT3 by interfering with phosphorylation. However, instead of preventing phosphorylation, it catalyzes the removal of the phosphate group from activated STAT3 (94; 272). Binding of IL-6 to the IL-6R results in recruitment of SHP2 to the gp130 domain of the receptor, where it dephosphorylates STAT3 (189; 272).

PIAS3

The negative regulator PIAS3 acts at one of the final steps in the STAT3 signaling pathway. PIAS3 binds to phosphorylated STAT3 (p-STAT3) and prevents it from binding to the promoter of IL-17 and other target genes The COOH-terminal acidic region of PIAS3 has been shown to interact with STAT3 and interfere with the transactivation of STAT3 (36). Importantly, Th17 cells have been found to lack expression of PIAS3 mRNA (257). siRNA-mediated inhibition of PIAS3 results in expansion of Th17 cells, which leads to exacerbation of EAE in mice (257). Taken together, these data suggest that PIAS3 is an important regulator of Th17 development. According to Akira *et al* (9), "The overall strength of STAT signaling for any given cell type may be largely influenced by the relative levels of STAT and PIAS3 protein expression."

TH17 DEVELOPMENTAL PATHWAYS IN HIV AND SIV INFECTION

Th17-promoting cytokines in HIV and SIV infection

It is not known whether the signaling pathways that are important in Th17 differentiation are altered during HIV and SIV infection. Several studies have shown that IL-6 (34; 96; 320) and TGF-β (226; 294) are both upregulated during infection. HIV

infection is characterized by high levels of IL-6, which is often used as a biomarker for chronic immune activation in HIV infection (267). Increased levels of TGF-β may be explained in part due to the expansion of regulatory T cells during infection (17; 59; 98; 191; 262), which are known to be a critical source of the cytokine. Additionally, IL-23 transcript levels were found to be elevated during the early stages of infection in the gastrointestinal tract (178).

The effect of HIV and SIV infection on expression of IL-21 is unclear. CD4+ T cells that produce IL-21, which include Th17 cells, are lost very early during the course of infection (166; 167; 245; 275). The loss of IL-21-producing CD4+ T cells may be compensated for by increased production of IL-21 from other cell types, namely CD8+ T cells, which have been shown to express IL-21 during infection (245; 274; 276; 323; 349). However, other studies have shown IL-21-producing CD4+ T cells are induced during acute and chronic HIV infection (358).

Receptors for Th17-promoting cytokines in HIV and SIV infection

There is little known about the expression of Th17-promoting cytokine receptors during HIV and SIV infection. Previous studies suggest that IL-6R is likely elevated during HIV infection based on sIL-6R expression (164) and CD126 (IL-6Rα) expression on monocytes, CD4+ T cells, and B cells during infection (334). Memory B cells also appear to express high levels of IL-21R during HIV infection (301). Conversely, studies have found that CD4+ T cells that express IL-23R are lost from the gastrointestinal tract during HIV infection (40). However, not much is known about the effect of HIV infection on expression of TGF-β receptor.

Th17 transcriptional pathways in HIV and SIV infection

There is evidence to suggest that the intracellular signaling pathways that are central to Th17 induction may be altered in HIV and SIV infection. High levels of STAT3 activation have been observed in the gastrointestinal tract during SIV infection, with the primary sources of p-STAT3 being macrophages and CD3+ T cells (250). As CD4+ T cells are depleted in the gastrointestinal tract during infection (238; 336), it is most likely that CD8+ T cells are the source of p-STAT3. The high levels of STAT3 activation are indicative of the pro-inflammatory environment and generalized immune activation that characterizes infection.

To date, no studies have examined the effect of PIAS3, SHP2, and SOCS3 on the STAT3 pathway in Th17 cells during SIV infection. While PIAS3 has not been examined in the context of HIV and SIV infection, studies on SOCS3 and SHP2 are limited. Studies have shown that the recruitment of SHP2 was increased in response to HIV signaling through two different mechanisms – CCR5 and C-type lectins as well as by gp120 binding (24).

SOCS3 expression has been previously examined in CD4+ T cells in the context of HIV infection; interestingly, while SOCS3 transcript levels were elevated, protein levels were decreased (246; 250). Several studies have shown that increased SOCS3 expression may be beneficial to the virus and detrimental to overall patient outcome. Elevated levels of SOCS3 are associated with increased permissiveness to infection at mucosal-associated lymphoid tissue sites (255) as well as with enhancement of HIV replication (8). Finally, elevated SOCS3 levels in patients infected with HIV/HCV also has been linked to a failure to respond to antiretroviral therapy (190).

Additionally, it appears that RORγt expression is likely unaltered during HIV infection. When the expression of various lineage-specific transcription factors was examined in the gastrointestinal tract of HIV-infected patients, RORγt expression remained constant in spite of infection (300). However, the ratio of FoxP3 to RORγt increased in infected patients, which may reflect the skewing towards a Treg phenotype during infection (300).

POTENTIAL MECHANISMS FOR TH17 LOSS IN HIV AND SIV INFECTION

Viral infection

The cause for the failure of Th17 cells to repopulate during HIV infection is not known. A role for direct viral involvement and subsequent depletion has been investigated. As they express the necessary receptor CD4 and co-receptor CCR5 that are required for viral entry, Th17 cells are capable of being infected by the virus (40). However, studies have shown that Th17 cells are not preferentially infected over other CD4+ T cell subsets such as Th1 cells (40). Other studies have suggested that direct viral infection is responsible for loss of Th17 cells in HIV-infected patients (95). Interestingly, other IL-17-producing subsets such as natural killer cells and CD8+ T cells are also lost in infection (113; 195; 261; 292); as these cells are not infected by HIV or SIV, it is likely that mechanisms other than direct viral involvement are responsible for the loss of these cells.

Regardless of the mechanisms contributing to Th17 depletion, there is substantial evidence to suggest that the extent of viral infection in patients is linked to the loss of Th17 cells. Several studies have shown that detection of IL-17 expression in HIV-infected patients was dependent on the degree of HIV infection, with IL-17 only present

in patients with plasma viral loads that remained nearly undetectable (259). Similarly, Th17 populations appear to be maintained in HIV patients who are long-term nonprogressors (62).

Indoleamine deoxygenase

One of the potential mechanisms that has been proposed to explain the failure of Th17 cells to develop is through indoleamine deoxygenase (IDO). IDO is the rate-limiting enzyme of the tryptophan catabolism pathway and is produced by macrophages and dendritic cells in response to TLR stimulation (105). Expression of IDO is upregulated during HIV and SIV infection (38; 104; 105; 149; 292), with high levels of IDO expression in HIV infection promoting regulatory T cell development through expression of the Treg transcription factor FoxP3 (179). Conversely, Th17 cells and expression of RORc, which serves as a master controller of RORγt expression, are inhibited in the presence of high levels of IDO (179).

Regulatory T cells

Other studies have suggested a role for regulatory T cells in the failure of Th17 cells to recover during infection. While the level of Tregs has been shown to be decreased in peripheral blood along with other CD4+ T cells (59; 288), expansion of Treg populations occurs in lymph nodes and mucosal tissues during HIV infection (17; 59; 98; 191; 262). Expansion of regulatory T cells during HIV infection is likely due to a combination of factors, including homeostatic response to immune activation, increased Treg development in the thymus, or prolonged survival of Tregs (59).

Numerous studies have demonstrated that the ratio of regulatory T cells to Th17 cells is altered in HIV infection (114; 202; 220; 309; 328). One such study showed that

the ratio of Th17 cells to Tregs is lower in HIV-infected patients and patients undergoing anti-retroviral therapy, and unchanged in elite controllers (38).

Whether the loss of Th17 cells allows for an expansion of regulatory T cells or whether high levels of Tregs may skew T cell development away from Th17 cells is not known (256). The developmental pathways that promote Th17 and Treg development are thought to be mutually exclusive and antagonistic as factors that promote Treg development are inhibitory to Th17 cells and vice versa (87; 172). The Treg transcription factor FoxP3 interacts with the Th17 transcription factor RORγt to inhibit Th17 differentiation (356; 368).

Loss of CD103+ dendritic cells

Other potential mechanisms to explain the failure of Th17 cells to recover include the loss of other supporting cell types that are believed to be important for IL-17 responses, namely CD103+ dendritic cells. CD103+ dendritic cells are lost during SIV infection and were found to be critical for promoting IL-17 and IL-22 responses (195). Upregulation of IL-17 and other Th17-associated genes occurred in response to co-culture of CD103+ DCs with naïve T cells (195). Interestingly, these studies also found a loss of other IL-17 responses, including natural killer cells that produce IL-17 (195).

CD103+ DCs are believed to promote Th17 development through their production of retinoic acid, a byproduct of vitamin A metabolism (256; 308). Retinoic acid (RA) may alter the composition of the gastrointestinal microbiome, thereby influencing Th17 differentiation in the GALT (53). Other studies suggested that RA might promote the homing of Th17 cells to the gastrointestinal tract through regulation of

the homing receptors CCR9 and α4β7 (342). Finally, RA leads to upregulation of the Th17 transcription factor RORγt, which is important for expression of IL-17 (195).

Loss of IL-21-producing cells

Similarly, other studies have suggested that the loss of IL-21-producing cells during infection may promote Th17 depletion during infection (245). IL-21 is thought to amplify the Th17 phenotype by a positive feedback mechanism, leading to increased production of IL-17. Although Th17 cells themselves are an important source of IL-21, the authors found that non-Th17 CD4+ T cells were the primary source of IL-21 and that these cells were lost, but not preferentially infected, during pathogenic SIV infection (245). Interestingly*, in vivo* treatment of SIV-infected rhesus macaques with recombinant IL-21 resulted in restoration of Th17 responses (245).

STATEMENT OF PROBLEM

Although several potential mechanisms have been suggested to explain the lack of Th17 cells during HIV infection, the true cause remains unknown. The majority of the mechanisms that have been previously examined in HIV and SIV infection involve extracellular factors, such as supporting cell types and inhibitory metabolic products. Few studies have sought to examine the developmental and intracellular pathways in these cells. Although it is not clear whether the exact induction pathways required for Th17 cells to develop properly are altered during infection, studies that have examined Th17-promoting cytokines in other contexts appear to suggest that a lack of abundance of these cytokines is likely not the source of the dysregulation observed in infection.

Although much has been gleaned in recent years about the requirements and development of these cells in humans and in mice, there have been few studies to-date

that have examined T cell differentiation in non-human primates. This study will provide important contributions to the current understanding about Th17 development in general, in addition to non-human primates.

The loss of Th17 cells in HIV infection is thought to be a critical event for pathogenesis that facilitates microbial translocation and immune activation during infection. Both phenomena are linked to faster disease progression and increased patient mortality. Understanding the mechanism(s) by which dysregulation of Th17 responses occurs during HIV infection is the first step in assessing how to restore this immune subset and its valuable contributions to the immune response, ultimately leading to improved patient prognosis.

HYPOTHESIS

Our hypothesis is that HIV infection alters the regulation of signaling pathways required for the development of Th17 responses. Using the Th17 differentiation pathway as a guide, we will first examine whether external factors required for differentiation, such as cytokine signaling, are altered during infection. Additionally, we will assess whether intracellular signaling pathways are dysregulated during infection.

SPECIFIC AIMS

Specific Aim 1) Characterize Th17 dysregulation during acute and chronic SIV infection

Subaim 1A: Determine the dynamics of peripheral and mucosal Th17 cells during acute and chronic SIV infection

Subaim 1B: Examine if Th17 dysregulation is associated with microbial translocation and immune activation during SIV infection

Specific Aim 2) Determine if Th17 dysregulation during SIV infection is due to a paucity of Th17-promoting cytokines and their receptors

Subaim 2A: Characterize expression of Th17-promoting cytokines during SIV infection

Subaim 2B: Determine if expression of Th17-promoting cytokine receptors on CD4+ memory T cells is altered during SIV infection

Subaim 2C: Characterization of *in vitro* Th17 differentiation in rhesus macaques

Specific Aim 3) Examine if altered intracellular signaling is the cause for Th17 dysregulation during SIV infection

Subaim 3A: Determine if the expression and activation of STAT3 are altered during SIV infection

Subaim 3B: Examine if factors that negatively regulate Th17 cells are altered during SIV infection

CHAPTER 2: Materials and Methods

ANIMALS, INFECTION, AND SAMPLES

Indian-origin rhesus macaques (*Macaca mulatta*) housed at Bioqual Inc., and Advanced Bioscience Laboratories seronegative for SIV, simian retrovirus (SRV), and simian T-cell leukemia virus (STLV)-1 were used in these studies. The animals were housed in accordance with the American Association for Accreditation of Laboratory Animal Care guidelines. Animals were infected with pathogenic SIVmac251 intravenously whereas samples from healthy animals were used as controls. For the long-term ART (LT) samples, animals were treated daily with a combination of PMPA (9-(R)-[2-(phosphonomethoxy)propyl]adenine) and FTC (emtricitabine) for 13 weeks. PMPA and FTC were obtained from Gilead Sciences, Inc. (Foster City, CA)

Peripheral blood samples were collected at acute and chronic stages of infection. Acute time points included days 0 (n=6), 7 (n=6), 14 (n=6), and 35 (n=6) post infection whereas chronic samples were obtained at necropsy from animals that had been infected for at least one year (n=8). Jejunum and mesenteric lymph node samples were obtained at necropsy from chronically infected and uninfected animals. Peripheral blood mononuclear cells (PBMC) were obtained from peripheral blood by density gradient centrifugation. Enzymatic digestion and subsequent Percoll gradient centrifugation were used to isolate total cells from the jejunum as described previously (238). Mesenteric lymph node cells were isolated by grinding and pushing tissue through 0.45 uM filters as described previously (238). Isolated cells were washed, counted and either used fresh or cryopreserved for later use.

VIRAL LOADS

Plasma viral loads were determined by real-time reverse transcription PCR (RT-PCR) as previously described (64). Briefly, viral RNA was isolated from plasma by centrifugation and chemical dissociation of RNA from protein-nucleic acid complexes. Viral RNA was reversed-transcribed in a thermocycler to generate cDNA, which was subsequently used as a template for real-time PCR.

ANTIBODIES AND FLOW CYTOMETRY

All antibodies used in this study (**Table 1**) were titrated using rhesus macaque PBMC and used in different combinations for the various experiments.

To assess the dynamics of Th17 cells in healthy and infected animals, total PBMC and jejunal cells that had been previously cryopreserved were stimulated for 4 hours with a combination of 50 ng/mL phorbol mystriate acetate (PMA) (Sigma-Aldrich, St. Louis, MO), 500 ng/mL ionomycin (Sigma-Aldrich), and Brefeldin-A (BD Biosciences). Cells were harvested and stained with CD4-Pacific Blue, CD8-Alexa Fluor 700, CD95-FITC, and CD45RA-Texas Red PE. After washing, cells were fixed and permeabilized in Cytofix/Perm buffer (BD Biosciences) and stained with anti-CD3-Cy7APC and anti-IL-17-Alexa Fluor 647. Labeled cells were fixed in 0.5% PFA and analyzed using a Becton Dickinson (BD) LSR II instrument. Th17 cells were identified by their expression of CD4 and the capacity to produce IL-17 in response to PMA stimulation.

To determine levels of immune activation during infection, expression of HLA-DR and Ki-67 on CD4+ and CD8+ T cells was assessed by flow cytometry. Cryopreserved total PBMC were labeled with CD4-PE, CD8-Alexa Fluor 700, CD95-Pacific Blue, and HLA- DR-Texas Red PE. After washing, cells were fixed and

Table 1. Antibodies used in flow cytometry experiments.

Antibody	Clone	Company	Experiment
CD3	SP34.2	BD Biosciences (San Diego, CA)	IL-17 dynamics Immune activation CD4+ memory sort Total CD4+ sort purity Naive CD4+ sort purity
CD4	OKT4	BD Biosciences (San Diego, CA)	CD4+ memory sort Total CD4+ sort purity Naive CD4+ sort purity IL-17 dynamics Immune activation
CD8	RPA-T8	BD Biosciences (San Diego, CA)	IL-17 dynamics Immune activation CD4+ memory sort Total CD4+ sort Naïve CD4+ sort
CD14	M5E2	BD Biosciences (San Diego, CA)	Total CD4+ sort Naive CD4+ sort CD14+ expression on monocytes CD14+ sort
CD20	2H7	Biolegend (San Diego, CA)	Total CD4+ sort Naive CD4+ sort
CD28	CD28.2	BD Biosciences (San Diego, CA)	CD4+ memory sort
CD45RA	2H4LDH11LDB9	Beckman Coulter (Brea, CA)	IL-17 dynamics
CD95	DX2	BD Biosciences (San Diego, CA)	IL-17 dynamics CD4+ memory sort Naive CD4+ sort Immune activation
HLA-DR	Immu-357	Beckman Coulter (Brea, CA)	Immune activation
IFNγ	B27	BD Biosciences (San Diego, CA)	Th17 differentiation
IL-17	eBio64CAP17	eBioscience (San Diego, CA)	IL-17 dynamics Th17 differentiation
Ki-67	B56	BD Biosciences (San Diego, CA)	Immune activation

APC, allophyocyanin
FITC, fluorescein isothiocyanate
PE, phycoerythrin
PB, Pacific Blue
A647, Alexa Fluor 647
TR-PE, Texas Red PE

permeabilized in Cytofix/Perm buffer (BD Biosciences) and stained with anti-CD3-Cy7APC and anti-Ki67-FITC. Labeled cells were analyzed using a BD LSR II instrument.

To examine expression of receptors for Th17-promoting cytokines, memory CD4+ T cells were positively sorted from cryopreserved PBMC using fluorescence-activated cell sorting (FACS). Total PBMC were stained with a combination of anti-CD3-Cy7APC, anti-CD4-APC, anti-CD8-Alexa 700, anti-CD95-FITC, anti-CD28-Cy5PE, and the live-dead marker and amine-reactive dye VIVID to exclude dead cells. Live CD3+CD4+CD95+ memory T cells were sorted using a BD FACSAria sorter based on expression of CD95 and CD28 to over 95% purity (**Figure 6**). Sorted cells were used in a relative qRT-PCR assay to determine Th17-promoting cytokine receptor expression.

For Western blots and relative RT-PCR experiments for acute infection, live total CD4+ T cells were negatively sorted from fresh total PBMC by FACS using the following panel: CD8-Pacific Blue, CD14-Pacific Blue, and CD20-Pacific Blue. A negative gating strategy was utilized in order to avoid activating the cells through CD3 and CD4. VIVID was included to exclude dead cells from our analysis. Live CD4+ T cells were sorted as negative for CD8, CD14, CD20 and VIVID. CD4 purity was confirmed by staining a small aliquot of sorted cells with anti-CD3-Cy7APC and anti-CD4-APC (**Figure 7**).

To assess if the capacity of CD4+ T cells to produce IL-17 was altered during acute infection, sorted CD4+ T cells were seeded into a 96 well tissue culture plate at a density of 5×10^5 cells per well. Cells were stimulated with human recombinant IL-6

Figure 6. Gating strategy used to sort memory CD4+ T cells from total PBMC by FACS sorting. Total PBMC were labeled with anti-CD3, anti-CD4, anti-CD8, anti-CD95, anti-CD28 and VIVID. Memory CD4+ T cells were identified based on expression of CD95 and CD28, with memory cells expressing CD95 (287)

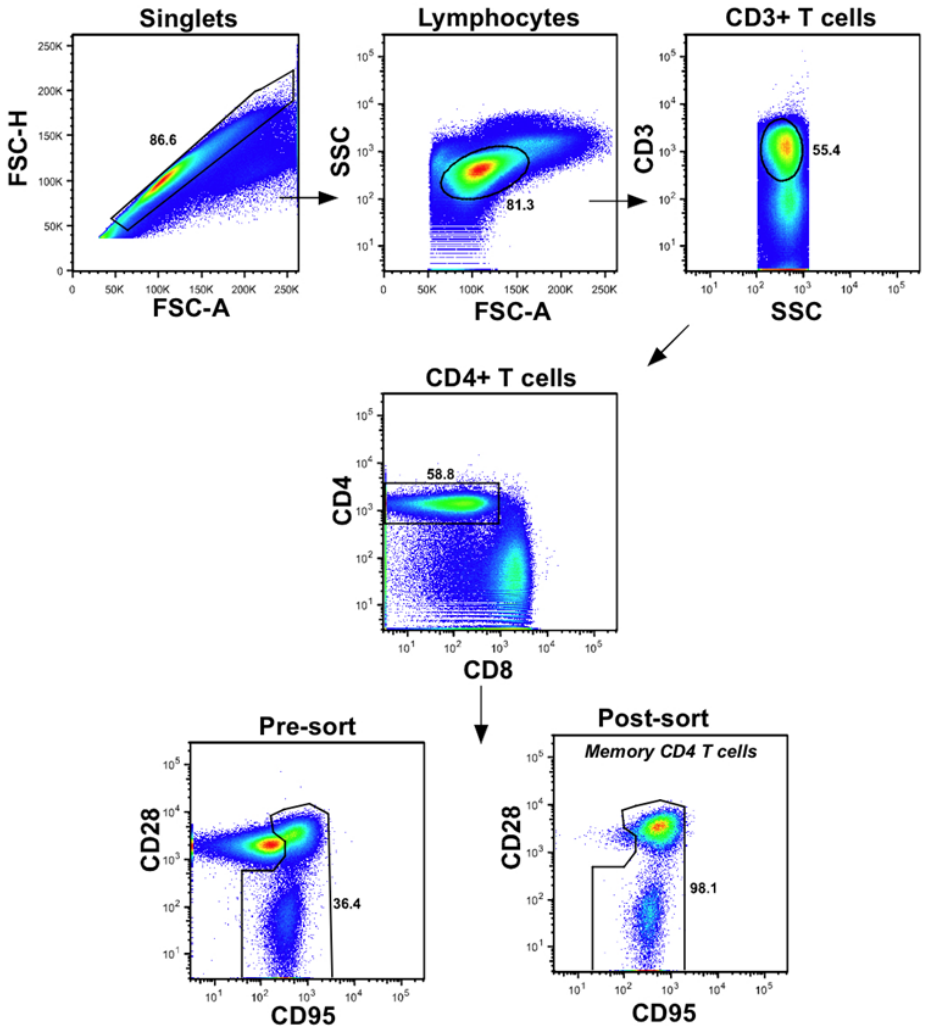
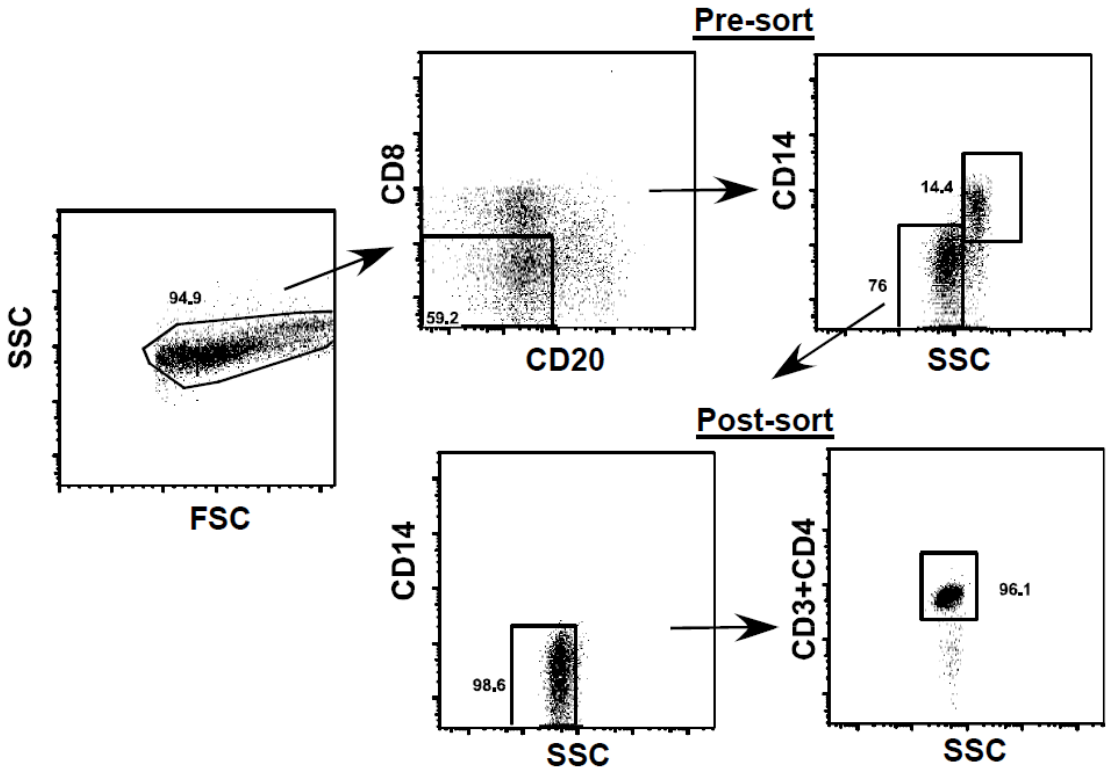

Singlets
FSC-H
FSC-A
86.6
Lymphocytes
SSC
FSC-A
81.3
CD3+ T cells
CD3
SSC
55.4
CD4+ T cells
CD4
CD8
58.8
Pre-sort
CD28
CD95
36.4
Post-sort
Memory CD4 T cells
CD28
CD95
98.1

Figure 7. Gating strategy used to sort total CD4+ T cells from peripheral blood using FACS sorting. Total PBMC were labeled with anti-CD20, anti-CD14, and anti-CD8. CD4+ T cells were negatively sorted by excluding CD20+/CD14+/CD8+ cells. Purity was confirmed by staining an aliquot of sorted cells with anti-CD3 and anti-CD4 antibodies. FSC, forward scatter, SSC, side scatter.

Previously published as (35)


Pre-sort
SSC
94.9
FSC
CD8
59.2
CD20
CD14
14.4
76
SSC
Post-sort
CD14
98.6
SSC
CD3+CD4
96.1
SSC

(rIL-6) (R&D Systems) in serum-free RPMI 1640 for 15 minutes at 37˚C as previously described (283). Unstimulated cells were set up simultaneously as negative controls.

CD14+ monocytes were positively sorted from cryopreserved PBMC at days 7 and 35 p.i., and used for relative qRT-PCR for *ex vivo* analysis of TLR4 and IL-23 expression (**Figure 8**).

RELATIVE QRT-PCR

RNA was isolated from cells using an RNeasy isolation kit (Qiagen Sciences, Gaithersburg, MD). Isolated RNA was treated with Ambion TurboDNase (Applied Biosystems, Austin, TX) to eliminate DNA contamination, which was confirmed by PCR for the housekeeping gene β-actin. Purified RNA was reverse transcribed using a Superscript III First Strand Synthesis kit (Invitrogen, Carlsbad, CA) to generate cDNA that was used to determine the relative expression of Th17 cytokines, cytokine receptors, and other associated genes by TaqMan qPCR using the ABI 7500 instrument (Applied Biosystems). TaqMan qPCR was performed using high-fidelity Platinum Taq polymerase (Invitrogen) with *Macaca mulatta*-specific primers and probes (**Table 2**) designed using Primer-3 software (299). Expression of the following genes was assessed: IL-17, IL-6, IL-21, IL-23, TGF-β, IL-6R, IL-21R, IL-23R, TGF-βR, STAT3, SOCS3, PIAS3, SHP2 and β-actin. Data were analyzed using the 2-CT (ddCT) method with ABI 7500 software and fold differences were calculated as previously described (307).

ELISA

Plasma levels of soluble CD14 (sCD14) (R&D Systems) and lipopolysaccharide (LPS) binding protein (LBP) (Cell Sciences) were assessed using commercially available

Figure 8. Gating strategy used to sort CD14+ monocytes. Total PBMC were labeled with anti-CD14 and sorted based on CD14 expression by FACS (pre-sort). Purity of CD14+ cells was assessed following sorting (post-sort).

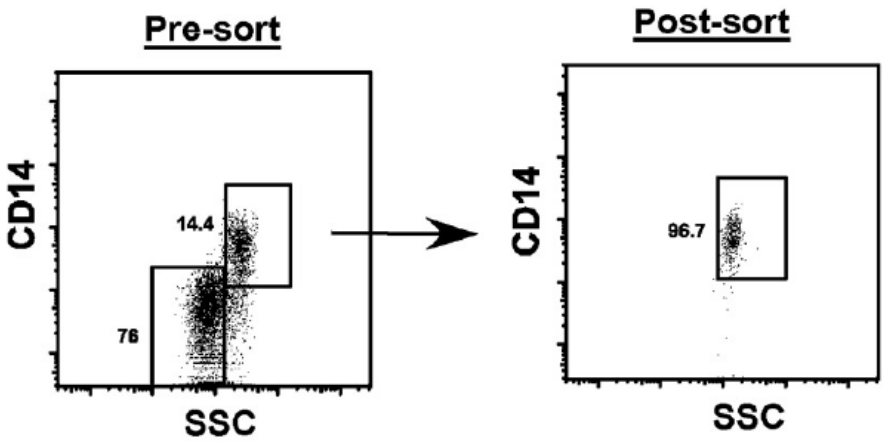

Pre-sort
Post-sort
CD14
SSC
14.4
76
96.7

Table 2. Primer and probe sequences used for qRT-PCR. All primers and probes were designed using the rhesus macaque genome and Primer3 software (299)

Gene	Primers and Probe
IL-17A	Forward : ACCAATCCCAAAAGGTCCTC Reverse: TCTCTCAGGGTCCTCATTGC Probe: FAM- CAACCGATCCACCTCACCTTGG-BHQ1
STAT3	Forward: GGAGGAGTTGCAGCAAAAAG Reverse: GATTCTCTCCTCCAGCATCG Probe: FAM-CCCCATTGTACAGCACCGGC-BHQ1
PIAS3	Forward: ACCATTGCCCTTCTATGAAGTC Reverse: AGGTAAAGTGCGCTTCCTCA Probe: FAM-ACCACCCTTGCATCCACTTCTA-BHQ1
SHP2	Forward: ATATGGCGTCATGCGTGTTA Reverse: TCCGTATTCCCTTGTCCAAC Probe: FAM-TGTCAAAGAAAGTGCTGCTCATGA-BHQ1
SOCS3	Forward: TTCTACTGGAGCGCAGTGAC Reverse: CTGTCGCGAATCAGAAAGGT Probe: FAM-AGGCGAACCTGCTGCTCAGC-BHQ1
IL-6	Forward: ATGCAATAACCACCCCTGAA Reverse: AAGAGCCCTCAGGTTGGACT Probe: FAM-TGCTGACGAAGCTGCAGGCA-BHQ1
IL-21	Forward: TGTGAATGACTTGGACCCTGAA Reverse: AAACAGGAAATAGCTGACCACTCA Probe: FAM- TCTGCCAGCTCCAGAAGATGTAGAGACAAACT-BHQ1
IL-23p19	Forward: CCAGCAGCTTTCACAGAAGC Reverse: TCTTAGATCCATGTGTCCCACT Probe: FAM-TGGCCTGGAGTGCACATCCA-BHQ1
TGFβ	Forward: TGTCATAGATTTCGTTGTGGGTTT Reverse: GTACAACAGCACCCGCGAC Probe: FAM-ACCATTAGCACGCGGGTGACCTCC-BHQ1
TLR4	Forward: CCTTTCAGCTCTGCCTTCAC Reverse: CACCTTTCGGCTTTTATGGA Probe: FAM-ATTCCCGGTGTGGCCATTGC-BHQ1
IL-6R	Forward: GGAGCAAGCTCAGCAAAACT Reverse: ACGGCAGTGACTGTGATGTT Probe: TCTTGCAGCCTGATCCGCCT
IL-21R	Forward: GCTTCTCCTGCTTGTCA Reverse: TTG TACAGGGGCACGAAGA Probe: CCCACCCAATGTGGAGGCTATG
IL-23R	Forward: CAGGCTTGGAGTTCACCATT Reverse: AGCCCAGAATTCCATGTGTT Probe: CCTGAAACAGTTCCCCAGGTCACA
TGFβR	Forward: AAGGCCAAATATCCCAAACA Reverse: TAGCTGCTCCGTTGGCATAC Probe: AAGGCTTCGCAGCTCTGCCA

β-actin	Forward: ATGCTTCTAGGCGGACTGTG Reverse: AAAGCCATGCCAATCTCATC Probe: FAM-TGCGTTACACCCTTTCTTGACAAAACC-BHQ1

enzyme-linked immunosorbent assay (ELISA) kits. Levels of the Th17-promoting cytokines IL-6, IL-23 and TGF-β were measured in plasma using Legend Max ELISA kits for huIL-6 and huTGF-β (BioLegend; San Diego, CA) or Ready-Set-Go huIL-23 ELISA kit from eBioscience (San Diego, CA).

IN VITRO STIMULATION OF NON-ADHERENT AND ADHERENT PBMC

Total PBMC were plated in a 96 well V-bottom plate and incubated overnight at 37°C. Supernatant containing non-adherent cells was transferred the following day to a fresh plate. The remaining adherent cells (APCs) were stimulated with the following combination of TLR ligands for 3 days at 37°C: polyI:C (50 μg/ml), CpG (4 μg/ml), LPS (5 μg/ml), and imiquimod (5 μg/ml). Non-adherent cells and total jejunal cells were also stimulated following the same protocol. Cells were subsequently washed and RNA was isolated as described earlier to be used for relative qRT-PCR.

WESTERN BLOTS

To examine the expression of Th17-associated genes, live sorted total CD4+ T cells were stimulated with human rIL-6 for 15 minutes at 37˚C in serum-free media as earlier described. Following stimulation, cells were harvested and lysed in high salt extraction buffer (400mM NaCl, 10mM Hepes pH 7.5, 1.5mM MgCl2, 0.1mM EGTA, 5% glycerol, 1mM DTT, DI water) containing 1x Halt Protease & Phosphatase Inhibitor Cocktail (Thermo Scientific, Rockford, IL). Lysates were prepared in NuPAGE LDS Sample Buffer (Invitrogen) and 2-mercaptoethanol (Sigma Aldrich) and boiled at 95˚C for 5 min. Proteins were separated on a NuPAGE 4-12% Bis-Tris gel (Invitrogen) with NuPAGE MOPS SDS Running Buffer (Invitrogen) and then transferred to nitrocellulose membranes. Membranes were blocked with SuperBlock T20 Blocking Buffer (Thermo

Scientific, Rockford, IL) and then probed with the following human primary antibodies: total STAT3, p-STAT3, SOCS3, SHP2, PIAS3, and GAPDH. All antibodies were obtained from Cell Signaling Technology, with the exception of SOCS3 which was obtained from Abcam (Cambridge, MA). For each antibody, a titration experiment was performed using rhesus macaque PBMC to confirm cross-reactivity and to determine the ideal number of cells required for detecting the various proteins being studied. For all Western blots shown, ~ 5 x 10^5 cells were loaded in each lane. Membranes were subsequently incubated with donkey anti-rabbit IgG secondary antibody conjugated to HRP (Millipore, Temecula, CA) and SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) was used for detection. Band intensity was quantified using ImageQuant software and was normalized to GAPDH for the same sample.

IN VITRO TH17 DIFFERENTIATION

To differentiate Th17 cells, live naïve CD4+ T cells from either total PBMC or mesenteric lymph node were negatively sorted using a combination of anti-CD8-Pacific Blue, anti-CD14-Pacific Blue, anti-CD20-Pacific Blue, and anti-CD95-FITC and VIVID. CD14+CD8+CD20+ cells were excluded along with all memory and dead cells. Sorted cells were stained with anti-CD3, anti-CD4, anti-CD28, and anti-CD95 to confirm purity.

Following sorting, cells were then plated in a 96 well plate under Th17 culture conditions using a protocol provided by John O'Shea. Cells were stimulated with plate-bound anti-CD3 (10 μg/ml) and soluble anti-CD28 (10 μg/ml) in the presence of anti-IFNγ antibody (10 μg/ml) and the following Th17-promoting cytokines: IL-6 (20 ng/ml), IL-1β (20 ng/ml), IL-23 (20 ng/ml), TGF-β (1 ng/ml), and IL-21 (20 ng/ml). As a control, a Th0 condition was set up with anti-CD3 and anti-CD28 only. Cells were

incubated at 37˚C for 5-14 days and split as necessary. In some cases, cells were additionally stimulated with IL-2 for 7 days after culture. At the end of culture, cells were stimulated with PMA/ionomycin in the presence of Brefeldin-A for 4 hours, fixed and permeabilized and labeled with anti-IL-17-Alexa Fluor 647 and anti-IFNγ-PE. Labeled cells were analyzed using a Becton Dickinson (BD) LSR II instrument.

CELL LINES

The HTLV CD4+ T cell line Hut102 was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. HEK293T and 3T3 cells were maintained in DMEM with 10% FBS and used for generating and testing lentiviral particles, respectively.

SEQUENCE ANALYSIS

The human (NM_006099.3) and rhesus macaque (XM_001095153.2) PIAS3 mRNA sequences were aligned using Clustal O multiple sequence alignment software to determine the extent of sequence homology. Alignments were prepared using T-Coffee and BoxShade software.

LENTIVIRAL CONSTRUCTS

The human PIAS3 Precision LentiORF construct was obtained from Thermo Scientific while lentivirus construct expressing GFP (pWPT-GFP) was obtained from Dr. Brian Schaefer at USUHS. DH5α bacteria (Invitrogen, Carlsbad, CA) were transformed with lentiviral constructs and grown overnight in Luria-Bertani (LB) broth with ampicillin at 37˚C. Plasmid purification was performed using a QiAmp isolation kit (Qiagen Sciences, Gaithersburg, MD). Expression of the PIAS3 gene and its cross-

reactivity with rhesus were confirmed by relative qPCR using primers and probe designed against the rhesus PIAS3 sequence.

GENERATION OF LENTIVIRAL PARTICLES

To generate lentiviral particles, calcium phosphate transfection was performed using 293T cells as the packaging cell line. Briefly, 6 x 10^5 cells were plated in DMEM in a 6-well tissue culture plate the day prior to transfection. On the day of transfection, media was replaced and cells were transfected with a combination of 3 plasmids: lentiviral plasmid containing either the PIAS3 construct or GFP only (1 μg), packaging plasmid pCMVR8.74 (1 μg), and the envelope plasmid pMD2.G (0.2 μg). The media was replaced 24 hours following transfection and transfection efficiency was confirmed by fluorescence microscopy. Supernatant containing viral particles was harvested at 48 hours post-transfection, centrifuged to remove cell debris, and passed through a 0.22 μM filter. Additional media was added to each well, with a second viral harvest performed at 72 hours post-transfection.

LENTIVIRAL TRANSDUCTION OF HUT102 CELLS

To determine the role of PIAS3 in suppression of IL-17 responses, lentiviral transduction of the HTLV cell line Hut102 was performed. The day of transduction, Hut102 cells were plated in a 6 well tissue culture plate at a density of 5 x 10^4 per well. 100 μl of either PIAS3 or GFP lentivirus was added to the cells and then centrifuged at 1200 x g for 2 hours at 25˚C to facilitate infection. Transduction efficiency was confirmed by fluorescence microcopy. At 24 hours post-transduction, rIL-6 (20ng/ml) was added to the wells for 24 hours to stimulate IL-17 expression. Lentiviral-infected

cells were harvested at 48 hours post-infection and used for RNA isolation for relative qRT-PCR for IL-17, PIAS3, and β-actin.

DATA AND STATISTICAL ANALYSIS

Flow cytometric data were analyzed using FlowJo version 8.6 (Tree Star, Inc., Ashland, OR). Statistical analysis was performed using the Mann-Whitney U test with GraphPad Prism version 4.0 software (GraphPad Prism Software, Inc., San Diego, CA). Error bars represent standard errors. Linear regression analysis was performed to determine line of fit and correlations were assessed using the Spearman correlation. A *P* value of <0.05 was considered significant.

CHAPTER 3: Dynamics of Th17 dysregulation, immune activation and microbial translocation during SIV infection

INTRODUCTION AND RATIONALE

HIV and SIV infection is characterized by massive viral replication, corresponding infection and loss of CD4+ memory T cells particularly at mucosal sites, and high levels of systemic immune activation (89; 217; 238; 242; 336). High levels of viral replication occur during the acute stage of infection and are accompanied by a significant loss of CD4+ memory T cells occurring between days 10 and 14 (238). Following acute infection, viral loads decrease to set point and CD4+ T cell counts partially recover. Low levels of viral replication persist throughout chronic infection, contributing to a slow, progressive decline in CD4+ T cells.

One of the hallmarks of infection is chronic, systemic activation of the immune system (19; 89; 152). Immune activation is a contributing factor to CD4+ T cell depletion, as activated T cells are targets for viral infection (42; 335; 339). While several causes of immune activation have been proposed, one of the contributing factors is thought to be the translocation of microbial products from the intestines into circulation due to breakdown of the intestinal epithelial barrier during infection (41; 101; 303). Several studies (15; 41; 51; 63; 234; 268; 303) in HIV and SIV have shown an increase in markers of microbial translocation such as LPS and sCD14 during infection, which are co-localized with breaks in the gastrointestinal epithelium (101).

The loss of CD4+ T cells in HIV and SIV infection includes the IL-17-producing subset Th17 cells (40; 52; 104; 175; 178; 245; 288; 290; 302). Th17 cells are lost during the acute stage of infection, coinciding with the massive generalized loss of CD4+ T cells

in multiple compartments (52; 178). Interestingly, Th17 cells fail to recover during the later stages of infection when other CD4+ T cells subsets such as Th1 cells are present (40; 52; 104).

Mucosal sites, such as the gastrointestinal tract, are critical sites not only for viral infection and CD4+ T cell loss, but also for Th17 cells and their role in immunity. As the gastrointestinal tract is rich in activated CD4+CCR5+ T cells which are viral targets, this site experiences a near complete ablation of all CD4+ T cells during the first two weeks of infection (238; 336). Importantly, CD4+ T cells remain depleted in the gastrointestinal tract throughout the course of infection (42; 138; 177; 242). Interestingly, Th17 cells are enriched at mucosal sites where they are thought to play a protective role at the host-pathogen interface, continual exposure to antigens while providing defense against pathogens that use mucosal sites to gain entry (256). Through regulation of tight junction proteins such as occludin and claudin, Th17 cells are also an important component of the maintenance of the epithelial barrier integrity in the gastrointestinal tract (75; 192). The loss of Th17 cells during HIV and SIV infection has been linked to microbial translocation and immune activation (40; 52; 101; 104; 195; 196; 229; 290; 303).

Using the SIV model in rhesus macaques, we first sought to determine the dynamics of peripheral and mucosal Th17 cells during SIV infection. We focused on both early and late time points during infection: day 0 to day 35 to capture acute infection and transition into clinical latency, and chronic infection, to capture later stages of disease. Previous studies (217; 238; 336) have shown that viral loads peak between 1 – 2 weeks p.i., with a massive loss of CD4+ memory T cells from multiple compartments occurring between day 10 and day 14 p.i. While previous studies (52) have shown that

the loss of CD4+ T cells during acute infection includes Th17 cells, it is not known how early in infection the suppression of IL-17 responses occurs. Previous studies (178) have indicated that Th17 cells are maintained through day 10 p.i., along with other CD4+ memory T cells (93; 238).

Additionally, we wanted to determine if Th17 dysregulation is associated with microbial translocation and immune activation during SIV infection. There is strong evidence to suggest that the loss of Th17 responses in chronic infection is associated with translocation of microbial products and subsequent immune activation (40; 52; 104; 196; 229; 290). However, it is not clear if microbial translocation occurs during the acute stage of infection and whether it is associated with the loss of Th17 cells. While elevated levels of immune activation have been observed during acute infection, it is not known if this increase is due to translocated microbial products or other factors.

RESULTS

Plasma viral loads and CD4+ T cell dynamics during SIV infection

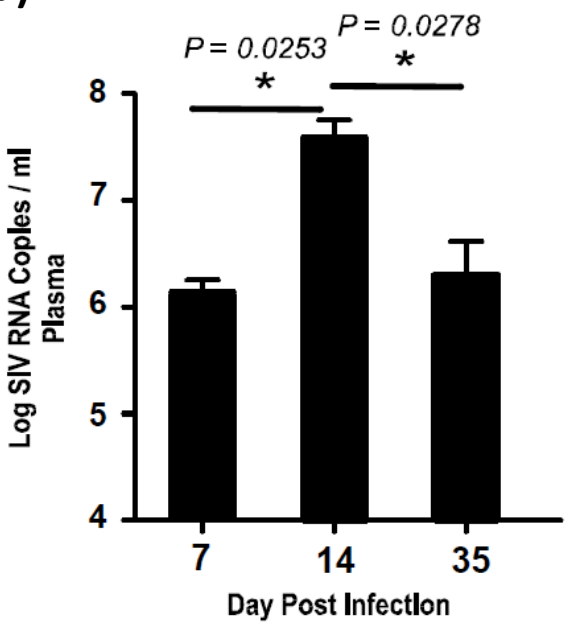
We first determined the viral loads in the plasma of SIV-infected animals during the acute and chronic stages of infection (**Figures 9 and 10**). By day 7 post-infection (p.i.), there were almost 10^6 copies of virus that peaked by day 14 p.i. at 10^8 copies and decreased to ~10^5 copies by day 35 p.i (**Figure 9a**). Viral loads remained high during chronic infection (**Figure 10a**).

Changes in plasma viral loads during the early stage of infection were accompanied by a significant loss of CD4+ memory T cells in peripheral blood at day 35 p.i. as compared to day 7 p.i. (**Figure 9b**). CD4+ T cell counts remained significantly

Figure 9. Acute SIV infection is characterized by high viral loads and loss of memory CD4+ T cells. (a) Plasma viral loads at days 7 (n=6), 14 (n=6), and 35 (n=6) post-infection (p.i.) as assessed by a qRT-PCR assay for SIV copies (64). Limit of detection = 30 copies/ml of plasma (b) Frequency of CD3+CD4+ memory T cells at days 0, 7, and 35 p.i. as determined by flow cytometry. Total PBMC were labeled with anti-CD3, anti-CD4, anti-CD8, anti-CD95, and anti-CD28. Memory T cells were identified based on expression of CD95 and CD28, with all memory cells expressing CD95 (287).

Previously published as (35)

a)


P = 0.0253
*
P = 0.0278
*
8
7
6
5
4
Log SIV RNA Copies / ml Plasma
7
14
35
Day Post Infection

b)

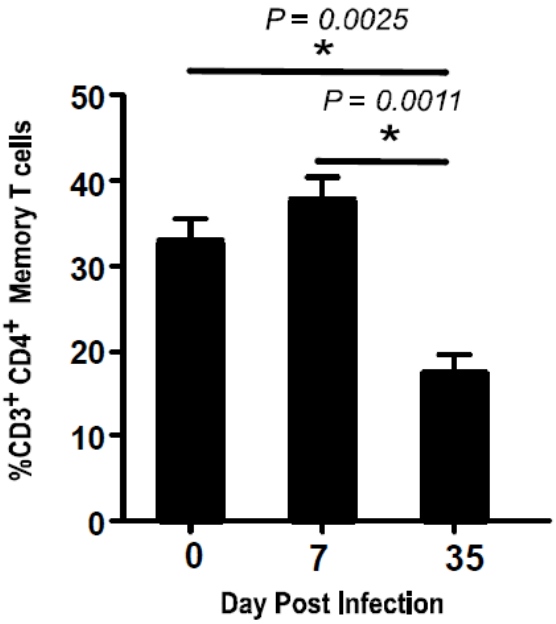
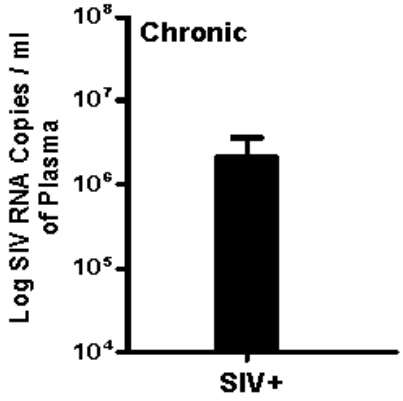
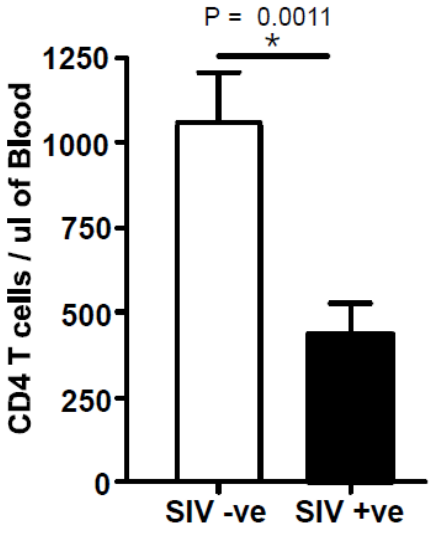

P = 0.0025
*
P = 0.0011
*
50
40
30
20
10
0
%CD3+ CD4+ Memory T cells
0
7
35
Day Post Infection

Figure 10. High viral loads and progressive loss of CD4+ T cells in multiple tissue compartments persist into chronic SIV infection. (a) Plasma viral loads (n =8) at necropsy as assessed by a qRT-PCR assay. Samples were obtained at necropsy from animals had been infected for at least one year. Limit of detection = 30 copies/ml of plasma. (b) Absolute counts of CD4+ T cells in peripheral blood of infected animals (n =8) as compared to healthy controls (n=8). Total PBMC were labeled with anti-CD3 and anti-CD4 and absolute numbers of CD4+ T cells were assessed by flow cytometry. (c) Frequency of total CD3+CD4+ T cells in jejunum during chronic infection (n =8). Total cells from the jejunum were labeled with anti-CD3 and anti-CD4 and frequency of CD4+ T cells was assessed by flow cytometry.

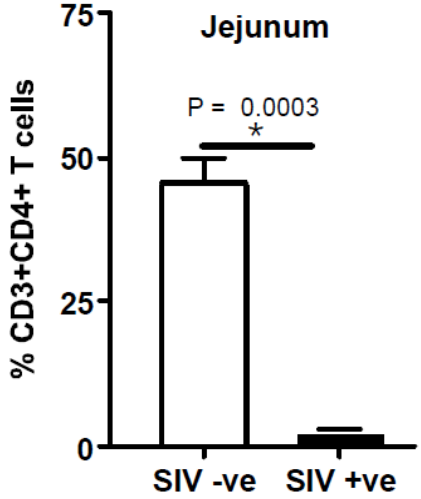
a)


Chronic
Log SIV RNA Copies / ml of Plasma
10^8
10^7
10^6
10^5
10^4
SIV+

b)


P = 0.0011
*
CD4 T cells / ul of Blood
1250
1000
750
500
250
0
SIV -ve
SIV +ve

c)


Jejunum
P = 0.0003
*
% CD3+CD4+ T cells
75
50
25
0
SIV -ve
SIV +ve

lower during chronic infection as compared to uninfected controls **(Figure 10b**). As previously reported (336), we found that CD4+ T cells from the jejunal mucosa were nearly completely ablated during chronic SIV infection (**Figure 10c**).

Th17 responses during SIV infection

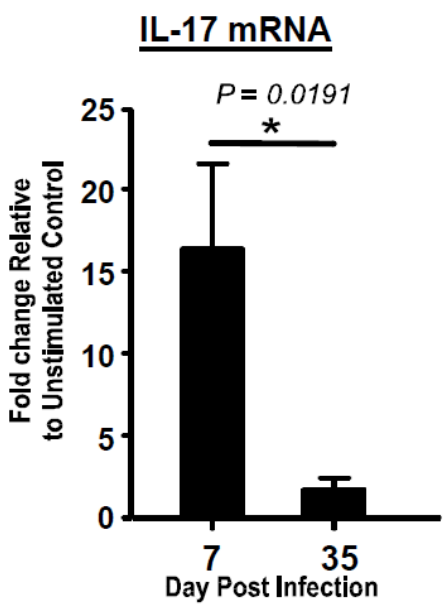
Th17 cells have been shown to be depleted during SIV infection (40; 52; 104; 175; 245; 288; 302). To determine if this loss occurred during the initial stages of infection, we examined the expression of IL-17 in negatively sorted peripheral blood CD4+ T cells that were stimulated with recombinant human IL-6 (rIL-6). Previous studies (283) had shown that human rIL-6 effectively stimulated rhesus macaque T cells. IL-17 expression was assessed by a relative qRT-PCR assay as described in the Materials and Methods section. IL-17 mRNA expression was significantly downregulated by day 35 p.i., as compared to day 7 p.i.(**Figure 11**), indicating that Th17 responses are suppressed very early during acute SIV infection.

Next we examined if Th17 responses were altered during chronic SIV infection. We used two tissue compartments: peripheral blood and jejunum, as previous studies (21; 40; 170) have shown that Th17 cells were enriched in the gastrointestinal tract. Total PBMC and cells from the jejunum were stimulated *ex vivo* with PMA/ionomycin in the presence of Brefeldin A. Th17 cells were identified based on their concurrent expression of CD4 and IL-17 by flow cytometry. We observed a significant decrease in the frequency of Th17 cells in PBMC from SIV-infected animals as compared to uninfected controls (**Figure 12**). Similar to peripheral blood, Th17 cells were significantly depleted in jejunum during chronic infection along with a loss of overall CD4+ T cells (**Figure 13**).

Figure 11. IL-17 expression in CD4+ T cells is significantly suppressed during acute SIV infection. (a) Expression of IL-17 mRNA in sorted CD4+ T cells from peripheral blood following short-term stimulation with human recombinant IL-6 (rIL-6). Total PBMC were labeled with anti-CD8, anti-CD20, anti-CD14 and VIVID and total CD4+ T cells were negatively sorted by FACS as previously described in **Figure 7**. 5 x 10^5 sorted CD4+ cells were stimulated with human rIL-6 in serum-free media for 15 minutes at 37°C. IL-17 expression was assessed by relative qRT-PCR and normalized using β-actin. Fold change was calculated using the ddCT method (307) and is shown relative to unstimulated controls at days 7 and 35 p.i. (b) ddCT values for IL-17 mRNA expression in unstimulated controls at days 7 and 35 p.i.

Previously published as (35)

a)


IL-17 mRNA
P = 0.0191
*
25
20
15
10
5
0
Fold change Relative to Unstimulated Control
7
35
Day Post Infection

b)

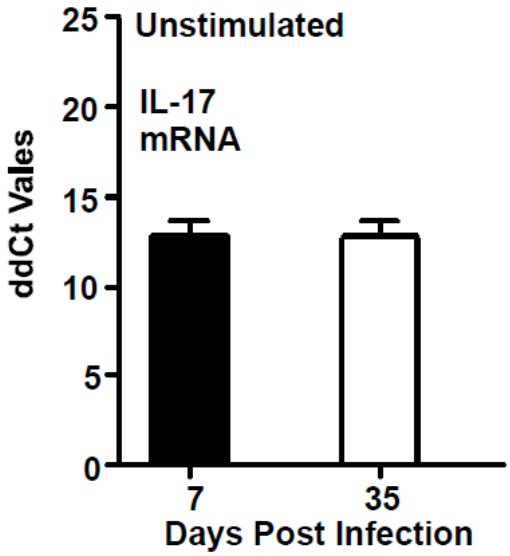
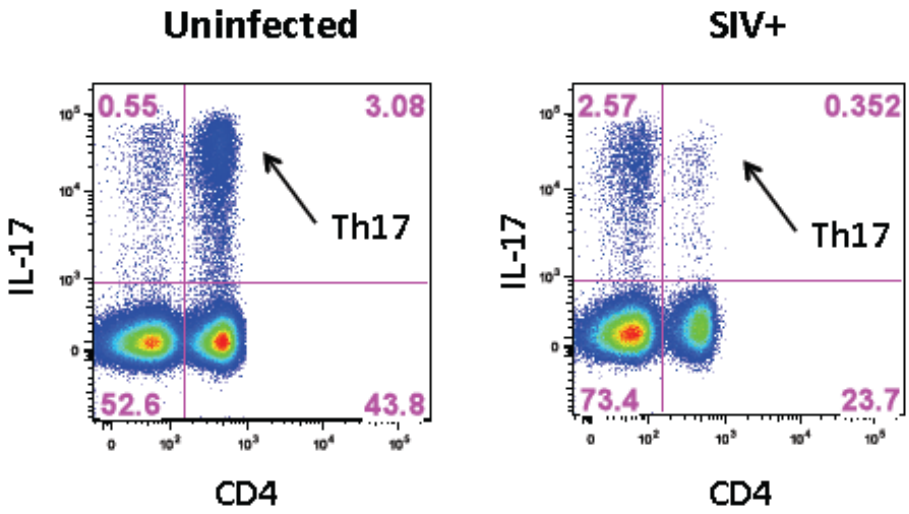

25
20
15
10
5
0
Unstimulated
IL-17 mRNA
ddCt Vales
7
35
Days Post Infection

Figure 12. Th17 cells are significantly depleted from peripheral blood during chronic SIV infection. (a) Gating strategy for Th17 cells in peripheral blood. Total PBMC were stimulated with PMA and ionomycin in the presence of Brefeldin-A and labeled with anti-CD3, anti-CD4, anti-CD8, anti-CD95, anti-CD45RA, and anti-IL-17 and analyzed using flow cytometry. Representative dot plots from one uninfected and one SIV-infected animal are shown. Th17 cells were identified by concurrent expression of CD4 and IL-17 in response to PMA/ionomycin stimulation. (b) Frequency of CD4+IL-17+ cells in SIV-infected and healthy animals.

a)


Uninfected
SIV+
0.55
3.08
52.6
43.8
2.57
0.352
73.4
23.7
Th17
Th17
IL-17
IL-17
CD4
CD4

b)

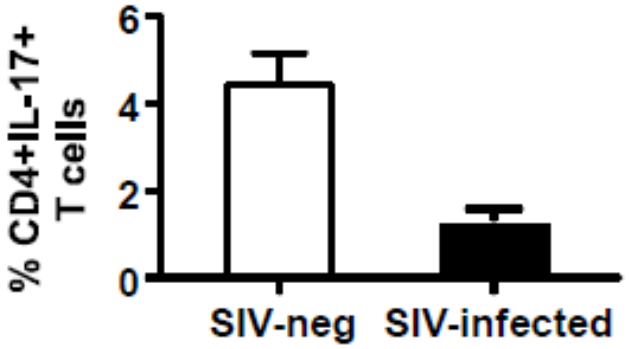
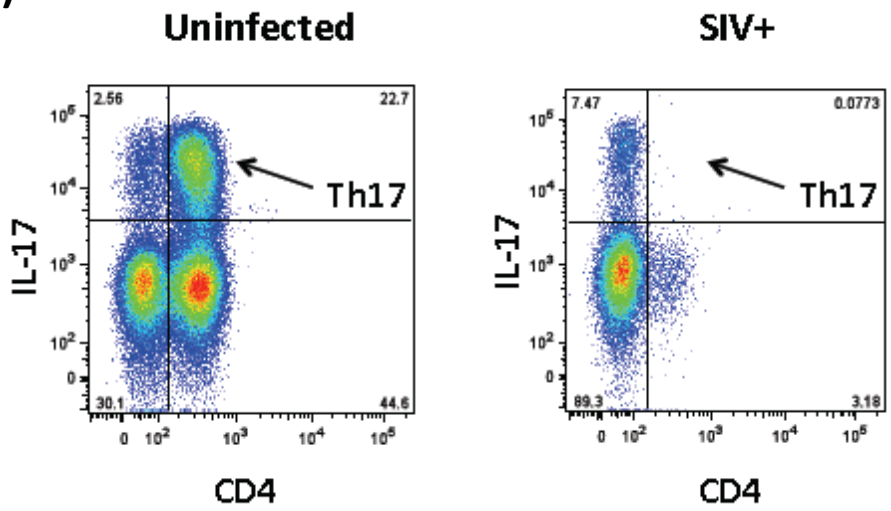
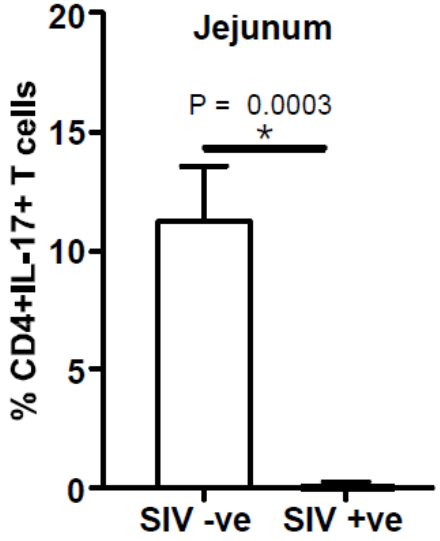

% CD4+IL-17+ T cells
6
4
2
0
SIV-neg
SIV-infected

Figure 13. Th17 cells are significantly depleted from jejunum during chronic SIV infection. (a) Gating strategy for Th17 cells in jejunum. Total jejunal cells were stimulated with PMA and ionomycin in the presence of Brefeldin-A and labeled with anti-CD3, anti-CD4, anti-CD8, anti-CD95, anti-CD45RA, and anti-IL-17 and analyzed using flow cytometry. Representative dot plots from one uninfected and one SIV-infected animal are shown. Th17 cells were identified by the concurrent expression of CD4 and expression of IL-17 in response to PMA/ionomycin stimulation. (b) Frequency of CD4+IL-17+ cells in SIV-infected and healthy animals.

a)


Uninfected
SIV+
Th17
Th17
IL-17
IL-17
CD4
CD4
2.56
22.7
30.1
44.6
7.47
0.0773
89.3
3.18

b)


Jejunum
P = 0.0003
*
% CD4+IL-17+ T cells
20
15
10
5
0
SIV -ve
SIV +ve

Immune activation in SIV infection

Several studies have previously shown that the loss of Th17 cells was accompanied by increased immune activation during infection (104; 229). To determine if the loss of Th17 cells leads to immune activation, we examined the expression of the nuclear proliferation marker Ki67 and the activation marker HLA-DR on CD4+ and CD8+ T cells during acute and chronic SIV infection (**Figure 14**). Previous studies (176) have used Ki67 and HLA-DR as markers of immune activation during HIV and SIV infection. Markers of immune activation were significantly elevated as early as day 7 p.i. (**Figure 14a**) and remained high during chronic stages of SIV infection (**Figure 14b-c**).

Markers of microbial translocation in SIV infection

Studies have suggested that Th17 cells play an important role in maintaining mucosal epithelial barrier integrity and that loss of Th17 cells is associated with increased translocation of microbial products (101; 290). To determine if loss of Th17 cells during early stages of infection was associated with microbial translocation, we examined the plasma levels of two commonly used markers of microbial translocation, sCD14 and LBP (**Figure 15**). Both are released in response to LPS. While there was no change in sCD14 or LBP levels between day 0 and day 7, there was a significant increase in the level of both markers by day 35 p.i. (**Figure 15a-b**). Markers of microbial translocation remained persistently high during chronic stages of SIV infection (**Figure 15c**).

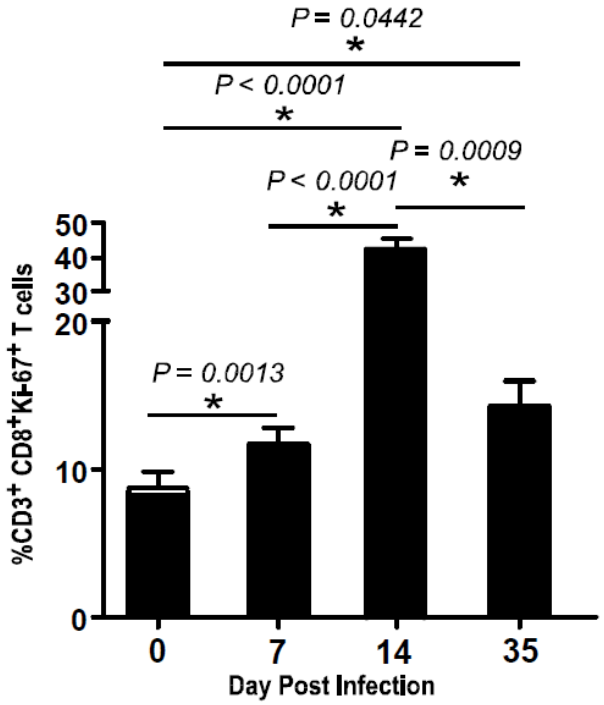
The loss of Th17 cells correlates with microbial translocation but not immune activation during acute infection

To determine if loss of Th17 cells was a cause for increased microbial translocation, we correlated the loss of IL-17 responses with markers of microbial

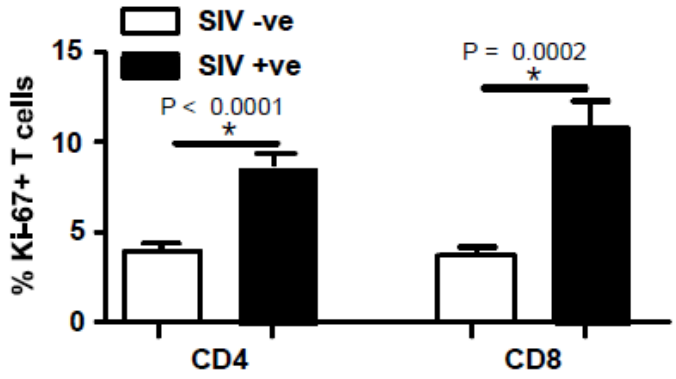
Figure 14. Immune activation is elevated during acute SIV infection and persists during chronic infection. Total PBMC were labeled with anti-CD3, anti-CD4, anti-CD8, anti-CD95, anti-HLA-DR, and anti-Ki67 and analyzed using flow cytometry. (a) Frequency of CD3+CD8+Ki-67+ memory T cells in peripheral blood at days 0, 7, 14, and 35 p.i. (b) Frequency of CD3+CD4+Ki-67+ and CD3+CD8+Ki-67+ T cells in peripheral blood from healthy and SIV-infected animals during chronic infection (c) Frequency of CD3+CD4+HLA-DR+ and CD3+CD8+HLA-DR+ T cells in peripheral blood from healthy and SIV-infected animals during chronic infection

Previously published as (35)

a)


P = 0.0442
*
P < 0.0001
*
P = 0.0009
P < 0.0001
*
*
P = 0.0013
*
%CD3+ CD8+Ki-67+ T cells
50
40
30
20
10
0
0
7
14
35
Day Post Infection

b)


SIV -ve
SIV +ve
% Ki-67+ T cells
15
10
5
0
P < 0.0001
*
P = 0.0002
*
CD4
CD8

c)

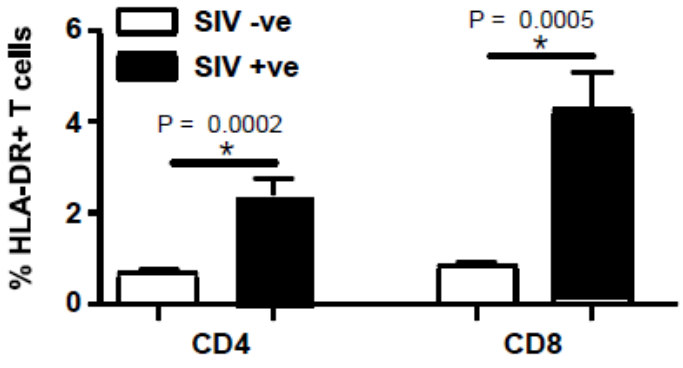
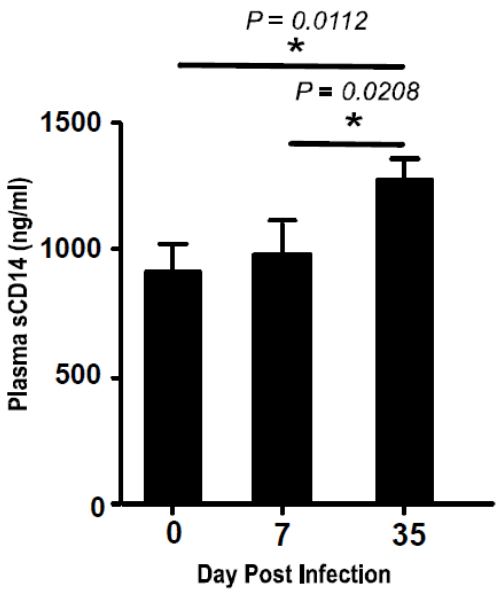

SIV -ve
SIV +ve
% HLA-DR+ T cells
6
4
2
0
P = 0.0002
*
P = 0.0005
*
CD4
CD8

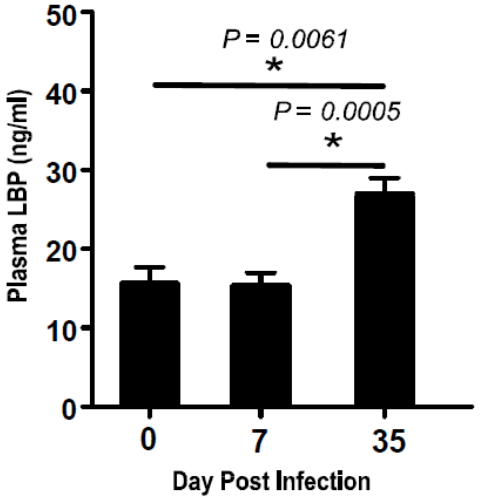
Figure 15. Markers of microbial translocation are elevated during acute and chronic SIV infection. Plasma (a) sCD14 and (b) LBP levels at days 0, 7, and 35 p.i. as measured by ELISA (c) Plasma sCD14 levels in uninfected animals and SIV-infected animals during chronic infection

Previously published as (35)

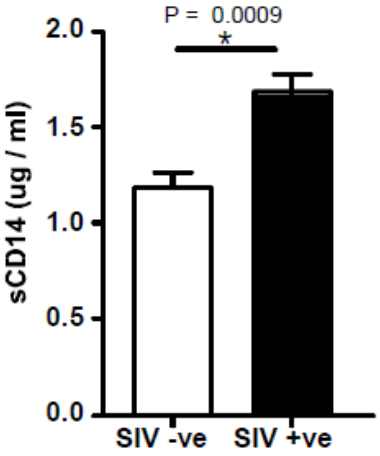
a)


P = 0.0112
*
P = 0.0208
*
1500
1000
500
0
Plasma sCD14 (ng/ml)
0
7
35
Day Post Infection

b)


50
40
30
20
10
0
Plasma LBP (ng/ml)
P = 0.0061
*
P = 0.0005
*
0
7
35
Day Post Infection

c)


P = 0.0009
*
2.0
1.5
1.0
0.5
0.0
sCD14 (ug / ml)
SIV -ve
SIV +ve

translocation. Plasma LBP and sCD14 levels were found to be negatively correlated with IL-17 expression during both acute (**Figure 16**) and chronic SIV infection (**Figure 17c**) suggesting that loss of Th17 cells likely contributes to increased translocation of microbial products.

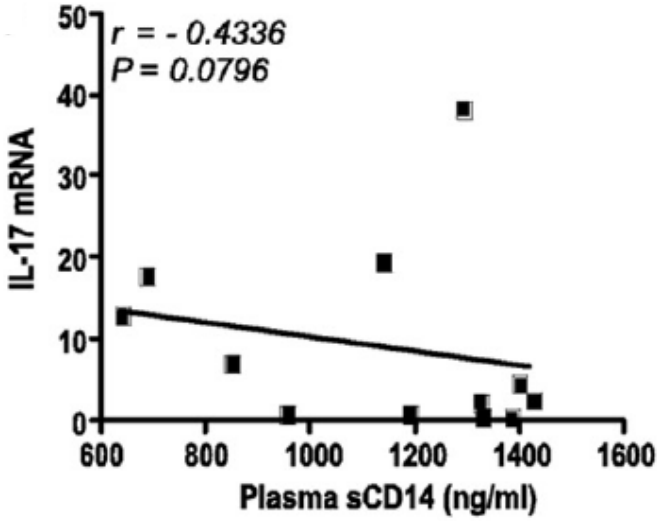
One of the leading hypotheses in the field is that translocation of microbial products into systemic circulation is likely responsible for immune activation during infection (41; 196; 303). To determine whether translocated microbial products were the cause for immune activation during SIV infection, we correlated the expression of markers of microbial translocation with levels of immune activation during acute (**Figure 18a-b**) and chronic stages of infection (**Figure 17a**). In keeping with previous results from other studies (41; 101; 303), we found a significant correlation between markers of microbial translocation and immune activation during chronic SIV infection (**Figure 17a**).

Interestingly, neither sCD14 nor LBP levels were found to correlate with immune activation during acute SIV infection (**Figure 18a-b**) suggesting that microbial translocation may not be the primary driving force behind the high levels of immune activation during early stages of infection. In contrast, we found a significantly high positive correlation between plasma viral loads and immune activation (**Figure 18d**), indicating that viral replication during acute infection was likely driving immune activation at these early time points. Similarly, we did not see a significant correlation between IL-17 mRNA expression and Ki67+ CD8+ T cells (Figure 18c), indicating that immune activation might not be linked to loss of Th17 activity in acute infection. Unlike

Figure 16. IL-17 is negatively correlated with markers of microbial translocation during acute infection. Correlation of IL-17 mRNA fold change with plasma (a) sCD14 and (b) LBP levels for days 7 and 35 p.i.

Previously published as (35)

a)


r = - 0.4336
P = 0.0796
IL-17 mRNA
50
40
30
20
10
0
600
800
1000
1200
1400
1600
Plasma sCD14 (ng/ml)

b)

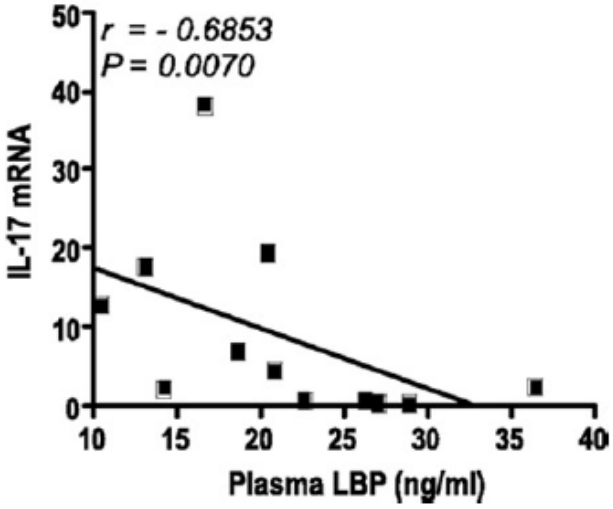
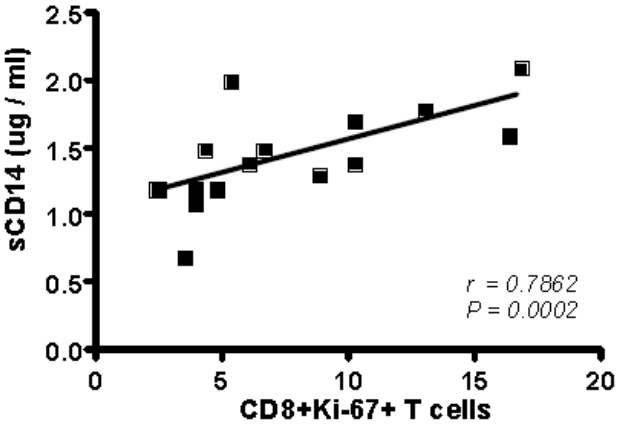
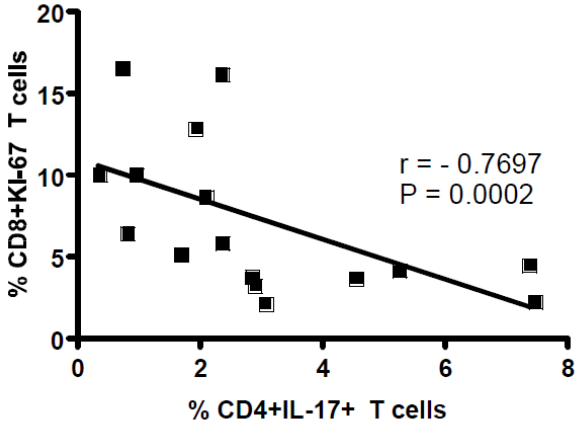

r = - 0.6853
P = 0.0070
IL-17 mRNA
50
40
30
20
10
0
10
15
20
25
30
35
40
Plasma LBP (ng/ml)

Figure 17. Markers of microbial translocation, immune activation, and Th17 frequency are correlated in chronic infection. (a) Plasma sCD14 levels are positively correlated with the frequency of CD8+Ki-67+ T cells. (b) CD8+Ki-67+ T cells and (c) plasma sCD14 levels negatively correlate with the frequency of CD4+IL-17+ T cells.

a)


sCD14 (ug / ml)
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
CD8+Ki-67+ T cells
r = 0.7862
P = 0.0002

b)


% CD8+KI-67 T cells
20
15
10
5
0
0
2
4
6
8
% CD4+IL-17+ T cells
r = - 0.7697
P = 0.0002

c)

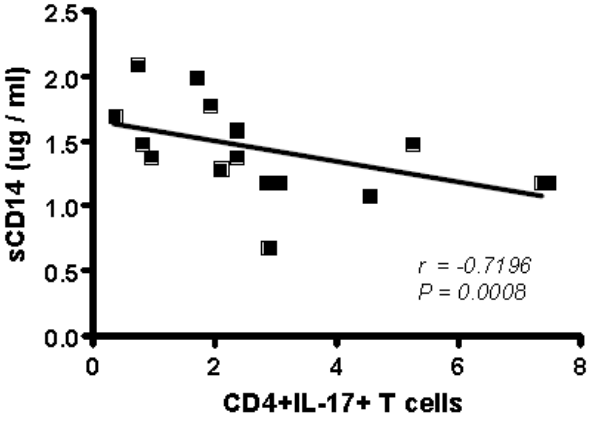
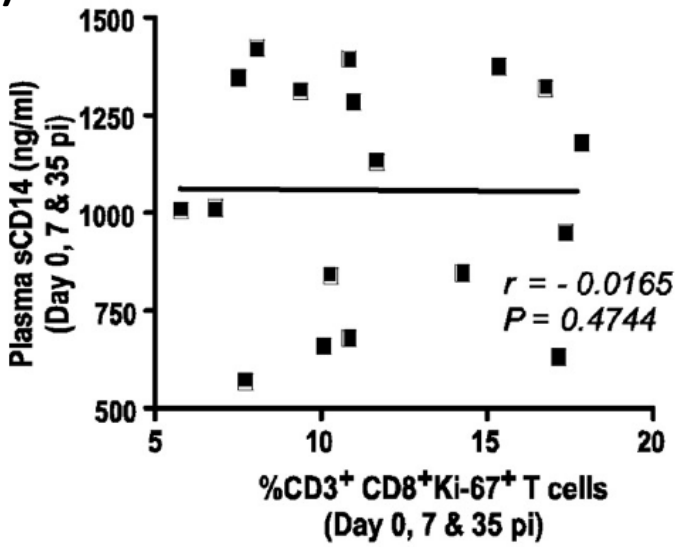

sCD14 (ug / ml)
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
CD4+IL-17+ T cells
r = -0.7196
P = 0.0008

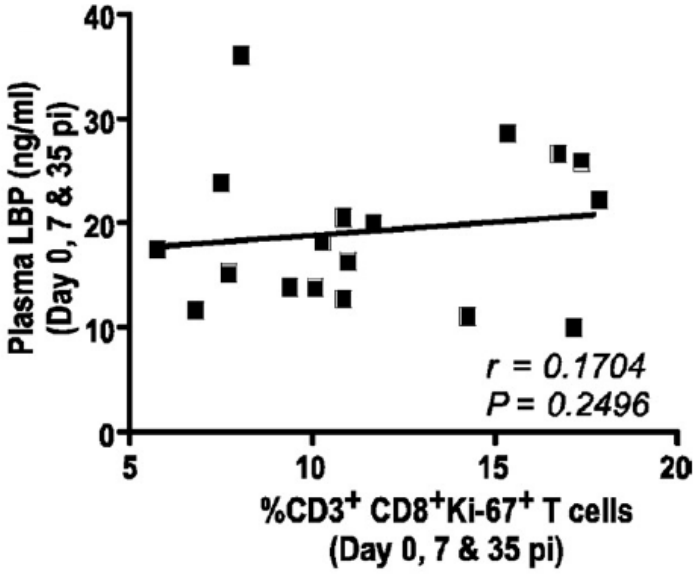
Figure 18. Immune activation is correlated with viral loads but not markers of microbial translocation or IL-17 expression in acute SIV infection. Correlations between frequency of CD3+CD8+Ki67+ T cells and (a) plasma sCD14 (b) plasma LBP (c) fold change in IL-17 mRNA expression by CD4+ T cells and (d) plasma viral loads

Previously published as (35)

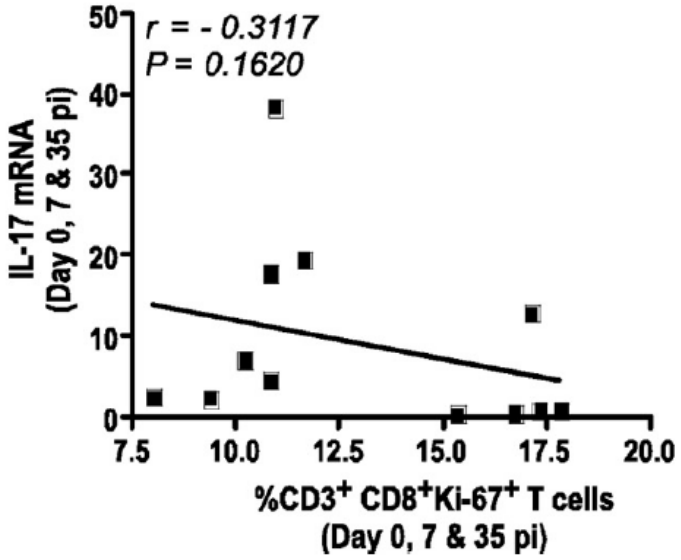
a)


1500
1250
1000
750
500
Plasma sCD14 (ng/ml)
(Day 0, 7 & 35 pi)
r = - 0.0165
P = 0.4744
5
10
15
20
%CD3+ CD8+Ki-67+ T cells
(Day 0, 7 & 35 pi)

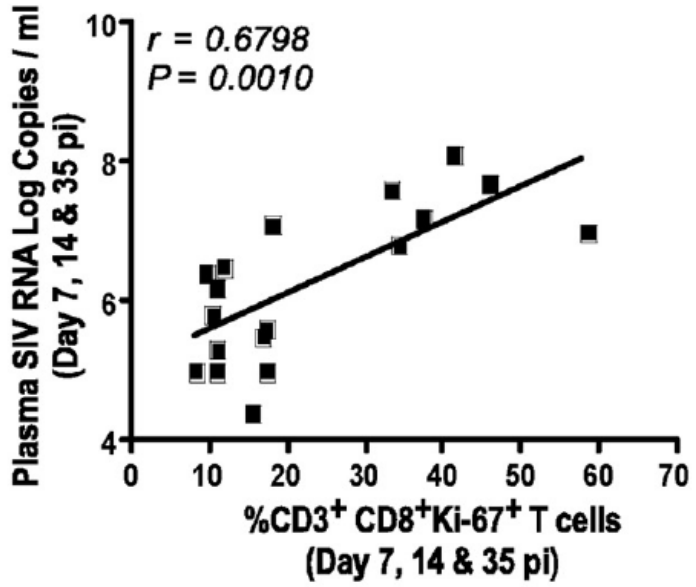
b)


40
30
20
10
0
Plasma LBP (ng/ml)
(Day 0, 7 & 35 pi)
r = 0.1704
P = 0.2496
5
10
15
20
%CD3+ CD8+Ki-67+ T cells
(Day 0, 7 & 35 pi)

c)


r = - 0.3117
P = 0.1620
IL-17 mRNA
(Day 0, 7 & 35 pi)
50
40
30
20
10
0
7.5
10.0
12.5
15.0
17.5
20.0
%CD3+ CD8+Ki-67+ T cells
(Day 0, 7 & 35 pi)

d)


r = 0.6798
P = 0.0010
Plasma SIV RNA Log Copies / ml
(Day 7, 14 & 35 pi)
10
8
6
4
0
10
20
30
40
50
60
70
%CD3+ CD8+Ki-67+ T cells
(Day 7, 14 & 35 pi)

acute SIV infection however, the loss of Th17 cells during chronic stages of infection was found to significantly correlate with immune activation (**Figure 17b**).

LPS and other microbial products bind to receptors such as TLR4 and CD14 that are expressed on monocytes and macrophages, leading to activation and release of pro-inflammatory mediators (15; 41; 303). To determine whether microbial products might be activating monocytes during infection, we measured mRNA expression of TLR4 on sorted CD14+ monocytes by PCR, and examined the density of CD14 expression on monocytes by flow cytometry. The expression of both TLR4 and CD14 was significantly upregulated during early stages of infection (**Figure 19a**).

Next we examined the expression of the pro-inflammatory cytokine IL-23 in sorted CD14+ monocytes to determine if monocytes were likely being activated by translocated microbial products. Expression of IL-23 mRNA was significantly elevated during acute stages of SIV infection (**Figure 19b**). Interestingly, both sCD14 and LBP levels positively correlated with IL-23 mRNA expression (**Figure 20a-b**), indicating that microbial products could potentially be driving IL-23 production by monocytes. Additionally, there was a significant positive correlation between LBP levels and CD14 density on monocytes (**Figure 20c**), supporting the above observation that microbial translocation likely contributes to monocyte activation during acute infection.

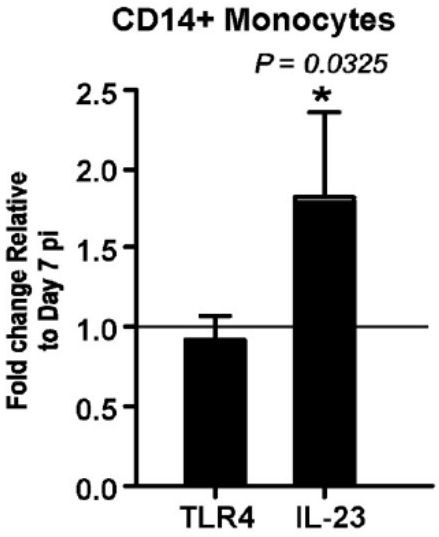
DISCUSSION

HIV and SIV infection are primarily infections of the immune system, wherein viral infection leads to destruction of the CD4+ T cell component of the cellular immune response. Some of the most severely affected sites during infection include mucosal tissues such as the gastrointestinal tract, where massive sustained loss of CD4+ T cells

Figure 19. Acute SIV infection is characterized by increased expression of CD14 and IL-23 in CD14+ monocytes. (a) *Ex vivo* expression of TLR4 and IL-23 expression in sorted CD14+ monocytes. Total PBMC were labeled with anti-CD14 and CD14+ monocytes were sorted by FACS. TLR4 and IL-23 expression in sorted CD14+ monocytes were assessed by relative qRT-PCR and normalized using β-actin. Fold change was calculated using the ddCT method (307), with fold change at day 35 p.i. shown relative to day 7. The dashed line indicates day 7 baseline. (b) Density of CD14 expression on sorted CD14+ monocytes at days 7 and 35 p.i. Histogram shows one representative animal. Also shown, MFI of CD14 expression on CD14+ monocytes at days 7 and 35 p.i.

Previously published as (35)

a)


CD14+ Monocytes
P = 0.0325
*
Fold change Relative to Day 7 pi
2.5
2.0
1.5
1.0
0.5
0.0
TLR4
IL-23

b)

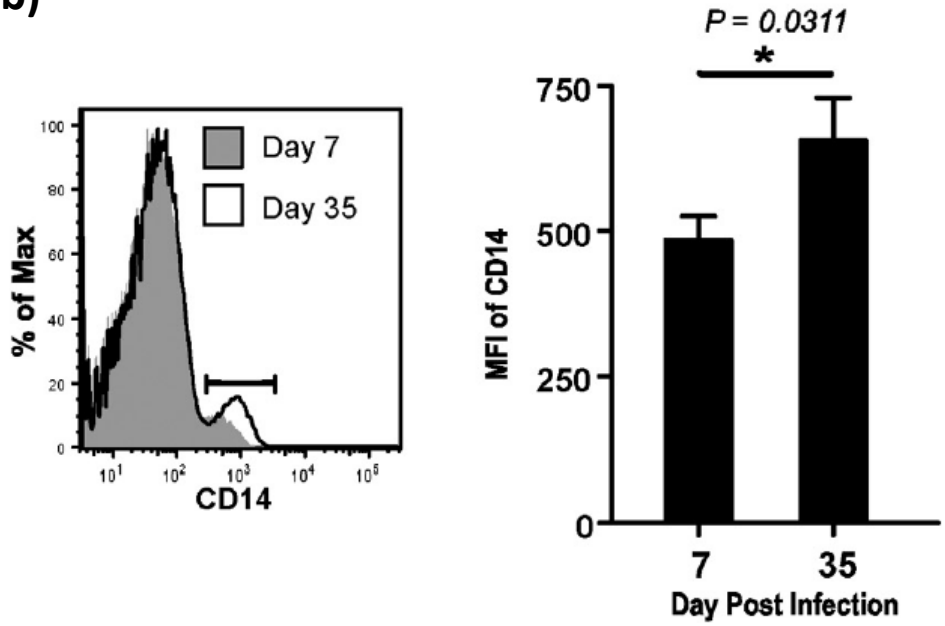
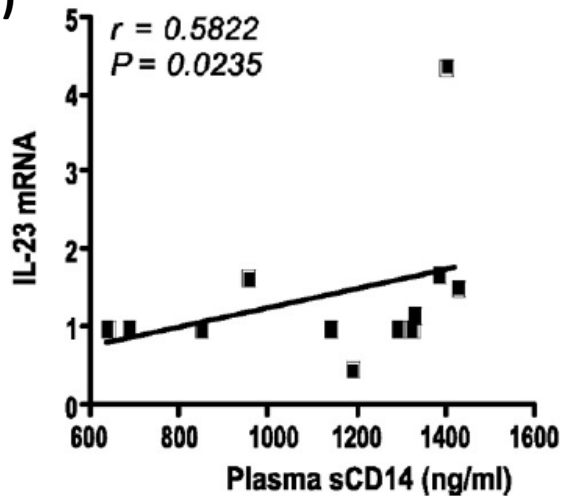

Day 7
Day 35
% of Max
CD14
P = 0.0311
*
MFI of CD14
750
500
250
0
7
35
Day Post Infection

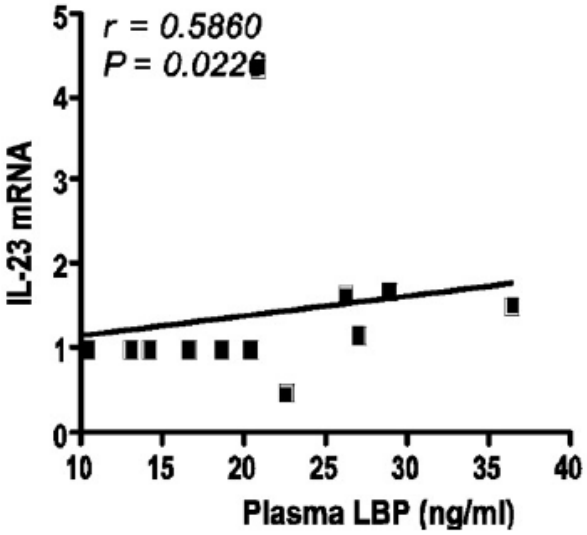
Figure 20. IL-23 mRNA and CD14 expression correlate with markers of microbial translocation during acute SIV infection. Correlations between fold change in IL-23 mRNA expression and plasma (a) sCD14 levels and (b) LBP levels. (c) Correlation between plasma LBP levels and MFI of CD14 on sorted CD14+ monocytes. Correlations are shown for samples from days 7 and 35 p.i.

Previously published as (35)

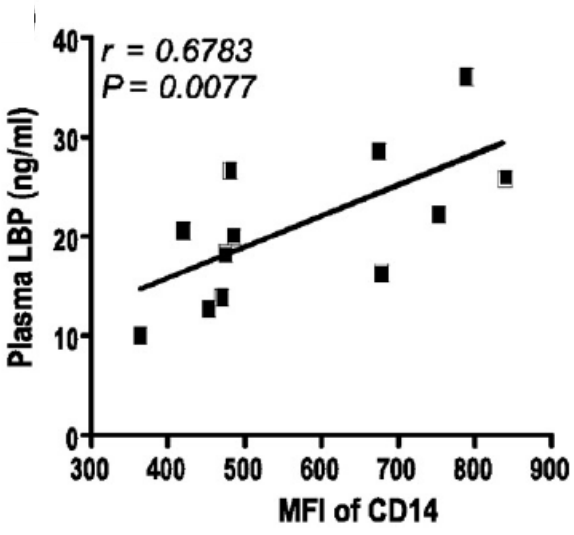
a)


r = 0.5822
P = 0.0235
IL-23 mRNA
Plasma sCD14 (ng/ml)
0
1
2
3
4
5
600
800
1000
1200
1400
1600

b)


r = 0.5860
P = 0.022
IL-23 mRNA
Plasma LBP (ng/ml)
0
1
2
3
4
5
10
15
20
25
30
35
40

c)


r = 0.6783
P = 0.0077
Plasma LBP (ng/ml)
MFI of CD14
0
10
20
30
40
300
400
500
600
700
800
900

and damage to the mucosal barrier occur (99; 101; 238; 336). It is at these sites that Th17 cells are thought to mediate immune homeostasis through their defense against extracellular pathogens and their role in the maintenance of the epithelial barrier's structural integrity (75; 192; 256; 286). The loss of Th17 cells during infection has implications for disease progression, as their failure to recover during infection has been linked to microbial translocation and immune activation (40; 196; 290).

Our results suggest that the loss of Th17 cells occurs during the early stages of infection and is accompanied by translocation of microbial products. While IL-17 responses remained detectable by day 7 following infection, expression of IL-17 was suppressed by day 35 p.i. Th17 cells are likely lost along with other CD4+ memory T cell subsets during the initial wave of CD4+ T cell depletion that occurs between days 10 and 14 p.i.

Importantly, early suppression of IL-17 responses in acute infection was associated with an increase in microbial translocation. While the loss of Th17 cells during chronic infection has been linked to microbial translocation and immune activation (40; 52; 101; 104; 229), it was not known if suppression of Th17 cells at early time points similarly influenced translocation of microbial products. The massive loss of CD4+ T cells from gastrointestinal tract from day 10 to day 14 p.i.(238) may incur inflammatory damage to the mucosal epithelium, which likely persists into and accumulates in chronic infection. Disruption of the mucosal barrier during chronic infection is similarly thought to facilitate entry of bacterial products into systemic circulation (101).

In line with previous studies, elevated levels of microbial translocation were correlated with increased immune activation (41) and the loss of Th17 cells. Interestingly,

unlike in chronic infection, translocation of microbial products during acute stages of infection did not correlate with immune activation. Instead, we found a significant correlation between immune activation and plasma viral loads suggesting that the high level of viral replication that occurs during the early phase of infection significantly contributes to immune activation during acute infection. Other studies (93) have also suggested that early viral replication may promote activation of the immune system, as evidenced by increased levels of pro-inflammatory cytokines such as IL-15. Additionally, it is possible that the increased viral replication that occurs during early infection may be masking the role of translocated bacterial products in acute immune activation.

Translocated microbial products such as LPS induce potent inflammatory cytokines by activating innate immune cells such as monocytes and macrophages. Our results were found to support this hypothesis as we observed a significant increase in CD14 expression on monocytes during acute infection that was accompanied by increased expression of IL-23 by these cells. Other studies have shown that IL-23 is produced by macrophages and dendritic cells in response to stimulation with bacterial and fungal pathogen-associated motifs such as zymosan and muramyl dipeptide (5; 125; 333). These data suggest that translocated microbial products likely contribute to the pro-inflammatory environment early during the course of infection.

In conclusion, our results suggest that SIV infection-mediated damage to the mucosal epithelium and Th17 cells occurs very early during infection and these changes have implications for chronic pathogenesis. The loss of Th17 cells in infection is accompanied by microbial translocation that contributes to the high levels of immune activation observed during infection. While microbial translocation may be associated

with immune activation early in infection, it is more likely that high levels of viral replication drive immune activation at early time points.

CHAPTER 4: Characterization of Th17 differentiation factors during acute and chronic SIV infection

INTRODUCTION AND RATIONALE

Differentiation of a naïve CD4+ T cell into a Th17 effector cell is dependent on a number of cytokines and cytokine signaling pathways (159; 322). Studies conducted in mice and humans have identified four critical cytokines for Th17 development: IL-6, IL-21, IL-23 and TGF-β. The combination of IL-6 and TGF-β appears to be important for the initial stage of Th17 differentiation (232; 289). IL-6 signaling through IL-6R activates the Th17-transcription factors STAT3 and RORγt, resulting in expression of IL-17 (264; 322; 341). The addition of TGF-β likely enhances Th17 development by two distinct mechanisms: inhibition of the Treg transcription factor FoxP3 and the negative regulator SOCS3 (289), both of which are inhibitory to IL-17 expression.

IL-21 and IL-23 play a role in the later stages of Th17 differentiation by amplifying and maintaining these cells. Production of IL-21 by Th17 cells initiates a positive feedback mechanism by which additional IL-17 transcription is induced (235; 269). Like TGF-β, IL-21 can inhibit expression of FoxP3 and IFNγ, which is known to favor Th1 development (163; 235; 269; 346). Terminal differentiation of Th17 cells is mediated by IL-23, which is essential for long-term maintenance of IL-17 production (6; 87; 172; 210; 232; 325). Previous studies have shown that Th17 cells lose the capacity to produce IL-17 following several weeks of culture in the absence of IL-23 signaling (325).

The critical requirement for IL-6, IL-21, IL-23, and TGF-β in Th17 development has been demonstrated in a number of earlier studies in mice. Inhibition of IL-6 signaling by either neutralizing antibody treatment (322; 341) or expression of mutant IL-6R gp130

(264) resulted in impairment of Th17 development. Similarly, severe defects in Th17 differentiation and IL-17 production were observed in TGF-β knockout mice (232) and mice that had been treated with anti-TGF-β antibody (289). Finally, IL-21 and IL-21R knockdown in mice resulted in a deficiency of Th17 cells that persisted despite treatment with other Th17-promoting cytokines such as IL-6 (235; 269; 346). Taken together, these data suggest that deficiency in any one of these four cytokine signaling pathways is sufficient to suppress IL-17 expression from CD4+ T cells.

However, recent studies have suggested that IL-21, IL-23 and TGF-β are dispensable for the initial differentiation of Th17 cells (4; 126; 318; 346; 355; 367). Several studies have found that TGF-β was not required for initial differentiation of Th17 cells, in which IL-6 and IL-1β were sufficient (4; 126). Additionally, as naïve T cells do not express IL-23R (280), IL-23 does not appear to be necessary for early Th17 differentiation steps (355; 367).

Th17 cells are lost very early during HIV and SIV infection and fail to recover during the later stages of infection unlike other CD4+ T cell subsets (40; 52; 104; 175; 178; 245; 288; 302). Although several mechanisms have been proposed to explain the failure of Th17 cells to recover during infection, the true cause remains unknown. It is possible that the factors required for Th17 cells to develop may be dysregulated during infection. We hypothesized that SIV infection may lead to altered expression of Th17-promoting cytokines and/or their receptors, thereby preventing development of Th17 cells during infection.

The first aim of this study was to determine whether SIV infection alters expression of the four cytokines, IL-6, IL-21, IL-23, and TGF-β, which promote Th17

development. Although previous studies have examined these four cytokines individually in the context of other research questions, analysis in the context of Th17 differentiation during infection has not been performed. As such, a complete Th17 cytokine profile has not been analyzed in the mucosal tissues where Th17 cells are essential for immune homeostasis.

Previous studies have found that IL-6 expression is upregulated during HIV infection (34; 96; 320). In fact, circulating IL-6 levels are commonly utilized as a biomarker for inflammation and immune activation in infection (267; 320). Similarly, TGF-β expression is elevated in HIV infection (226; 294), and IL-23 mRNA expression is increased in the mucosa during acute SIV infection (176).

However, it is not clear how HIV and SIV infection may affect expression of IL-21. Several studies have demonstrated a loss of IL-21-producing CD4+ T cells (166; 167; 245; 275) whereas other studies have shown IL-21-producing CD4+ T cells to be induced during acute and chronic HIV infection (358). It also appears that alternative cell types, such as CD8+ T cells, may be capable of producing IL-21 during infection (245; 274; 276; 323; 349).

The second aim was to assess whether the receptors for Th17-promoting cytokines are expressed on the surface of memory CD4+ T cells. The failure of cells to express any one of these necessary receptors required for cytokine signaling could be a potential mechanism to explain the failure of Th17 cells to develop during infection.

To date, there have been very few studies that have examined the expression of Th17-promoting cytokine receptors on CD4+ T cells in the context of HIV and SIV infection. Studies have shown sIL-6R levels to be increased in HIV infection (164) while

the CD126 component of the IL-6R was found to be elevated on monocytes, CD4+ T cells, and B cells during infection (334). Similarly, expression of IL-21R was increased on memory B cells during HIV infection (301). However, little is known about the effect of HIV infection on expression of the receptors for IL-23 and TGF-β.

The third and final aim was to determine whether T cells from SIV-infected animals are capable of differentiating into Th17 cells *in vitro*. If CD4+ T cells from SIV-infected animals differentiate into Th17 cells when provided with the appropriate cytokine cocktail, it is likely that one of the cytokine signaling pathways may be defective during SIV infection. However, the failure of cells from SIV-infected animals to produce IL-17 even when provided with the appropriate signals would likely suggest that mechanisms other than cytokine signaling, such as intracellular pathways, are involved.

RESULTS

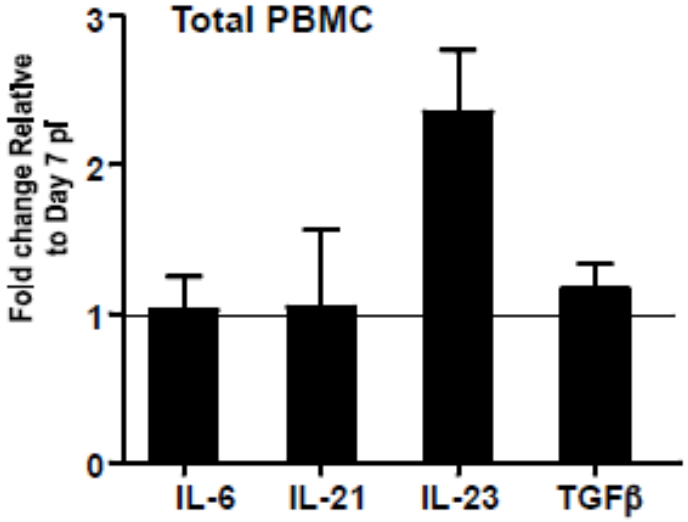
Ex vivo expression of Th17-promoting cytokines in peripheral blood and jejunum during SIV infection

IL-17 expression in CD4+ T cells was significantly suppressed during acute SIV infection and persisted into chronic infection. To determine if a lack of Th17-promoting cytokines was responsible for suppression of the Th17 subset during infection, we assessed expression levels of the four Th17-promoting cytokines IL-6, IL-21, IL-23, and TGF-β in total PBMC and jejunum by relative qRT-PCR (**Figure 21**). There was no significant change in expression levels of these four cytokine genes in peripheral blood or in the mucosa during either acute or chronic SIV infection (**Figure 21a-c**). Interestingly, IL-23 expression was elevated in peripheral blood and in mucosal tissues during infection

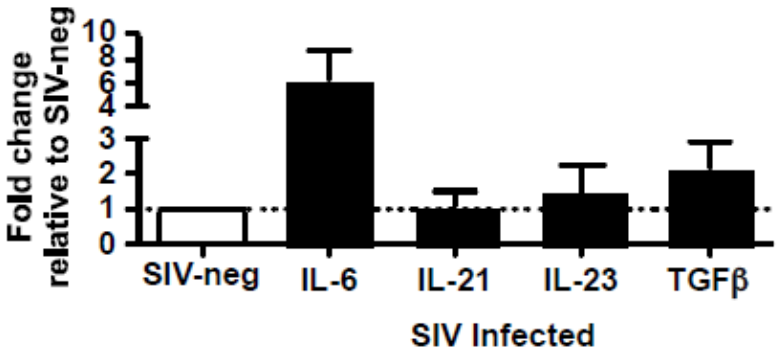
Figure 21. There is no deficiency in *ex vivo* expression of Th17-promoting cytokines during SIV infection. (a) Expression of Th17-promoting cytokines in peripheral blood during acute infection. Expression of IL-6, IL-21, IL-23, and TGF-β was assessed by relative qRT-PCR using RNA isolated from total PBMC and normalized using β-actin. Fold changes were calculated using the ddCT method (307), with fold changes in mRNA expression at day 35 p.i. are shown relative to day 7 p.i. The dashed line represents baseline expression. Expression of Th17-promoting cytokines in (b) total PBMC and (c) jejunal cells during chronic infection. Fold changes in SIV-infected animals are shown relative to uninfected controls. The dashed line represents baseline expression of healthy controls.

Previously published as (35)

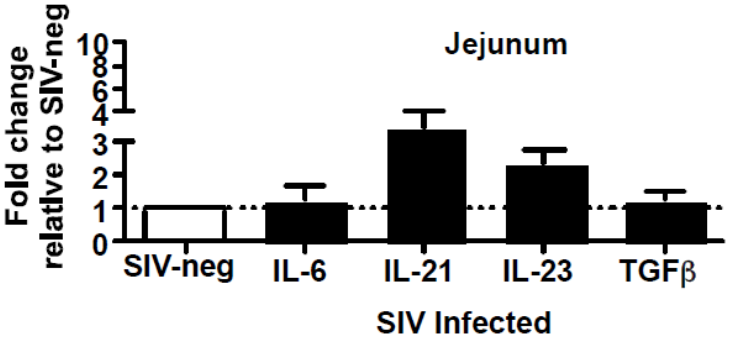
a)


Total PBMC
Fold change Relative to Day 7 pi
3
2
1
0
IL-6
IL-21
IL-23
TGFβ

b)


Fold change relative to SIV-neg
10
8
6
4
3
2
1
0
SIV-neg
IL-6
IL-21
IL-23
TGFβ
SIV Infected

c)


Jejunum
Fold change relative to SIV-neg
10
8
6
4
3
2
1
0
SIV-neg
IL-6
IL-21
IL-23
TGFβ
SIV Infected

(**Figure 21a and c**). Additionally, we found high levels of IL-6 expression in PBMC (**Figure 21b**), as well as increased expression of IL-21 in the jejunal mucosa (**Figure 21c**). Taken together, these data suggest that the essential Th17-promoting cytokines were present in elevated levels during SIV infection and that a paucity of Th17-promoting cytokines during SIV infection was likely not a cause of suppression of Th17 cells during infection.

Production of Th17-promoting cytokines in response to TLR stimulation

Next we assessed if the capacity of PBMC and jejunal cells to produce Th17 cytokines was altered during SIV infection. Adherent and non-adherent fractions of PBMC, along with total jejunal cells, were stimulated with a cocktail of TLR ligands in order to induce cytokine production, and the levels of IL-6, IL-21, IL-23 and TGF-β mRNA were determined by qRT-PCR assay (**Figure 22**). Adherent cells likely contain cells such as monocyte/macrophages and dendritic cells that produce IL-6, IL-23 and TGF-β, while non-adherent cells contained predominantly lymphocytes which are known to be important source of IL-21 and TGF-β.

Our data showed that there was no deficiency in the capacity of the adherent cells from either PBMC or jejunum to produce mRNA of any of the four cytokines (**Figure 23**). However, TGF-β expression was slightly downregulated in the non-adherent fraction (**Figure 23b**), potentially due to the loss of regulatory T cells in peripheral blood during infection (59; 288) which are known to be important sources of TGF-β. Taken together, these data indicate that the functional capacity of cells to produce Th17-promoting cytokine mRNA is not altered during SIV infection and support the above observation

Figure 22. Experimental strategy for *in vitro* stimulation of cells using TLR ligands. Total PBMC were split into adherent and non-adherent fractions. Adherent and non-adherent fractions, along with total jejunal cells, were stimulated with a cocktail of TLR ligands (CpG, imiquimod, LPS, and polyI:C) for 3 days at 37˚C. Total RNA was subsequently isolated from cells and used for qRT-PCR for cytokine expression.

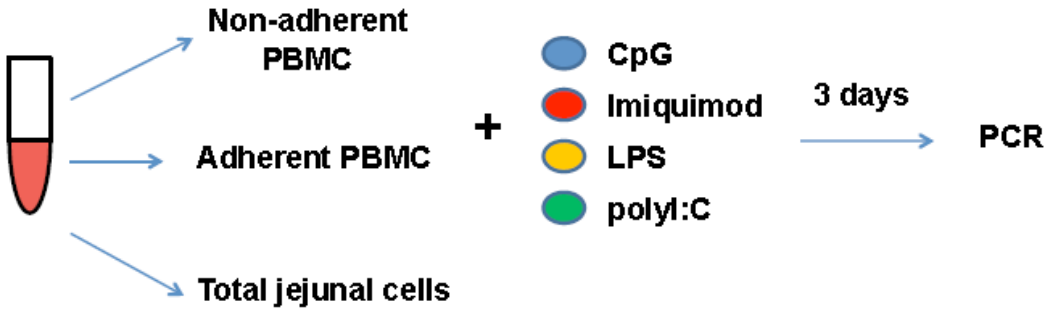
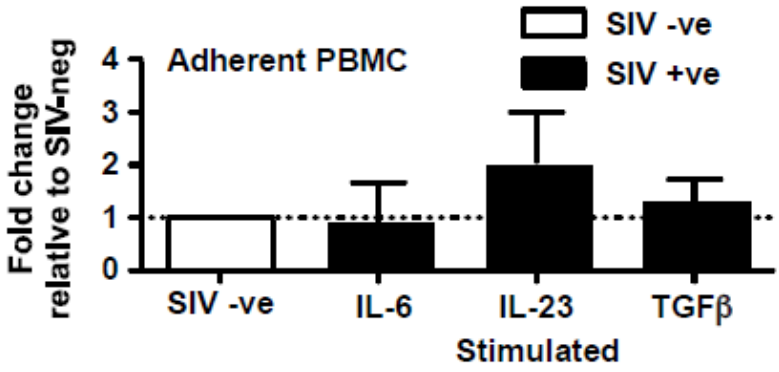
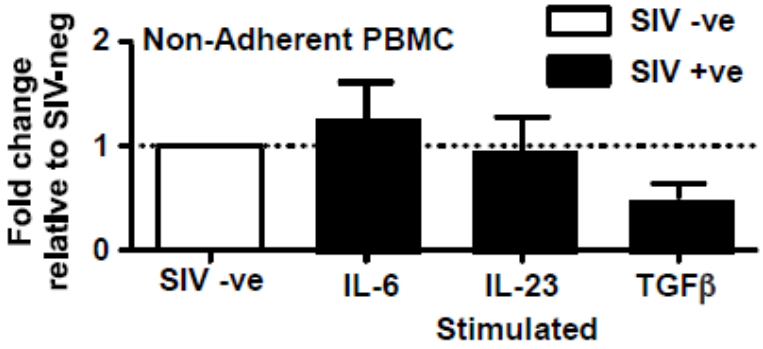

Non-adherent
PBMC
Adherent PBMC
Total jejunal cells
+
CpG
Imiquimod
LPS
polyI:C
3 days
PCR

Figure 23. Cells retain the functional capacity to produce Th17-promoting cytokines in response to stimulation during SIV infection. Expression of Th17-promoting cytokines in (a) adherent and (b) non-adherent PBMC and (c) total cells from the jejunum following stimulation with TLR ligands. Cells were stimulated with TLR ligands for 3 days and total RNA was isolated from cells and used to assess expression of IL-6, IL-21, IL-23, and TGF-β by relative qRT-PCR as normalized to β-actin. Fold changes were calculated using the ddCT method (307), with fold change in expression in stimulated samples shown relative to uninfected controls. The dashed line represents baseline expression.

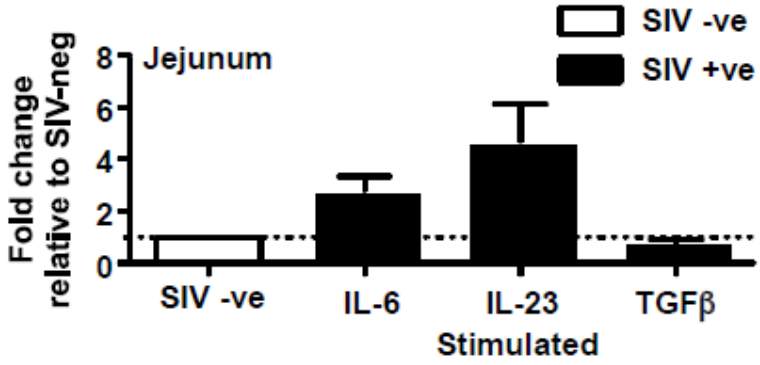
a)


Adherent PBMC
SIV -ve
SIV +ve
Fold change relative to SIV-neg
4
3
2
1
0
SIV -ve
IL-6
IL-23
TGFβ
Stimulated

b)


Non-Adherent PBMC
SIV -ve
SIV +ve
Fold change relative to SIV-neg
2
1
0
SIV -ve
IL-6
IL-23
TGFβ
Stimulated

c)


Jejunum
SIV -ve
SIV +ve
Fold change relative to SIV-neg
8
6
4
2
0
SIV -ve
IL-6
IL-23
TGFβ
Stimulated

that paucity of Th17 cytokines was likely not the cause of Th17 deficiency during SIV infection.

Evaluation of expression of Th17-promoting cytokines by ELISA

The previous results suggested that the message levels of the Th17-promoting cytokines IL-6, IL-21, IL-23, and TGF-β were not altered during SIV infection. However, it is not known if these four cytokines are present in circulation. To evaluate circulating protein levels of Th17-promoting cytokines, we used three commercially available ELISA kits for human IL-6, IL-23 and TGF-β to determine cytokine expression in plasma samples from acute and chronically infected animals. We were unable to determine the expression of IL-21 due to the lack of reliable assays.

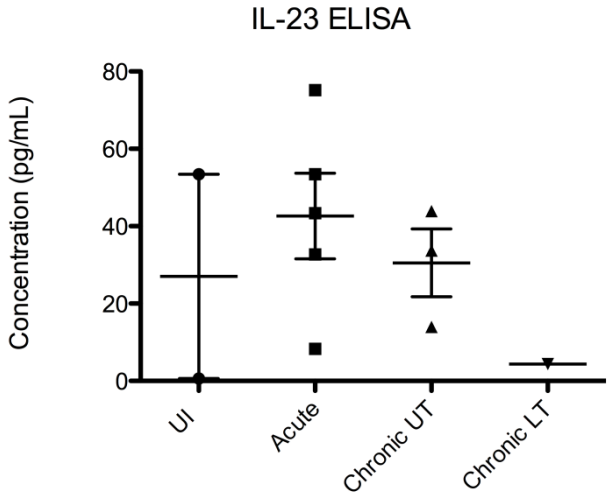
The expression of all three Th17-promoting cytokines examined was highly variable (**Figure 24**). However, IL-6 and IL-23 levels were elevated during both acute and chronic infection (**Figure 24a-b**) with a detectable level of TGF-β in SIV-infected animals (**Figure 24c**). Surprisingly, not all animals had detectable responses suggesting that cross-reactivity concerns may have played a role. Although the expression of Th17-promoting cytokines appeared to be variable during infection, the trend in cytokine levels appeared to support the mRNA expression levels determined by qRT-PCR.

Expression of Th17-promoting cytokine receptors on memory CD4+ T cells during SIV infection

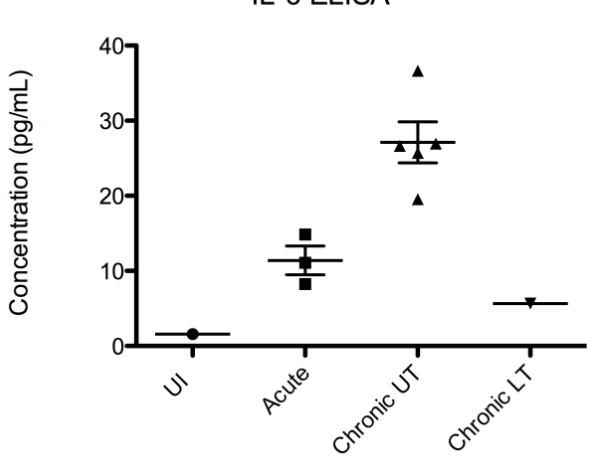
Our results suggested that deficiency in cytokine expression was likely not a cause for the failure of Th17 cells to develop during SIV infection. Although Th17-promoting cytokine expression was not deficient, we hypothesized that suppressed IL-17 expression may be due to the altered expression of receptors required for adequate

Figure 24. Expression of Th17-promoting cytokines in plasma by ELISA mirror PCR results. Expression of (a) IL-23 (b) IL-6 and (c) TGF-β was assessed in plasma using commercially available ELISA kits for human IL-23, IL-6, and TGF-β. Plasma samples were evaluated from uninfected (UI) and SIV-infected animals during acute (Acute) and chronic (Chronic UT) infection, as well as those that had undergone long-term ART (Chronic LT) therapy with a combination of FTC and PMPA.

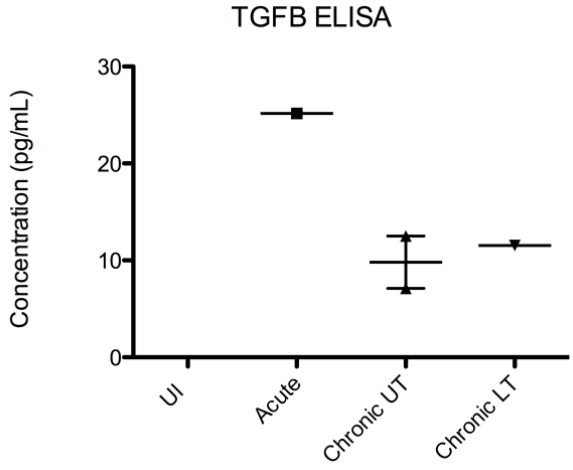
a)


IL-23 ELISA
Concentration (pg/mL)
80
60
40
20
0
UI
Acute
Chronic UT
Chronic LT

b)


IL-6 ELISA
Concentration (pg/mL)
40
30
20
10
0
UI
Acute
Chronic UT
Chronic LT

c)


TGFB ELISA
Concentration (pg/mL)
30
20
10
0
UI
Acute
Chronic UT
Chronic LT

signaling by the Th17-promoting cytokines. To assess whether the receptors for these cytokines were changed during SIV infection, live memory CD4+ T cells were sorted with high purity from uninfected and SIV-infected animals and used to determine the expression of IL-6R, IL-21R, IL-23R, and TGF-βR mRNA by relative qRT-PCR as described in the Materials and Methods.

We found that there was no major difference in the expression of Th17-promoting cytokine receptor mRNA in CD4+ memory T cells from SIV-infected animals as compared to healthy controls, except for IL-21R, which appeared to be downregulated (**Figure 25**). This is likely due to the loss of IL-21-producing CD4+ T cells during infection (245), which have been shown to play an important role in upregulation of IL-21R (365).

In vitro Th17 differentiation in rhesus macaques

Our data indicated that the key Th17-promoting cytokines and their cognate receptors were not deficient during SIV infection, suggesting that other mechanisms were likely responsible for the failure of Th17 cells to recover during infection. We hypothesized that SIV infection may alter the ability of CD4+ T cells to differentiate into Th17 cells even when all Th17-promoting cytokines were provided.

To determine if CD4+ T cells from SIV-infected rhesus macaques had a differentiation defect, live naïve CD4+ T cells were negatively sorted from total PBMC and cultured *in vitro* under Th17-polarizing conditions as described in the Materials and Methods (**Figure 26**). Memory CD4+ T cells were excluded as these cells were likely to include pre-existing Th17 cells.

Figure 25. Expression of Th17-promoting cytokine receptors on memory CD4+ cells is not altered during SIV infection. Memory CD4+ T cells were sorted using the gating strategy previously described in **Figure 6**. Total RNA was isolated and used to evaluate expression of the Th17-promoting cytokine receptors IL-6R, IL-21R, IL-23R, and TGF-βR by relative qRT-PCR as normalized to β-actin. Fold changes were calculated using the ddCT method (307), with fold change in expression in SIV-infected samples shown relative to uninfected controls. The dashed line represents baseline expression of healthy controls.

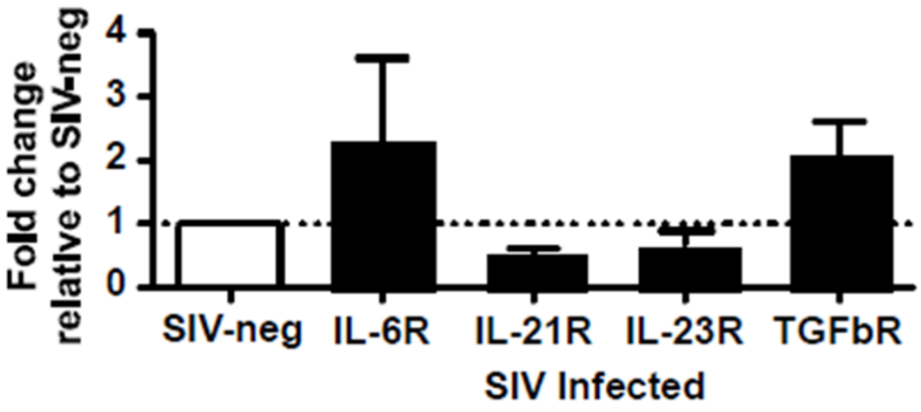
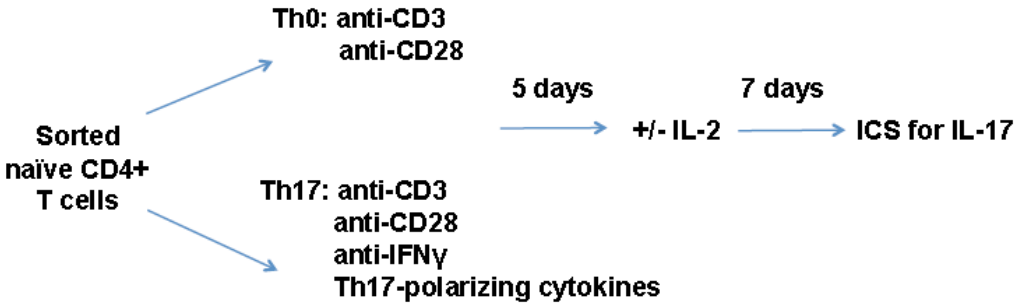
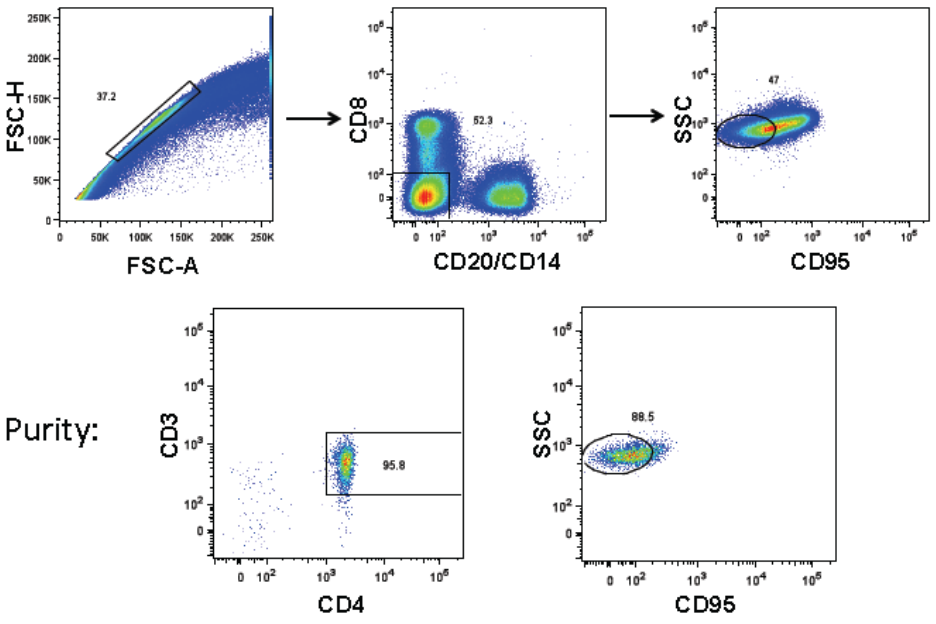

Fold change relative to SIV-neg
4
3
2
1
0
SIV-neg
IL-6R
IL-21R
IL-23R
TGFbR
SIV Infected

Figure 26. *In vitro* Th17 differentiation protocol for rhesus macaques. (a) Overview of Th17 differentiation protocol for rhesus macaques. The protocol was adapted from a human Th17 *in vitro* differentiation protocol developed in the laboratory of Dr. John O'Shea at NIH. (b) Gating strategy used for sorting naive CD4+ T cells for *in vitro* Th17 differentiation experiments. To avoid activating the cells by labeling with CD4 and CD95, naïve CD4+ T cells were sorted from total PBMCs using a negative gating strategy. Total PBMC were labeled with anti-CD8, anti-CD20, anti-CD14, anti-CD95, and VIVID and naïve CD4+ T cells were identified based on negative staining for the lineage markers CD8, CD20 and CD14 and negative staining for the memory marker CD95. Naïve CD4+ T cell purity was assessed by staining a small aliquot of sorted cells with anti-CD3 and anti-CD4.

a)


Th0: anti-CD3
anti-CD28
5 days
7 days
Sorted
naïve CD4+
T cells
+/- IL-2
ICS for IL-17
Th17: anti-CD3
anti-CD28
anti-IFNγ
Th17-polarizing cytokines

b)


FSC-H
FSC-A
37.2
CD8
CD20/CD14
52.3
SSC
CD95
47
Purity:
CD3
CD4
95.8
SSC
CD95
88.5

Initial differentiation experiments were not successful in that cells did not form blasts indicative of activation and proliferation in response to stimulation, suggesting that the cells were not responding to the initial simulation with anti-CD3 and anti-CD28. However, CD3 in rhesus macaques is highly polymorphic, unlike in humans (345). Previous studies have shown that the FN18 clone of anti-CD3 that was used for stimulation recognizes one of the polymorphic regions; therefore, not all animals are responsive to FN18 (345). It is possible that the rhesus macaques used in our studies were not responsive to the FN18 clone. To overcome this limitation, an additional clone of anti-CD3, 6G12, was obtained from the Non Human Primate Reagent Resource and used along with clone FN18 for stimulation. Interestingly, optimal activation of naïve CD4+ T cells occurred when stimulation was performed using a combination of the two anti-CD3 antibodies.

However, during initial experiments we failed to detect any IL-17 expression by flow cytometry in response to polarization, even in cells from healthy control animals despite the fact that cells were responding to anti-CD3 and anti-CD28 stimulation. Interestingly, IFNγ expression was also absent in cultured CD4+ T cells, potentially as a result of the neutralizing activity of the anti-IFNγ antibodies.

The differentiation protocol utilized for these studies was based on conditions used for *in vitro* differentiation of human Th17 cells (58). However, it lacked two cytokines that have been shown to play important roles in the development of Th17 cells: IL-21 and TGF-β (31; 172; 232; 235; 269; 289; 341; 346; 368). It is possible that the absence of one or both of these cytokines was the reason for the lack of Th17 induction even in cells from healthy rhesus macaques. To test this hypothesis, we set up cultures

that included TGF-β or TGF-β and IL-21 in addition to the other Th17-promoting cytokines; however, we did not observe IL-17-producing cells in these cultures.

We next assessed if cell death was a cause for the lack of IL-17 production in these cultures by staining for the live/dead maker VIVID. Our results appeared to show that a large number of cells in these stimulated cultures were likely undergoing apoptosis due to continuous overstimulation with anti-CD3 antibodies. To rule this out, we avoided restimulating the cells after they were split following proliferation. However, when we analyzed these cultures in parallel with restimulated cultures, cell death persisted and IL-17 production remained undetectable.

As the cells were stimulated with PMA/ionomycin and Brefeldin-A for 4 hours after culture, we sought to determine if PMA and ionomycin stimulation was a cause for the death of these cells. We examined IL-17 production immediately after culture in the absence of PMA and ionomycin. When these cells were compared to those Th17-conditioned cells that had been restimulated with PMA/ionomycin, we did not observe any difference in either cell viability or Th17 differentiation.

The above Th17 differentiation experiments were performed using cells from peripheral blood, due to their availability and cell numbers. However, Th17 cells differentiate in the lymph nodes, where antigen-presenting cells present antigen to naïve T cells. To examine if cells from lymph nodes were more responsive to Th17-polarizing conditions, cells isolated from mesenteric lymph nodes of healthy animals were cultured under Th17-promoting conditions, after which IL-17 responses were assessed by flow cytometry. However, as with peripheral blood, no Th17 differentiation was observed using lymph node cells.

DISCUSSION

HIV and SIV infection are characterized by a dysfunction of Th17 cells that begins in acute infection and persists into chronic infection (40; 52; 104; 175; 178; 245; 288; 302). It is possible that the failure of Th17 cells to develop in SIV and HIV infection is due to external factors that promote a Th17 phenotype. While other studies have examined the role of inhibitory molecules such as indoleamine deoxygenase in suppression of Th17 cells (105; 292), the aim of this study was to determine if the failure of Th17 cells to repopulate during SIV infection was due to the deficiency of Th17-promoting cytokines or their receptors on CD4+ T cells.

Th17-promoting cytokines were normally expressed *ex vivo* in PBMC and jejunum during acute and chronic SIV infection, suggesting that alterations in the Th17-promoting cytokine profile are likely not responsible for the failure of Th17 cells to recover during infection. These data suggest that the factors that promote the differentiation of Th17 cells are present during infection. Interestingly, expression of IL-23 was elevated in PBMC during acute infection, potentially as a result of monocyte activation by translocated microbial products that occurs very early during infection. As such, our previous results showed that microbial products such as sCD14 and LBP are increased early in infection and activated CD14+ monocytes significantly upregulated the expression of IL-23 during acute SIV infection. We have previously shown that IL-23 was significantly elevated in mucosal tissues during acute infection (176). Additionally, IL-6, a critical Th17-promoting cytokine, was significantly enhanced during SIV infection, which supports previous studies that demonstrated high levels of plasma IL-6 during chronic HIV infection (34; 96; 320).

Other studies have demonstrated elevated levels of the Th17-promoting cytokines IL-6 and TGF-β in plasma during HIV and SIV infection (34; 96; 226; 294; 320). There is some debate about whether the expression of IL-21 is increased. Some studies have shown that plasma IL-21 levels were downregulated during infection, which may be due to loss of Th17 cells which are an important source of IL-21 (166; 167; 245; 275). However, other studies (366) have found that IL-21 levels were significantly higher during infection, most likely due to the expansion of T follicular helper cells (76; 221; 283) which are major producers of IL-21. It is also possible that CD8+ T cells, which have been shown to be an alternative source of IL-21, may compensate for the loss of Th17 cells by producing IL-21 during infection (245; 274; 276; 323; 349). As such, IL-21 was found to be elevated in the mucosa during chronic SIV infection. As loss of CD4+ T cells in the mucosa is accompanied by a significant increase in CD8+ T cells (206), it is likely that the increased expression of IL-21 observed in the jejunum was due to increased production from CD8+ T cells.

Circulating protein levels of Th17-promoting cytokines in plasma were also assessed during infection. Although expression of IL-6, IL-23, and TGF-β appeared to be highly variable in the samples analyzed, the trend observed in the ELISA data was comparable to the results obtained from PCR analysis. It is possible that the labile nature of cytokines in plasma may be at least partially responsible for the technical challenges in the ELISA experiments. Alternatively, it is likely that the human ELISA reagents were not highly cross-reactive with non-human primate samples.

Although we did not observe any major changes in the expression of receptors for the various Th17-promoting cytokines in SIV-infected animals, IL-21R expression

appeared to be slightly downregulated. The exact reason for suppression of IL-21R is not clear. IL-21R is induced by IL-21 in an autocrine manner (365) and it is possible that the loss of certain subsets of IL-21-producing CD4+ T cells as reported previously (245) may be a contributing factor.

It was surprising that the *in vitro* differentiation experiments under Th17-polarizing conditions failed to induce Th17 cells. Although many studies have been published using *in vitro* differentiation of Th17 cells for humans and mice, there are no reports characterizing *in vitro* differentiation of non-human primate Th17 cells. It is possible that the human *in vitro* differentiation protocol we used for differentiating rhesus macaque Th17 cells was not adequate. Due to the lack of access to human cells and tissues, we were unable to perform *in vitro* differentiation experiments in human cells as a positive control.

Additional studies such as the use of an APC-T cell co-culture strategy to promote Th17 differentiation from naïve cells or including neutralizing antibodies against other cytokines such as IL-4 may be yield better results. Antigen-presenting cells such as myeloid cells are likely a critical supporting cell type for Th17 differentiation, not only through antigen presentation but also through cytokine production. The use of the co-culture model may also provide a more physiologically relevant means of T cell differentiation.

In conclusion, the studies reported here demonstrate that Th17-promoting cytokines and their cognate receptors are not deficient during SIV infection. This suggests that mechanisms other than extracellular factors are likely contributing to the suppression of IL-17 responses during infection.

CHAPTER 5: Altered intracellular signaling may be a cause for Th17 dysregulation during SIV infection

INTRODUCTION AND RATIONALE

Interactions between Th17-promoting cytokines and their cognate receptors induce a series of intracellular signaling events that lead to production of IL-17. The most critical signaling pathway for the induction of IL-17 is the Janus-associated kinase (JAK)/ Signal transducer and activator-3 (STAT3) pathway (158; 270). During initial differentiation of a naïve CD4+ T cell, binding of IL-6 to its receptor induces phosphorylation of STAT3 by JAKs. Following phosphorylation, activated STAT3 dimerizes and translocates to the nucleus, where it binds to the promoter of IL-17 and other target genes, inducing transcription (270). In addition to IL-6, other Th17-promoting cytokines such as IL-21 and IL-23 also induce activation of the STAT3 signaling cascade (57; 172; 346).

The importance of the STAT3 signaling pathway in IL-17 production is evident from both mouse and human models. The immune disorder Job's Syndrome is characterized by high levels of circulating IgE and a complete absence of Th17 cells (133; 247). The lack of Th17 cells in these patients has been associated with mutations in STAT3 that render it non-functional; as such, the clinical picture in patients with Job's Syndrome is characterized by recurrent bacterial and fungal infections (133; 247). Likewise, conditional knockdown of STAT3 in mouse models prevented expression of IL-17 by CD4+ T cells (147; 355).

As our previous data suggested that the necessary cytokines and receptors required for Th17 differentiation were normally expressed in SIV infection, we

hypothesized that dysregulation of the intracellular STAT3 signaling pathway may be suppressing the induction of Th17 cells during SIV infection.

STAT3 signaling is regulated by 3 proteins, namely Suppressor of Cytokine Signaling 3 (SOCS3), protein tyrosine phosphatase SHP2, and Protein Inhibitor of Activated STAT3 (PIAS3). SOCS3 targets the earliest steps in the JAK/STAT3 signaling pathway by interacting with JAKs and inhibiting the phosphorylation and activation of STAT3 (94). Knockdown of SOCS3 in mice is accompanied by enhancement of Th17 responses (289) while overexpression of SOCS3 resulted in suppression of IL-17 production.

Unlike SOCS3, SHP2 possesses phosphatase activity and dephosphorylates STAT3 after it has been activated (189; 272). Dephosphorylation of STAT3 by SHP2 has been shown to occur in response to engagement of the IL-6R (189; 272) leading to the suppression of IL-17.

PIAS3 is an E3 SUMO-protein ligase that acts further downstream in the signaling cascade. PIAS3 inhibits IL-17 production by interacting with p-STAT3, thereby preventing its binding to the IL-17 promoter (9; 61; 354). Interestingly, expression of PIAS3 message is largely absent in Th17 cells, while further knockdown of PIAS3 via siRNA resulted in enhanced Th17 development (257).

While each of these regulatory proteins has been shown to play an important role in STAT3 regulation and subsequent production of IL-17, it is not known if they affect the induction of Th17 cells during HIV and SIV infection. It is possible that SIV infection alters either STAT3 activation or the proteins that regulate STAT3, thereby suppressing IL-17 production during infection.

The first aim was to assess whether the expression and activation of STAT3 were altered during SIV infection. Little is known about the JAK/STAT3 signaling pathway in Th17 cells during HIV and SIV infection. Previous studies have shown that STAT3 activation was increased in the gastrointestinal tract of SIV-infected animals, as p-STAT3 levels were found to be significantly elevated (250). Further analysis suggested that CD3+ T cells and macrophages were likely the main sources of p-STAT3 (250). However, as most of the CD4+ T cells in the gastrointestinal tract are depleted during SIV infection it is possible that the increased p-STAT3 levels were restricted to CD8+ T cells.

The second aim was to determine whether the regulation of STAT3 was altered during SIV infection. Previous studies have found that SOCS3 message levels were upregulated in HIV infection, while protein levels were downregulated (246; 250). Elevated SOCS3 expression was associated with increased HIV replication and infection at mucosal sites (8; 255). Additionally, SHP2 was shown to be recruited to IL-6R gp130 during HIV infection (24).

RESULTS

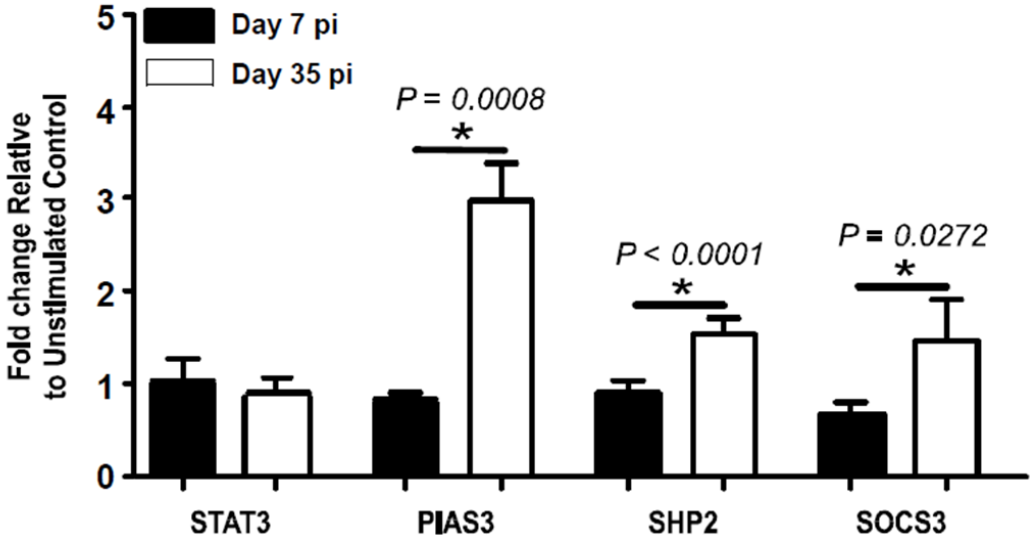
Expression of negative regulators of STAT3 during acute SIV infection

To determine if the negative regulatory factors played a role in decreased IL-17 production during SIV infection, total CD4+ T cells were negatively sorted from PBMC from infected animals and stimulated with recombinant IL-6 (rIL-6) for 15 minutes. Following short-term stimulation we assessed the expression levels of PIAS3, SHP2, and SOCS3 by qRT-PCR. The mRNA levels of all three factors were upregulated during infection whereas the expression of STAT3 mRNA remained unchanged (**Figure 27**).

Figure 27. Negative regulators of STAT3 are upregulated during acute SIV infection.

Total PBMC were labeled with anti-CD8, anti-CD20, anti-CD14 and total CD4+ T cells were negatively sorted by FACS as previously described in **Figure 7**. Sorted CD4+ T cells were stimulated with rIL-6 for 15 minutes and total RNA was isolated and used to assess expression of STAT3, PIAS3, SOCS3, and SHP2 by relative qRT-PCR as normalized to β-actin. Fold changes were calculated using the ddCT method (307), with fold change in expression in stimulated samples shown relative to unstimuated controls for days 7 and 35 p.i. The dashed line represents baseline expression of healthy controls.

Previously published as (35)


Day 7 pi
Day 35 pi
Fold change Relative to Unstimulated Control
5
4
3
2
1
0
P = 0.0008
*
P < 0.0001
*
P = 0.0272
*
STAT3
PIAS3
SHP2
SOCS3

To determine if the suppression of IL-17 production during SIV infection was due to the negative regulation of STAT3 signaling, we correlated the expression of PIAS3, SHP2, and SOCS3 with IL-17 expression (**Figure 28**). We found that each of three negative regulators had a significantly high negative correlation with expression of IL-17, suggesting that the increased expression of these regulatory genes may contribute to the suppression of IL-17 during SIV infection.

Expression and activation of STAT3 in SIV infection

Our results indicated that negative regulation of the STAT3 signaling pathway may be playing a role in SIV infection. To determine if specific regulatory pathways were involved, we first evaluated the phosphorylation status of STAT3 in CD4+ T cells during SIV infection.

As no antibodies specific for rhesus macaque total STAT3 and p-STAT3 were commercially available, we first screened human antibodies to assess cross-reactivity with rhesus macaque proteins, and titrated the amount of antibody and number of cells required for optimal staining using rhesus macaque PBMC. We identified anti-human total STAT3 and p-STAT3 antibodies that cross-reacted with rhesus macaque proteins and were capable of detecting both the alpha and beta isoforms of STAT3 (**Figure 29**). We also found that total STAT3 and p-STAT3 bands were detectable at cell concentrations as low as ~1 x 10^5; however ~5 x 10^5 cells appeared to be the optimal cell number to obtain a clear signal. Therefore, all subsequent experiments involving Western blots were performed using ~5 x 10^5 cells. Using these data we screened and identified anti-human PIAS3, SHP2, and SOCS3 antibodies that cross-reacted with rhesus macaques as well (**Figure 30**).

Figure 28. Expression of negative regulators of STAT3 correlates with IL-17 expression during acute SIV infection. Correlations between fold change in IL-17 mRNA expression and fold change in (a) PIAS3 (b) SHP2 and (c) SOCS3 mRNA expression in CD4+ T cells for days 7 and 35 p.i.

Previously published as (35)

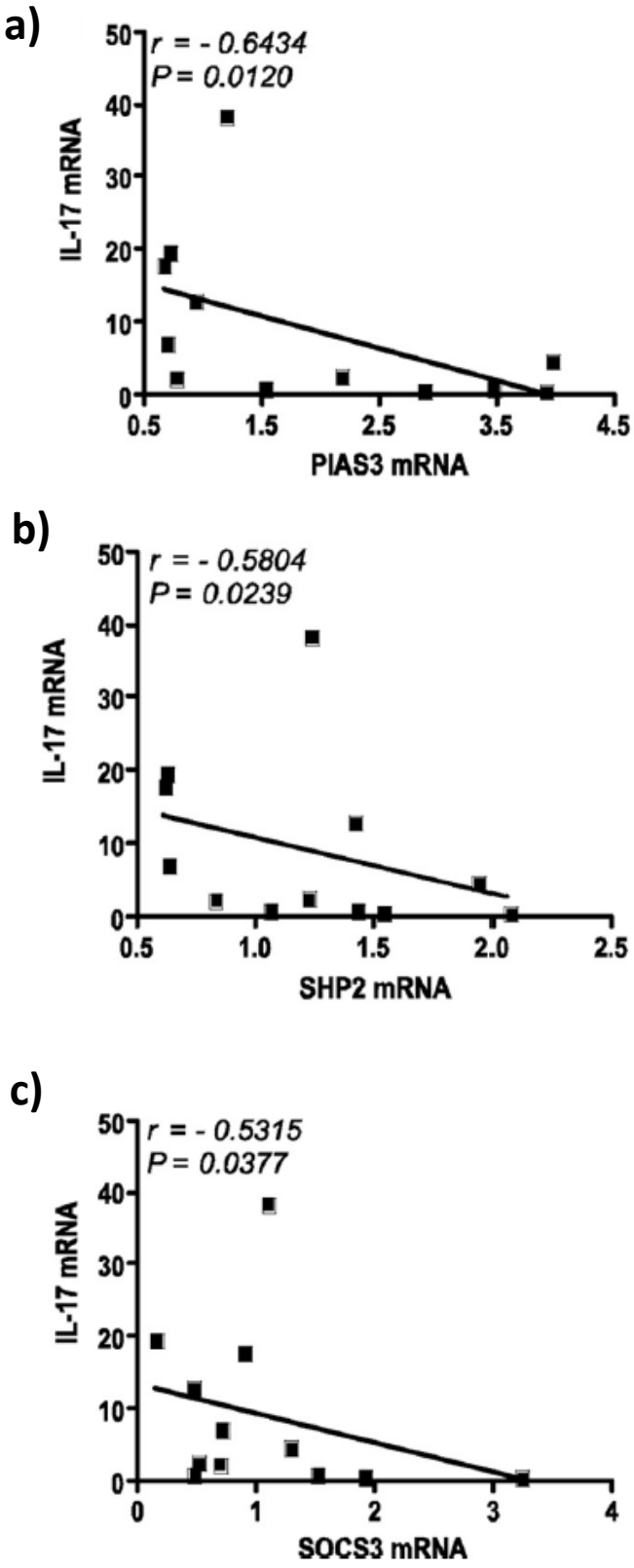

a)
r = - 0.6434
P = 0.0120
IL-17 mRNA
PIAS3 mRNA
b)
r = - 0.5804
P = 0.0239
IL-17 mRNA
SHP2 mRNA
c)
r = - 0.5315
P = 0.0377
IL-17 mRNA
SOCS3 mRNA

Figure 29. Human total STAT3 and p-STAT3 antibodies are cross-reactive with rhesus macaques. Western blots for human total STAT3 and p-STAT3 were performed using total PBMC lysates from rhesus macaques to determine if human total STAT3 and p-STAT3 antibodies were cross-reactive with non-human primate samples. To assess the optimal number of cells to be used for subsequent experiments, a titration was performed using 1×10^6, 5×10^5, 2×10^5, 1×10^5, 5×10^4, and 1×10^4 cells per lane. The alpha (86 kDa) and beta (79 kDa) isoforms of total STAT3 and p-STAT3 were detectable in rhesus cells and at various cell concentrations.

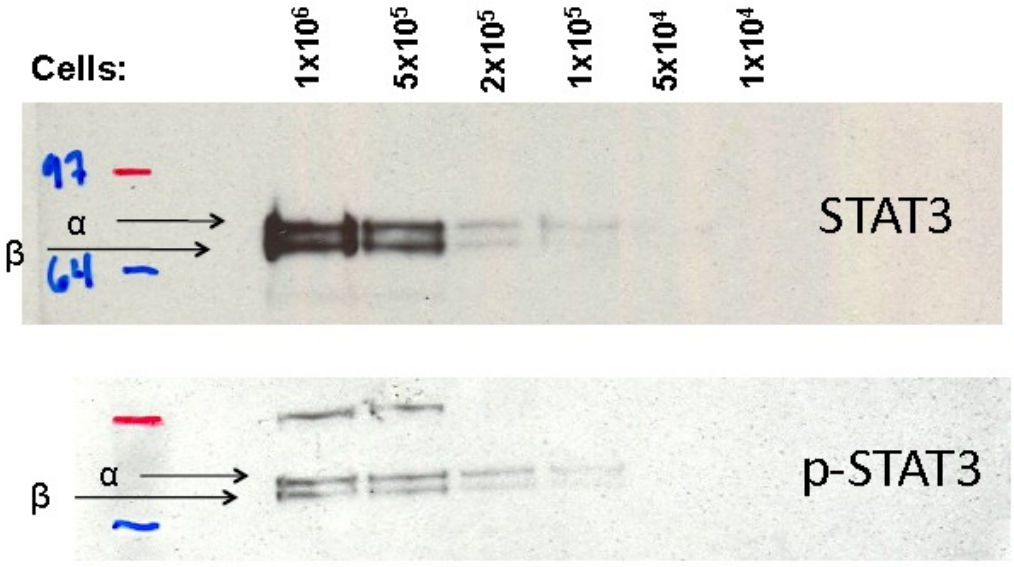
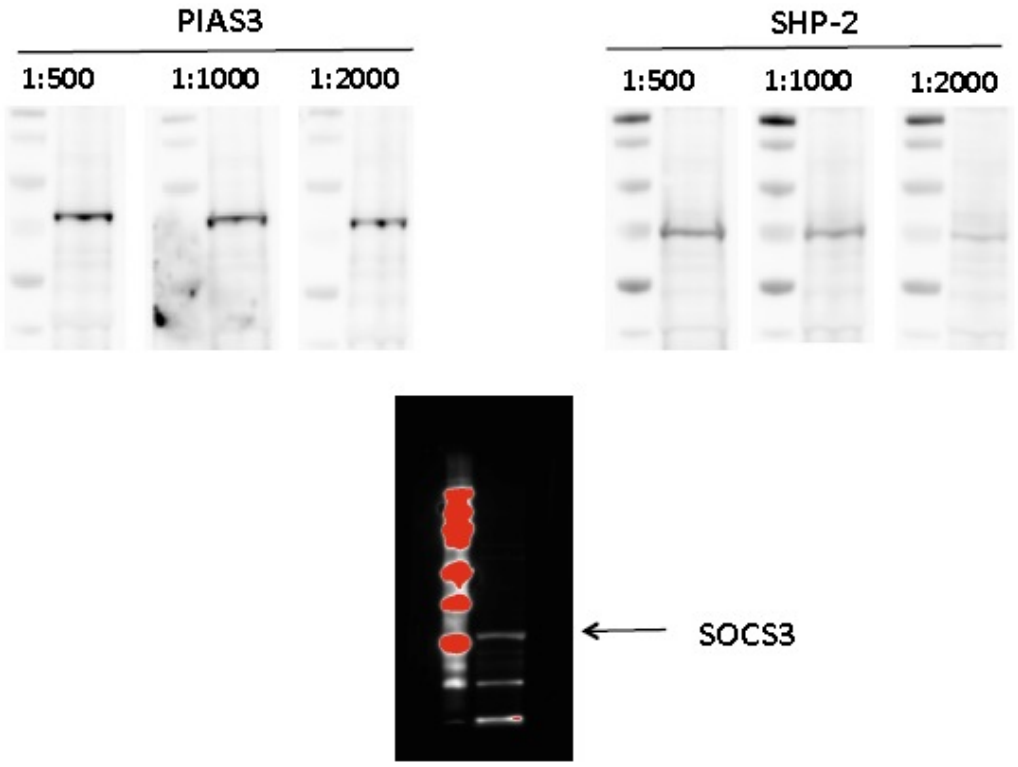

Cells:
1x10^6
5x10^5
2x10^5
1x10^5
5x10^4
1x10^4
97
64
α
β
STAT3
α
β
p-STAT3

Figure 30. Evaluation of human PIAS3, SHP2 and SOCS3 antibodies for cross-reactivity with rhesus macaques. Western blots for human PIAS3, SHP2, and SOCS3 were performed using total PBMC lysates from rhesus macaques to determine if human antibodies were cross-reactive with non-human primate samples. For PIAS3 and SHP2, titration of the antibody dilutions 1:2000, 1:1000, and 1:500 is shown. For SOCS3, the 1:500 dilution is shown.


PIAS3
1:500
1:1000
1:2000
SHP-2
1:500
1:1000
1:2000
SOCS3

We first examined the effect of SIV infection on total STAT3 and p-STAT3 using negatively sorted total CD4+ T cells from PBMC following short-term stimulation with rIL-6. We found that STAT3 phosphorylation occurred in CD4+ T cells in response to IL-6 stimulation in both uninfected and SIV-infected animals, as there was no significant difference in expression of either total STAT3 or p-STAT3 between uninfected and infected animals (**Figure 31**). These data indicated that there was no defect in STAT3 phosphorylation during SIV infection yet IL-17 expression remained suppressed. Taken together, these data suggested that the STAT3 signaling pathway may be defective downstream of STAT3 phosphorylation.

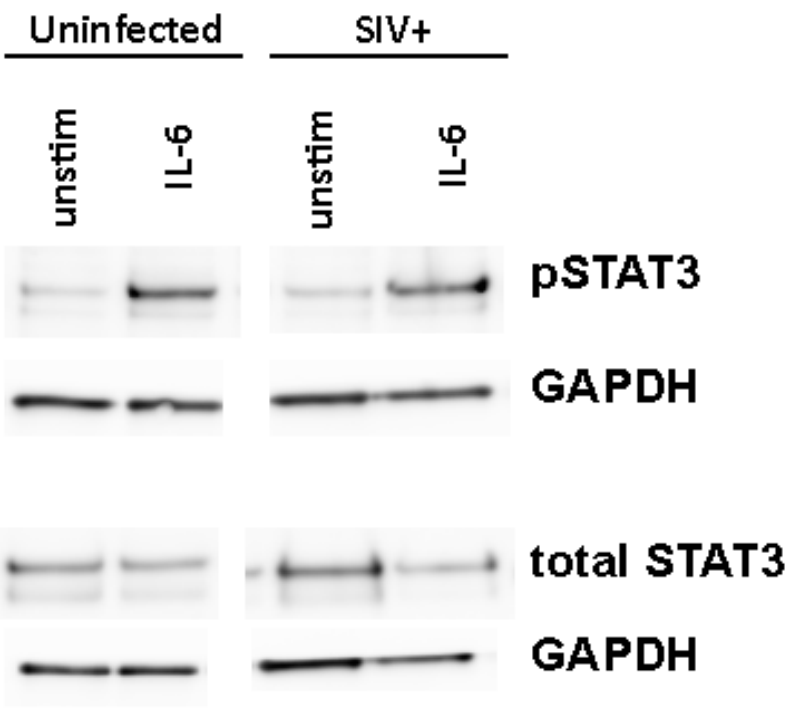
Expression of STAT3 negative regulators during SIV infection

Our data suggested that STAT3 activation was not defective in CD4+ T cells during SIV infection. Two of the negative regulatory factors, SOCS3 and SHP2, prevent STAT3 activation at the phosphorylation stage – SOCS3 prevents phosphorylation of STAT3 by JAKs, while SHP2 dephosphorylates activated STAT3. However, the third negative regulator, PIAS3, prevents binding of p-STAT3 to the IL-17 promoter. We hypothesized that increased PIAS3 expression during SIV infection may be preventing STAT3-mediated expression of IL-17.

To address this hypothesis, sorted CD4+ T cells from uninfected and SIV-infected animals were stimulated with rIL-6 and assessed for PIAS3 protein expression by Western blot (**Figure 32**). PIAS3 levels were significantly elevated in CD4+ T cells from SIV-infected animals as compared to uninfected animals (**Figure 32b**). Our results suggested that upregulation of PIAS3 during SIV infection may contribute to the dysregulation of STAT3 signaling.

Figure 31. Phosphorylation of STAT3 in CD4+ T cells in response to stimulation is not altered during SIV infection. (a) Expression of total STAT3 and p-STAT3 in sorted CD4+ T cells. Total CD4+ T cells were negatively sorted from total PBMC as previously described in **Figure 7**. Sorted cells were stimulated with rIL-6 and expression of p-STAT3 and STAT3 was assessed by Western blot in cell lysates. Blots for one representative uninfected and infected animal are shown, with 5 x 10^5 cells per lane. Densitometry was performed and b) fold change in rIL-6 stimulated samples was calculated relative to unstimulated controls after normalization to the housekeeping gene GAPDH.

a)


Uninfected
SIV+
unstim
IL-6
unstim
IL-6
pSTAT3
GAPDH
total STAT3
GAPDH

b)

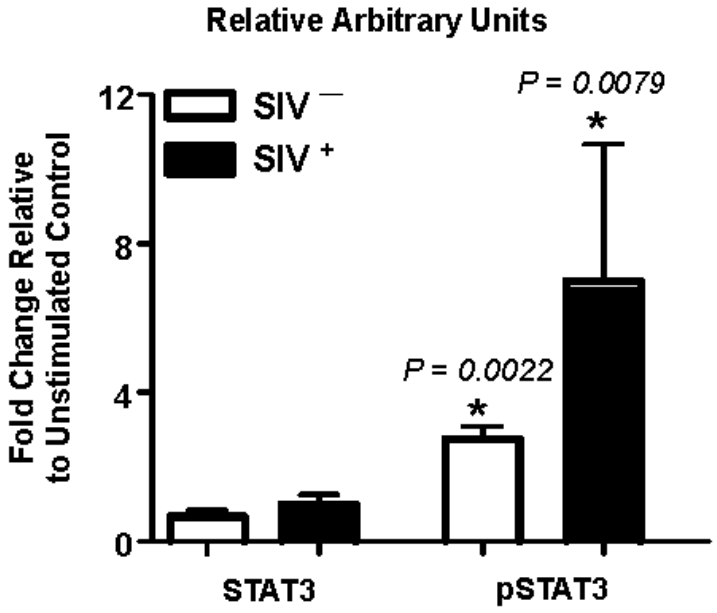
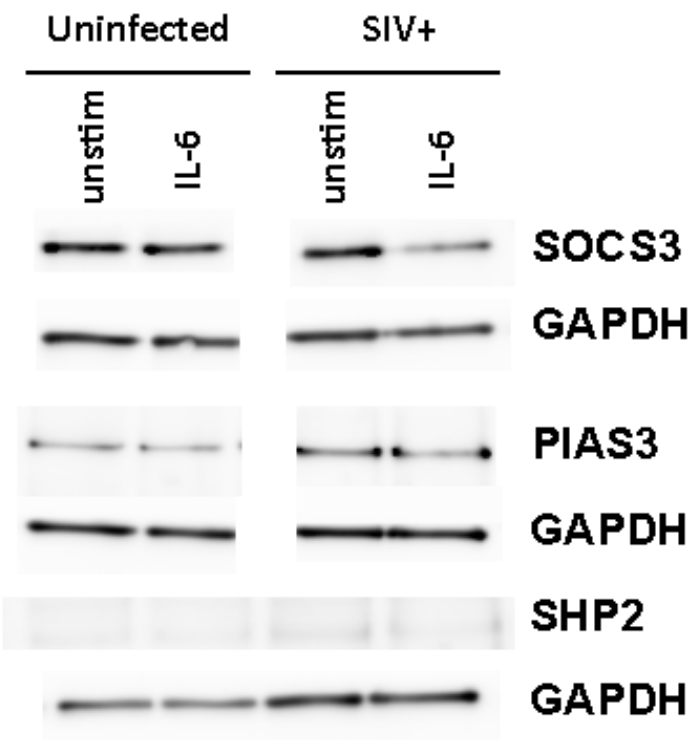
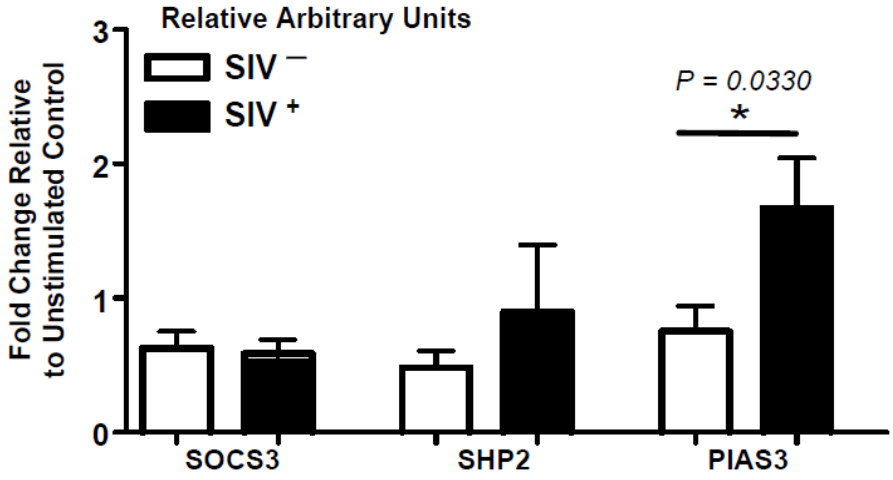

Relative Arbitrary Units
Fold Change Relative to Unstimulated Control
12
8
4
0
SIV −
SIV +
P = 0.0079
*
P = 0.0022
*
STAT3
pSTAT3

Figure 32. Expression of the STAT3 negative regulator PIAS3 is increased during SIV infection. (a) Expression of PIAS3, SOCS3, and SHP2 in sorted CD4+ T cells. Total CD4+ T cells were negatively sorted from total PBMC as previously described in **Figure 7**. Sorted cells were stimulated with rIL-6 and expression of PIAS3, SHP2, and SOCS3 was assessed by Western blot in cell lysates. Blots for one representative uninfected and infected animal are shown, with 5 x 10^5 cells per lane. Densitometry was performed and (b) fold change in rIL-6 stimulated samples was calculated relative to unstimulated controls after normalization to the housekeeping gene GAPDH.

a)


Uninfected
SIV+
unstim
IL-6
unstim
IL-6
SOCS3
GAPDH
PIAS3
GAPDH
SHP2
GAPDH

b)


Relative Arbitrary Units
SIV −
SIV +
Fold Change Relative to Unstimulated Control
3
2
1
0
P = 0.0330
*
SOCS3
SHP2
PIAS3

Characterization of IL-17 expression in Hut102 cells

Our results suggested a role for negative regulation of IL-17 by PIAS3 during SIV infection. To confirm a role for PIAS3 in Th17 dysregulation, we overexpressed PIAS3 in a human CD4+ T cell line (Hut102) that is stably infected with human T lymphotropic virus (HTLV). Hut102 cells constitutively produce IL-17 and IL-17 expression in these cells can be further enhanced in response to various stimuli such as PMA (224; 293). Accordingly, these cells demonstrate constitutive activation of STAT3, as evidenced by high levels of p-STAT3 (324).

We first confirmed that Hut102 cells produced IL-17 using flow cytometry (**Figure 33**) and qRT-PCR (**Figure 34**) following stimulation with rIL-6 or PMA/ionomycin in comparison to unstimulated cells. As previously reported, Hut102 cells constitutively produced significant amounts of IL-17 in the absence of stimulation (**Figure 33**), which was further enhanced following stimulation with rIL-6 and PMA/ionomycin (**Figure 34**).

We also evaluated the expression of Th17-associated genes, including STAT3, PIAS3, SHP2, and SOCS3, by qRT-PCR (**Figure 34**). As expected, Hut102 cells expressed high levels of STAT3, likely due to their constitutive activation and production of IL-17. Two of the STAT3 negative regulatory factors, SOCS3 and SHP2, were either expressed at very low levels or undetectable by PCR. Most importantly, these cells expressed very low levels of PIAS3. Based on these characteristics, the Hut102 cell line was used for subsequent optimization of *in vitro* experiments involving PIAS3 overexpression.

Figure 33. Hut102 cells constitutively express IL-17. Hut102 cells were labeled with anti-CD3, anti-CD4, and anti-IL-17 and analyzed using flow cytometry. Analysis of Hut102 cells demonstrate that these cells are CD3+ CD4+ and produce high amounts of IL-17 in the absence of stimulation.

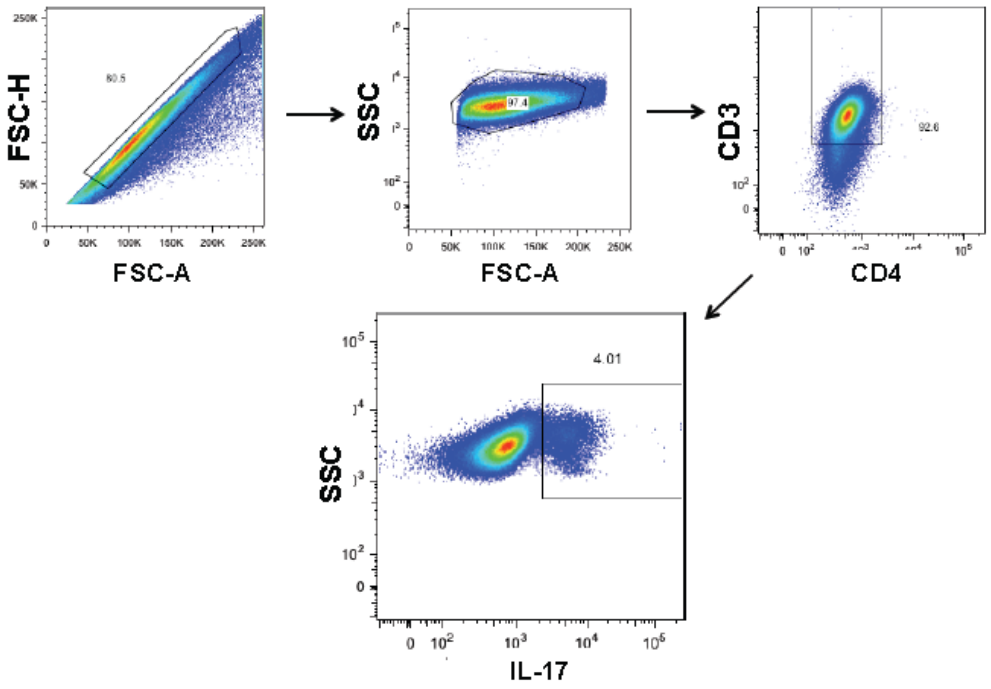
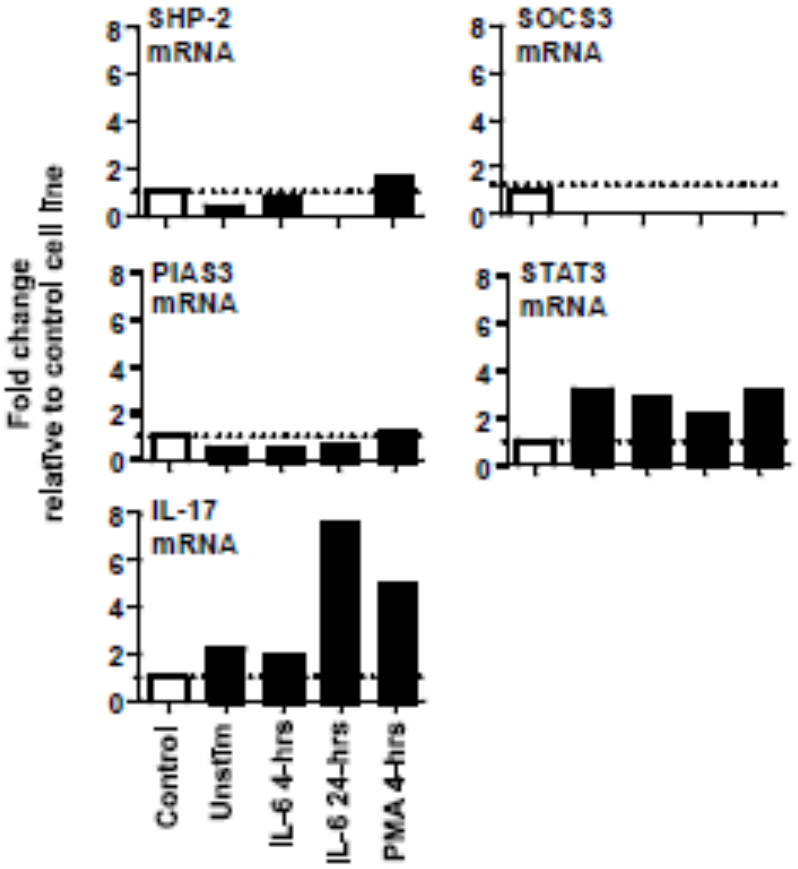

FSC-H
FSC-A
80.5
SSC
FSC-A
97.4
CD3
CD4
92.6
SSC
IL-17
4.01

Figure 34. mRNA expression of IL-17 and the Th17-associated genes SHP2, SOCS3, PIAS3, and STAT3 in Hut102 cells. Hut102 cells were left unstimulated or stimulated with PMA and ionomycin in the presence of Brefeldin-A for 4 hours or stimulated with rIL-6 for 4 hours or overnight. Total RNA was isolated and used to evaluate expression of the Th17-associated genes PIAS3, SHP2, SOCS3, and STAT3 by relative qRT-PCR as normalized to β-actin. Fold changes were calculated using the ddCT method (307), with fold changes shown relative to the control cell line CEMx174 which are negative for IL-17 expression. The dashed line represents baseline expression of control cells.


SHP-2
mRNA
SOCS3
mRNA
PIAS3
mRNA
STAT3
mRNA
IL-17
mRNA
Fold change
relative to control cell line
0
2
4
6
8
Control
Unstim
IL-6 4-hrs
IL-6 24-hrs
PMA 4-hrs

Overexpression of PIAS3 in Hut102 cell line

To test the role of PIAS3 in Th17 dysregulation, we used PIAS3 overexpression to demonstrate suppression of IL-17 in Hut102 cells. As very few rhesus-specific reagents are available, a pLOC vector expressing human PIAS3 was commercially obtained (**Figure 35**). In addition to the human PIAS3 ORF that is expressed under the control of an hCMV promoter, the vector also contained GFP for visualization of expression and a Blasticidin S resistance marker for selection of positive cells. Human and rhesus PIAS3 share greater than 98% homology (**Figure 36**), suggesting that the human PIAS3-expressing vector would be suitable for *in vitro* experiments. We confirmed this using primers and a probe designed against the rhesus macaque PIAS3 sequence in a qRT-PCR experiment with the human PIAS3 plasmid vector.

To overexpress PIAS3 in Hut102 cells, we generated lentiviral particles expressing human PIAS3 and VSV-G using calcium phosphate transfection of 293T cells as the packaging cell line. A lentiviral expression system was chosen as it is ideal for achieving overexpression in difficult-to-transduce cells, such as primary cells. Although initial experiments were aimed at determining whether PIAS3 overexpression resulted in IL-17 knockdown in a CD4+ T cell line, future experiments will be performed using primary cells from rhesus macaques to confirm a role for PIAS3 in the context of SIV infection. Unlike cell lines, primary cells are highly heterogeneous and in different stages of cell cycling, making efficient transfection of these cells difficult to achieve.

The 293T cells were transfected with the lentiviral construct along with the packaging and envelope plasmids (**Figure 37**). Transfection efficiency was confirmed using fluorescent microscopy at 24 and 48 hours post-transfection (**Figure 38**).

Figure 35. Plasmid map for LentiOrf expression vector for human PIAS3 (ORF = human PIAS3; Thermo Scientific)

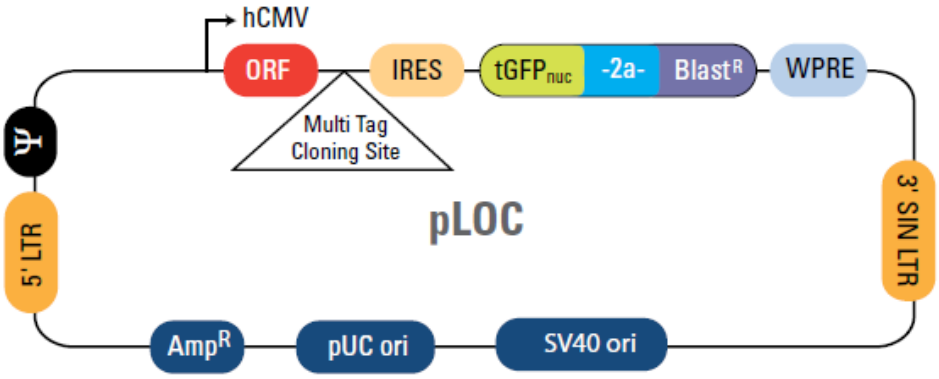
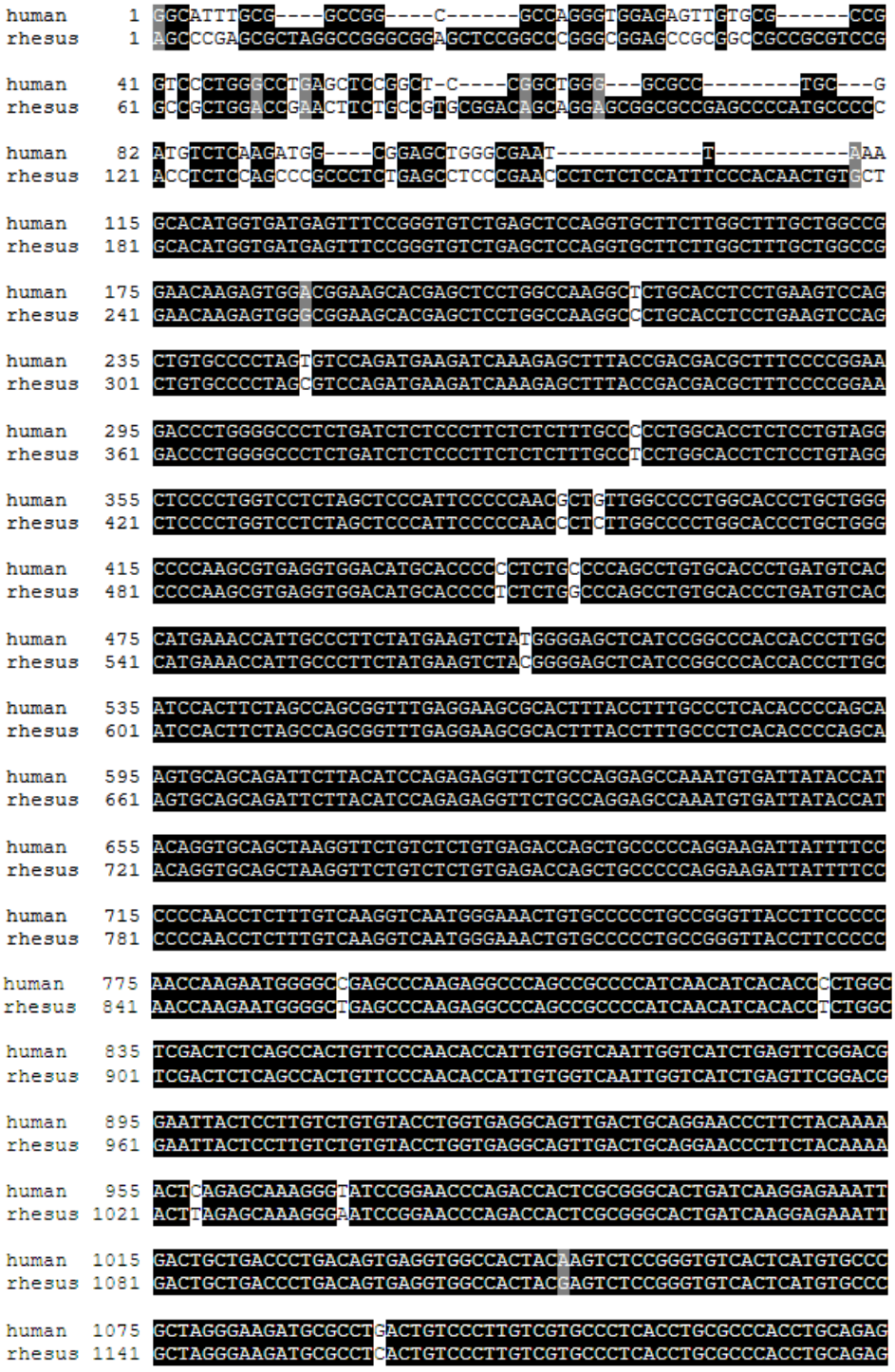


Figure 1. pLOC lentiviral ORF clone.

Figure 36. Sequence alignment showing homology between human and rhesus macaque PIAS3 mRNA. Alignments of the human (NM_006099.3) and rhesus macaque (XM_001095153.2) PIAS3 mRNA sequences were performed using Clustal O software and prepared using T-Coffee and BoxShade programs.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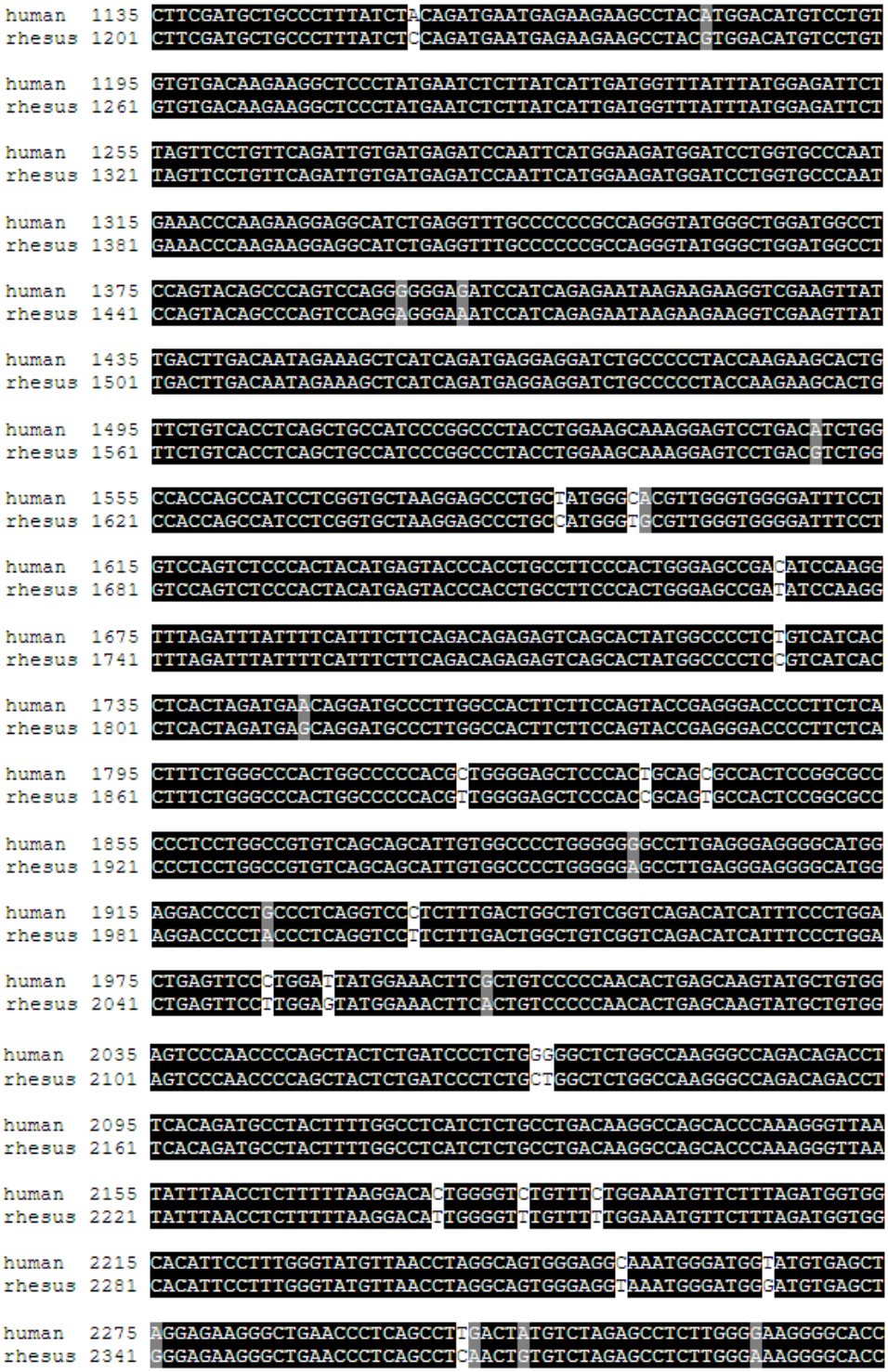
rhesus 1 AGCCCGAGCGCTAGGCCGGGCGGAGCTCCGGCCCGGGCGGAGCCGCGGCCGCCGCGTCCG
human 41 GTCCCTGGGCCTGAGCTCCGGCT-C----CGGCTGGG---GCGCC--------TGC---G
rhesus 61 GCCGCTGGACCGAACTCTCTGCCGTGCGGACAGCAGGAGCGGCGCCGAGCCCCATGCCCCC
human 82 ATGTCTCAAGATGG----CGGAGCTGGGCGAAT----------T----------AAA
rhesus 121 ACCTCTCCAGCCCGCCCTCTGAGCCTCCCGAACCCTCTCTCCATTTCCCACAACTGTGCT
human 115 GCACATGGTGATGAGTTTCCGGGTGTCTGAGCTCCAGGTGCTTCTTGGCTTTGCTGGCCG
rhesus 181 GCACATGGTGATGAGTTTCCGGGTGTCTGAGCTCCAGGTGCTTCTTGGCTTTGCTGGCCG
human 175 GAACAAGAGTGGACGGAAGCACGAGCTCCTGGCCAAGGCTCTGCACCTCCTGAAGTCCAG
rhesus 241 GAACAAGAGTGGCGGAAGCACGAGCTCCTGGCCAAGGCCCTGCACCTCCTGAAGTCCAG
human 235 CTGTGCCCCTAGTGTCCAGATGAAGATCAAAGAGCTTTACCGACGACGCTTTCCCCGGAA
rhesus 301 CTGTGCCCCTAGCGTCCAGATGAAGATCAAAGAGCTTTACCGACGACGCTTTCCCCGGAA
human 295 GACCCTGGGGCCCTCTGATCTCTCCCTTCTCTCTTTGCCCCCTGGCACCTCTCCTGTAGG
rhesus 361 GACCCTGGGGCCCTCTGATCTCTCCCTTCTCTCTTTGCCTCCTGGCACCTCTCCTGTAGG
human 355 CTCCCCTGGTCCTCTAGCTCCCATTCCCCCAACGCTGTTGGCCCCTGGCACCCTGCTGGG
rhesus 421 CTCCCCTGGTCCTCTAGCTCCCATTCCCCCAACCCTCTTGGCCCCTGGCACCCTGCTGGG
human 415 CCCCAAGCGTGAGGTGGACATGCACCCCCCTCTGCCCCAGCCTGTGCACCCTGATGTCAC
rhesus 481 CCCCAAGCGTGAGGTGGACATGCACCCCTCTCTGGCCCAGCCTGTGCACCCTGATGTCAC
human 475 CATGAAACCATTGCCCTTCTATGAAGTCTATGGGGAGCTCATCCGGCCCACCACCCTTGC
rhesus 541 CATGAAACCATTGCCCTTCTATGAAGTCTACGGGGAGCTCATCCGGCCCACCACCCTTGC
human 535 ATCCACTTCTAGCCAGCGGTTTGAGGAAGCGCACTTTACCTTTGCCCTCACACCCCAGCA
rhesus 601 ATCCACTTCTAGCCAGCGGTTTGAGGAAGCGCACTTTACCTTTGCCCTCACACCCCAGCA
human 595 AGTGCAGCAGATTCTTACATCCAGAGAGGTTCTGCCAGGAGCCAAATGTGATTATACCAT
rhesus 661 AGTGCAGCAGATTCTTACATCCAGAGAGGTTCTGCCAGGAGCCAAATGTGATTATACCAT
human 655 ACAGGTGCAGCTAAGGTTCTGTCTCTGTGAGACCAGCTGCCCCCAGGAAGATTATTTTCC
rhesus 721 ACAGGTGCAGCTAAGGTTCTGTCTCTGTGAGACCAGCTGCCCCCAGGAAGATTATTTTCC
human 715 CCCCAACCTCTTTGTCAAGGTCAATGGGAAACTGTGCCCCCTGCCGGGTTACCTTCCCCC
rhesus 781 CCCCAACCTCTTTGTCAAGGTCAATGGGAAACTGTGCCCCCTGCCGGGTTACCTTCCCCC
human 775 AACCAAGAATGGGGCCGAGCCCAAGAGGCCCAGCCGCCCCATCAACATCACACCCCTGGC
rhesus 841 AACCAAGAATGGGGCTGAGCCCAAGAGGCCCAGCCGCCCCATCAACATCACACCTCTGGC
human 835 TCGACTCTCAGCCACTGTTCCCAACACCATTGTGGTCAATTGGTCATCTGAGTTCGGACG
rhesus 901 TCGACTCTCAGCCACTGTTCCCAACACCATTGTGGTCAATTGGTCATCTGAGTTCGGACG
human 895 GAATTACTCCTTGTCTGTGTACCTGGTGAGGCAGTTGACTGCAGGAACCCTTCTACAAAA
rhesus 961 GAATTACTCCTTGTCTGTGTACCTGGTGAGGCAGTTGACTGCAGGAACCCTTCTACAAAA
human 955 ACTCAGAGCAAAGGGTATCCGGAACCCAGACCACTCGCGGGCACTGATCAAGGAGAAATT
rhesus 1021 ACTTAGAGCAAAGGGAATCCGGAACCCAGACCACTCGCGGGCACTGATCAAGGAGAAATT
human 1015 GACTGCTGACCCTGACAGTGAGGTGGCCACTACAAGTCTCCGGGTGTCACTCATGTGCCC
rhesus 1081 GACTGCTGACCCTGACAGTGAGGTGGCCACTACGAGTCTCCGGGTGTCACTCATGTGCCC
human 1075 GCTAGGGAAGATGCGCCTGACTGTCCCTTGTCGTGCCCTCACCTGCGCCCACCTGCAGAG
rhesus 1141 GCTAGGGAAGATGCGCCTCACTGTCCCTTGTCGTGCCCTCACCTGCGCCCACCTGCAGAG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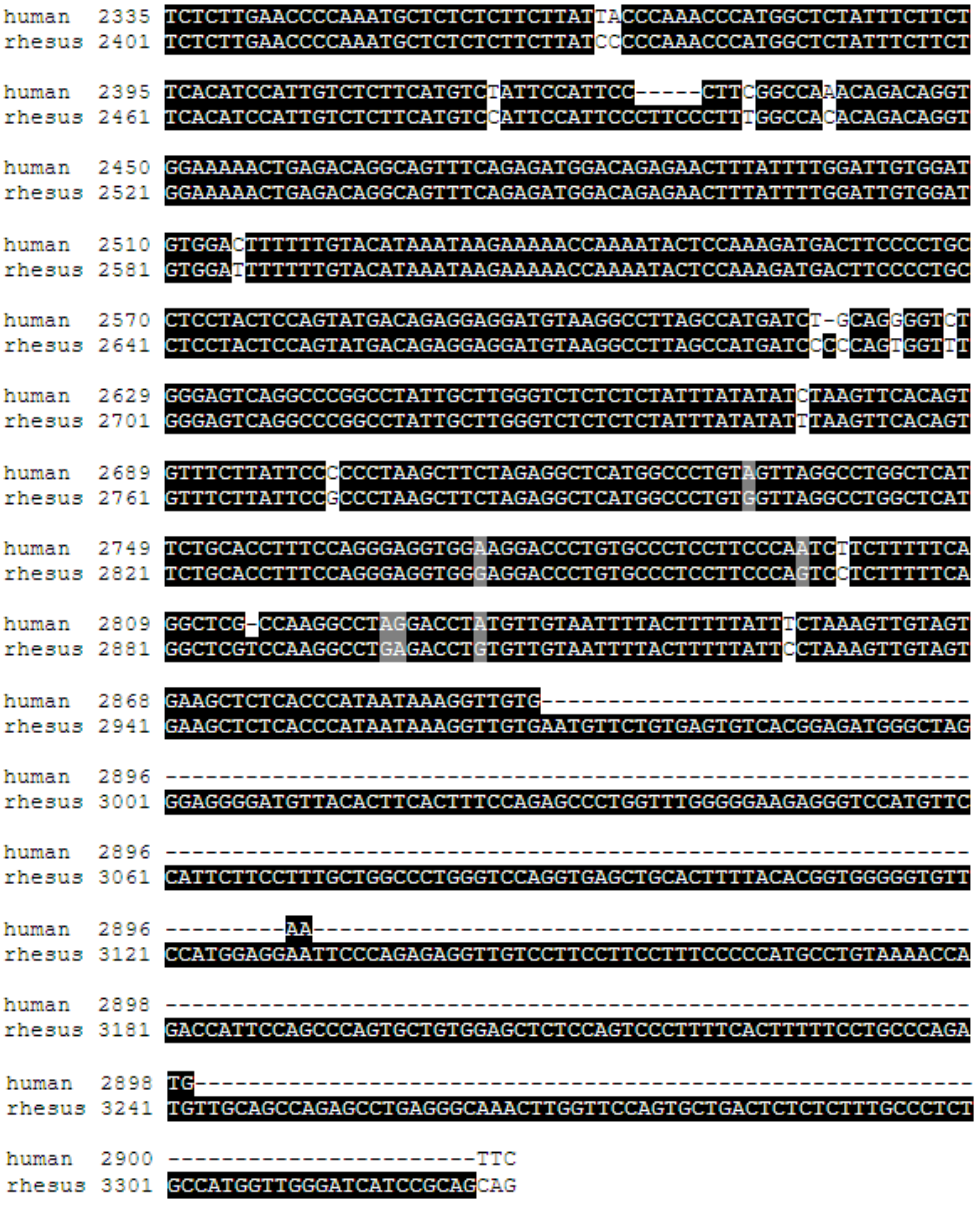
rhesus 1201 CTTCGATGCTGCCCTTTATCTCCAGATGAATGAGAAGAAGCCTACGTGGACATGTCCTGT
human 1195 GTGTGACAAGAAGGCTCCCTATGAATCTCTTATCATTGATGGTTTATTTATGGAGATTCT
rhesus 1261 GTGTGACAAGAAGGCTCCCTATGAATCTCTTATCATTGATGGTTTATTTATGGAGATTCT
human 1255 TAGTTCCTGTTCAGATTGTGATGAGATCCAATTCATGGAAGATGGATCCTGGTGCCCAAT
rhesus 1321 TAGTTCCTGTTCAGATTGTGATGAGATCCAATTCATGGAAGATGGATCCTGGTGCCCAAT
human 1315 GAAACCCAAGAAGGAGGCATCTGAGGTTTGCCCCCCCGCCAGGGTATGGGCTGGATGGCCT
rhesus 1381 GAAACCCAAGAAGGAGGCATCTGAGGTTTGCCCCCCCGCCAGGGTATGGGCTGGATGGCCT
human 1375 CCAGTACAGCCCAGTCCAGGGGGGAGATCCATCAGAGAATAAGAAGAAGGTCGAAGTTAT
rhesus 1441 CCAGTACAGCCCAGTCCAGGAGGGAAATCCATCAGAGAATAAGAAGAAGGTCGAAGTTAT
human 1435 TGACTTGACAATAGAAAGCTCATCAGATGAGGAGGATCTGCCCCCTACCAAGAAGCACTG
rhesus 1501 TGACTTGACAATAGAAAGCTCATCAGATGAGGAGGATCTGCCCCCTACCAAGAAGCACTG
human 1495 TTCTGTCACCTCAGCTGCCATCCCGGCCCTACCTGGAAGCAAAGGAGTCCTGACATCTGG
rhesus 1561 TTCTGTCACCTCAGCTGCCATCCCGGCCCTACCTGGAAGCAAAGGAGTCCTGACGTCTGG
human 1555 CCACCAGCCATCCTCGGTGCTAAGGAGCCCTGCTATGGGCACGTTGGGTGGGGATTTCCT
rhesus 1621 CCACCAGCCATCCTCGGTGCTAAGGAGCCCTGCCATGGGTGCGTTGGGTGGGGATTTCCT
human 1615 GTCCAGTCTCCCACTACATGAGTACCCACCTGCCTTCCCACTGGGAGCCGACATCCAAGG
rhesus 1681 GTCCAGTCTCCCACTACATGAGTACCCACCTGCCTTCCCACTGGGAGCCGATATCCAAGG
human 1675 TTTAGATTTATTTTCATTTCTTCAGACAGAGAGTCAGCACTATGGCCCCTCTGTCATCAC
rhesus 1741 TTTAGATTTATTTTCATTTCTTCAGACAGAGAGTCAGCACTATGGCCCCTCCGTCATCAC
human 1735 CTCACTAGATGAACAGGATGCCCTTGGCCACTTCTTCCAGTACCGAGGGACCCCTTCTCA
rhesus 1801 CTCACTAGATGAGCAGGATGCCCTTGGCCACTTCTTCCAGTACCGAGGGACCCCTTCTCA
human 1795 CTTTCTGGGCCCACTGGCCCCCACGCTGGGGAGCTCCCACTGCAGCGCCACTCCGGCGCC
rhesus 1861 CTTTCTGGGCCCACTGGCCCCCACGTTGGGGAGCTCCCACCGCAGTGCCACTCCGGCGCC
human 1855 CCCTCCTGGCCGTGTCAGCAGCATTGTGGCCCCTGGGGGGGCCTTGAGGGAGGGGCATGG
rhesus 1921 CCCTCCTGGCCGTGTCAGCAGCATTGTGGCCCCTGGGGGAGCCTTGAGGGAGGGGCATGG
human 1915 AGGACCCCTGCCCTCAGGTCCCTCTTTGACTGGCTGTCGGTCAGACATCATTTCCCTGGA
rhesus 1981 AGGACCCCTACCCTCAGGTCCTTCTTTGACTGGCTGTCGGTCAGACATCATTTCCCTGGA
human 1975 CTGAGTTCCCTGGATTATGGAAACTTCGCTGTCCCCCAACACTGAGCAAGTATGCTGTGG
rhesus 2041 CTGAGTTCCTTGGAGTATGGAAACTTCACTGTCCCCCAACACTGAGCAAGTATGCTGTGG
human 2035 AGTCCCAACCCCAGCTACTCTGATCCCTCTGGGGGCTCTGGCCAAGGGCCAGACAGACCT
rhesus 2101 AGTCCCAACCCCAGCTACTCTGATCCCTCTGCTGGCTCTGGCCAAGGGCCAGACAGACCT
human 2095 TCACAGATGCCTACTTTTGGCCTCATCTCTGCCTGACAAGGCCAGCACCCAAAGGGTTAA
rhesus 2161 TCACAGATGCCTACTTTTGGCCTCATCTCTGCCTGACAAGGCCAGCACCCAAAGGGTTAA
human 2155 TATTTAACCTCTTTTTAAGGACACTGGGGTCTGTTTCTGGAAATGTTCTTTAGATGGTGG
rhesus 2221 TATTTAACCTCTTTTTAAGGACATTGGGGTTTGTTTTTGGAAATGTTCTTTAGATGGTGG
human 2215 CACATTCCTTTGGGTATGTTAACCTAGGCAGTGGGAGGCAAATGGGATGGTATGTGAGCT
rhesus 2281 CACATTCCTTTGGGTATGTTAACCTAGGCAGTGGGAGGTAAATGGGATGGGATGTGAGCT
human 2275 AGGAGAAGGGCTGAACCCTCAGCCTTGACTATGTCTAGAGCCTCTTGGGGAAGGGGCACC
rhesus 2341 GGGAGAAGGGCTGAACCCTCAGCCTCAACTGTGTCTAGAGCCTCTTGGGAAAGGGGCACC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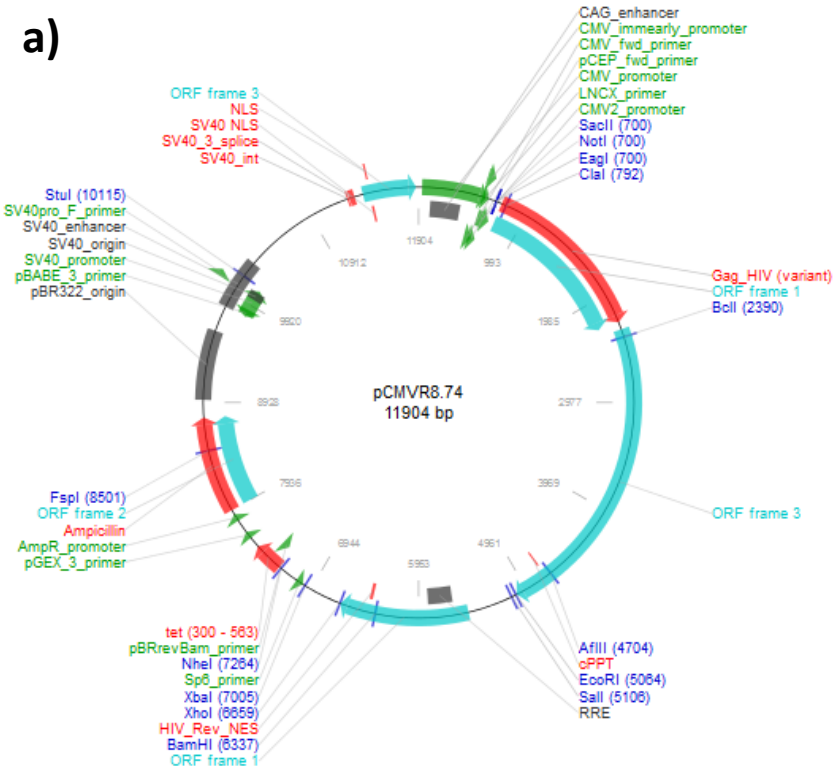
rhesus 2401 TCTCTTGAACCCCAAATGCTCTCTCTTCTTATCCCCCAAACCCATGGCTCTATTTCTTCT
human 2395 TCACATCCATTGTCTCTTCATGTCTATTCCATTCC-----CTTCGGCCAAACAGACAGGT
rhesus 2461 TCACATCCATTGTCTCTTCATGTCCATTCCATTCCCTTCCCTTTGGCCACACAGACAGGT
human 2450 GGAAAAACTGAGACAGGCAGTTTCAGAGATGGACAGAGAACTTTATTTTGGATTGTGGAT
rhesus 2521 GGAAAAACTGAGACAGGCAGTTTCAGAGATGGACAGAGAACTTTATTTTGGATTGTGGAT
human 2510 GTGGACTTTTTTGTACATAAATAAGAAAAACCAAAATACTCCAAAGATGACTTCCCCTGC
rhesus 2581 GTGGATTTTTTTGTACATAAATAAGAAAAACCAAAATACTCCAAAGATGACTTCCCCTGC
human 2570 CTCCTACTCCAGTATGACAGAGGAGGATGTAAGGCCTTAGCCATGATCT-GCAGGGGTCT
rhesus 2641 CTCCTACTCCAGTATGACAGAGGAGGATGTAAGGCCTTAGCCATGATCCCCCAGTGGTTT
human 2629 GGGAGTCAGGCCCGGCCTATTGCTTGGGTCTCTCTCTATTTATATATCTAAGTTCACAGT
rhesus 2701 GGGAGTCAGGCCCGGCCTATTGCTTGGGTCTCTCTCTATTTATATATTTAAGTTCACAGT
human 2689 GTTTCTTATTCCCCCCTAAGCTTCTAGAGGCTCATGGCCCTGTAGTTAGGCCTGGCTCAT
rhesus 2761 GTTTCTTATTCCGCCCTAAGCTTCTAGAGGCTCATGGCCCTGTGGTTAGGCCTGGCTCAT
human 2749 TCTGCACCTTTCCAGGGAGGTGGAAGGACCCTGTGCCCTCCTTCCCAATCTTCTTTTTCA
rhesus 2821 TCTGCACCTTTCCAGGGAGGTGGGAGGACCCTGTGCCCTCCTTCCCAGTCCTCTTTTTCA
human 2809 GGCTCG-CCAAGGCCTAGGACCTATGTTGTAATTTTACTTTTTATTTCTAAAGTTGTAGT
rhesus 2881 GGCTCGTCCAAGGCCTGAGACCTGTGTTGTAATTTTACTTTTTATTCCTAAAGTTGTAGT
human 2868 GAAGCTCTCACCCATAATAAAGGTTGTG--------------------------------
rhesus 2941 GAAGCTCTCACCCATAATAAAGGTTGTGAATGTTCTGTGAGTGTCACGGAGATGGGCTAG
human 2896 --
rhesus 3001 GGAGGGGATGTTACACTTCACTTTCCAGAGCCCTGGTTTGGGGGAAGAGGGTCCATGTTC
human 2896 --
rhesus 3061 CATTCTTCCTTTGCTGGCCCTGGGTCCAGGTGAGCTGCACTTTTACACGGTGGGGGTGTT
human 2896 ---------AA--
rhesus 3121 CCATGGAGGAATTCCCAGAGAGGTTGTCCTTCCTTCCTTTCCCCCATGCCTGTAAAACCA
human 2898 --
rhesus 3181 GACCATTCCAGCCCAGTGCTGTGGAGCTCTCCAGTCCCTTTTCACTTTTTCCTGCCCAGA
human 2898 TG--
rhesus 3241 TGTTGCAGCCAGAGCCTGAGGGCAAACTTGGTTCCAGTGCTGACTCTCTCTTTGCCCTCT
human 2900 ------------------------TTC
rhesus 3301 GCCATGGTTGGGATCATCCGCAGCAG

Figure 37. Plasmid maps. Maps for (a) packaging plasmid pCMVR8.74 and (b) envelope plasmid pMD2.G. (pCMVR8.74 from http://www.addgene.org/22036/ and pMD2.G from http://www.addgene.org/12259/)

a)


CAG_enhancer
CMV_immearly_promoter
CMV_fwd_primer
pCEP_fwd_primer
CMV_promoter
LNCX_primer
CMV2_promoter
SacII (700)
NotI (700)
EagI (700)
ClaI (792)
ORF frame 3
NLS
SV40 NLS
SV40_3_splice
SV40_int
StuI (10115)
SV40pro_F_primer
SV40_enhancer
SV40_origin
SV40_promoter
pBABE_3_primer
pBR322_origin
Gag_HIV (variant)
ORF frame 1
BclI (2390)
pCMVR8.74
11904 bp
ORF frame 3
FspI (8501)
ORF frame 2
Ampicillin
AmpR_promoter
pGEX_3_primer
tet (300 - 563)
pBRrevBam_primer
NheI (7264)
Sp6_primer
XbaI (7005)
XhoI (6659)
HIV_Rev_NES
BamHI (6337)
ORF frame 1
AflII (4704)
cPPT
EcoRI (5064)
SalI (5106)
RRE

b)

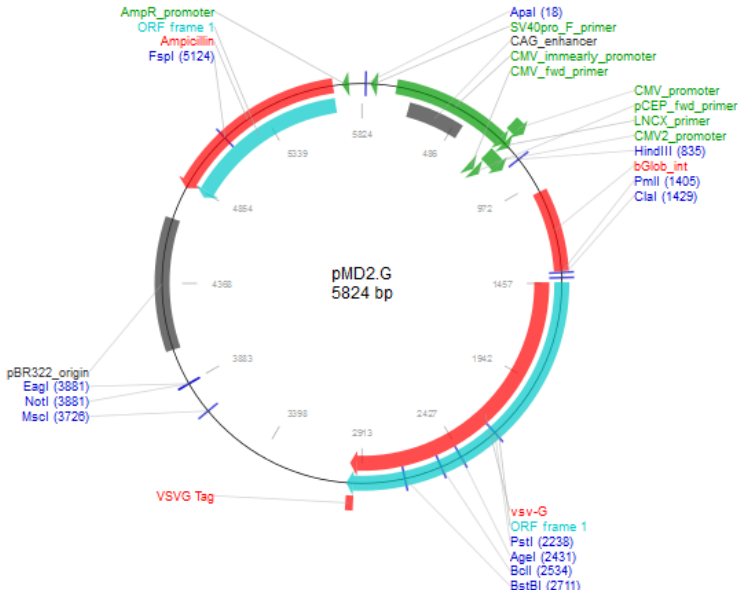
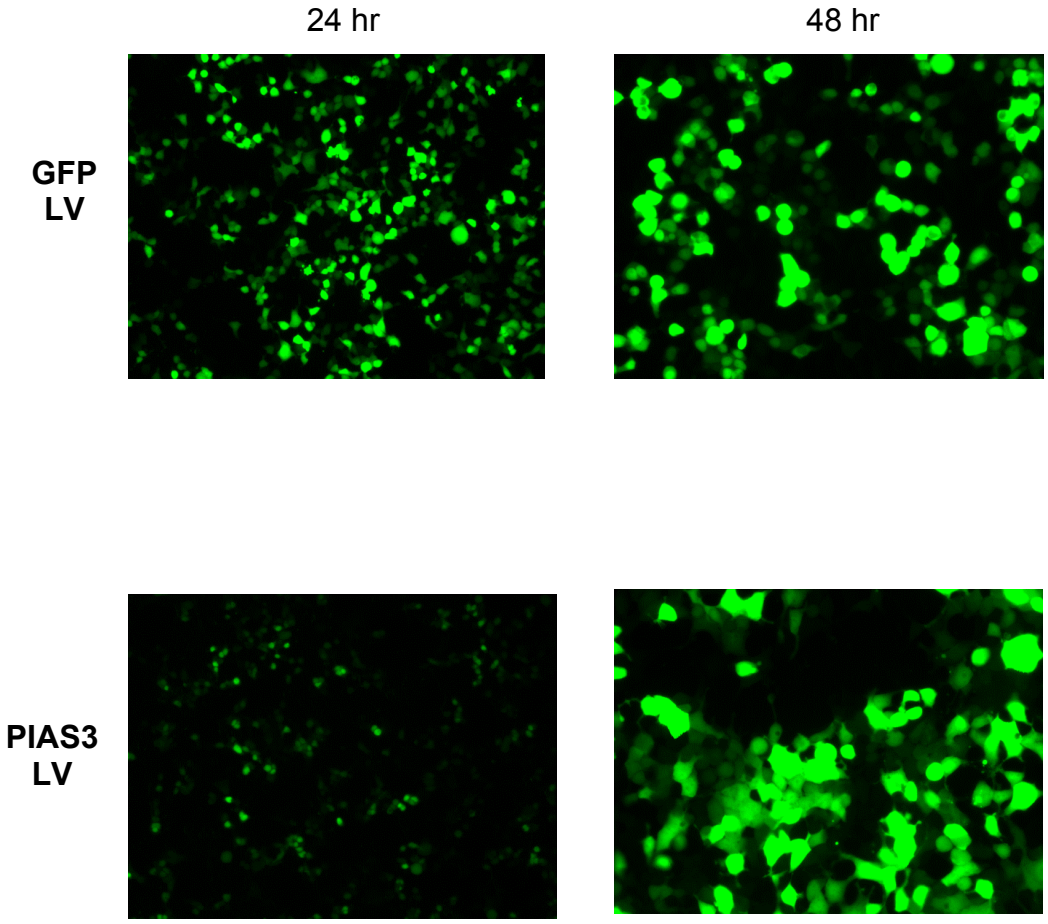

AmpR_promoter
ORF frame 1
Ampicillin
FspI (5124)
ApaI (18)
SV40pro_F_primer
CAG_enhancer
CMV_immearly_promoter
CMV_fwd_primer
CMV_promoter
pCEP_fwd_primer
LNCX_primer
CMV2_promoter
HindIII (835)
bGlob_int
PmlI (1405)
ClaI (1429)
pMD2.G
5824 bp
pBR322_origin
EagI (3881)
NotI (3881)
MscI (3726)
VSVG Tag
vsv-G
ORF frame 1
PstI (2238)
AgeI (2431)
BclI (2534)
BstBI (2711)

Figure 38. Generation of PIAS3 lentivirus. Lentiviruses expressing the PIAS3 LentiORF plasmid (PIAS3 LV) or a control plasmid expressing GFP only (GFP LV) were generated in 293T cells by calcium phosphate transfection with packaging plasmid, envelope plasmid, and lentiviral plasmid. GFP expression was assessed at 24 and 48 hours post-transfection by fluorescent microscopy.


24 hr
48 hr
GFP
LV
PIAS3
LV

Supernatants containing lentiviral particles was harvested at 48 and 72 hours post-transfection while the 293T packaging cells were lysed for Western blot analysis to determine PIAS3 protein levels as compared to cells only or GFP lentivirus controls. Our results showed that PIAS3 was efficiently expressed at 48 hours after transfection (**Figure 39**).

To test the infectivity of the lentiviral particles, the NIH 3T3 cell line was infected with the supernatant harvested from PIAS3 LV-transfected 293T cells and infection efficiency was assessed by GFP expression. Nearly all the cells were GFP-positive by 24 hours post-transduction, indicating that the viral particles were infectious (**Figure 40a)**.

Previous work by other groups using similar lentiviral systems suggested that 50-100 ul of lentiviral particles was sufficient for efficient transduction of cells. To test this, we infected 293T cells with 100 ul of lentivirus and assessed infectivity by GFP expression and found that nearly all cells were infected (**Figure 40a).** To confirm overexpression of PIAS3, transduced 293T cells were collected at 48 hours post-transduction and qRT-PCR was performed for PIAS3. Expression of PIAS3 was significantly increased in cells infected with PIAS3 lentivirus as compared to a control lentivirus expressing GFP only (**Figure 40b)**.

Next we transduced the Hut 102 cell line with lentivirus expressing either PIAS3 or GFP alone. Both GFP- and PIAS3-expressing lentiviral particles were infectious in Hut102 cells as indicated by microscopy (**Figure 41a**). As IL-17 expression by Hut 102 cells appeared to increase in response to 24-hour stimulation with rIL-6 (**Figure 34**), Hut102 cell cultures were stimulated with rIL-6 24 hours following lentiviral transduction; unstimulated transduced cultures were set up in parallel. We evaluated the

Figure 39. PIAS3 is overexpressed following generation of PIAS3 lentivirus. Following lentivirus production by calcium phosphate transfection in 293T cells, PIAS3 expression was assessed by Western blot in cell lysates from a cells only control (cells only), cells used to generate lentiviruses expressing GFP (GFP LV) and PIAS3 (PIAS3 LV) and cells transfected with PIAS3 only (PIAS3). GAPDH expression is shown as a loading control for each sample.

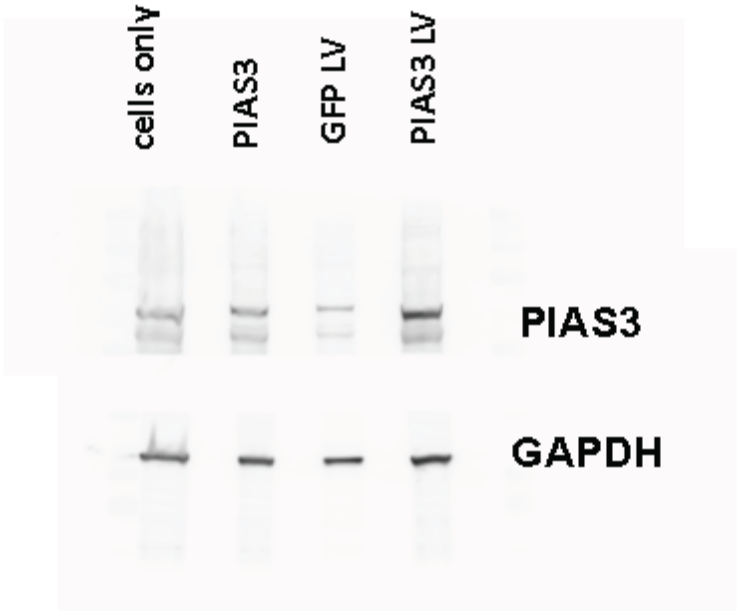
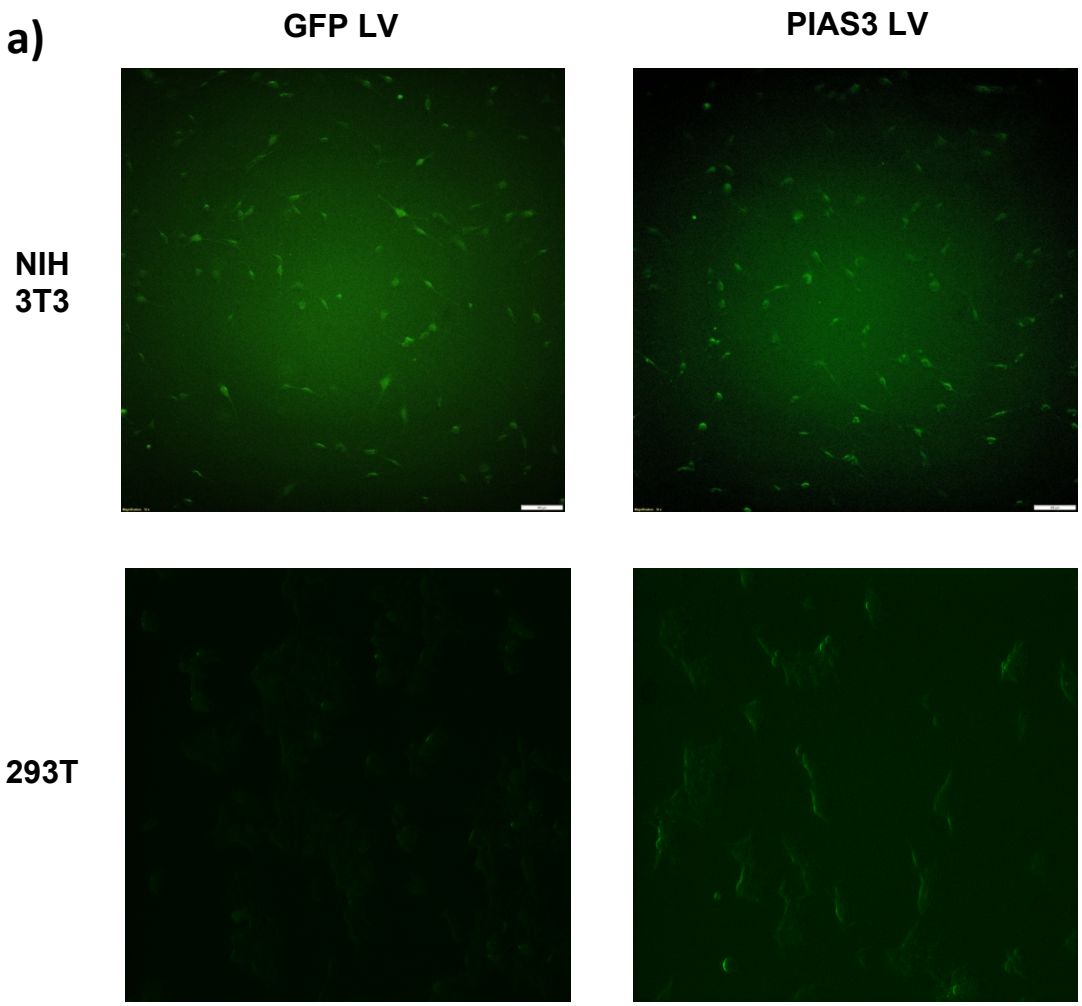

cells only
PIAS3
GFP LV
PIAS3 LV
PIAS3
GAPDH

Figure 40. Lentiviruses expressing GFP and PIAS3 are infectious in 3T3 and 293T cells. Following production of lentiviruses, 3T3 or 293T cells were transduced with lentivirus for 48 hours (a) GFP expression in 3T3 or 293T cells 24 hours following addition of either control lentivirus expressing GFP only (GFP LV) or lentivirus expressing PIAS3 (PIAS3 LV). (b) PIAS3 mRNA expression in 293T cells following transduction with GFP LV or PIAS3 LV. Total RNA was isolated from cells 48 hours after transduction and used to assess expression of PIAS3 by relative qRT-PR as normalized to β-actin. Fold changes were calculated using the ddCT method (307), with fold change in PIAS3 LV-infected cells shown relative to control GFP LV-infected cells. Fold change is PIAS3 expression is shown for cells infected with two different preparations of PIAS3 LV.


a)
GFP LV
PIAS3 LV
NIH 3T3
293T

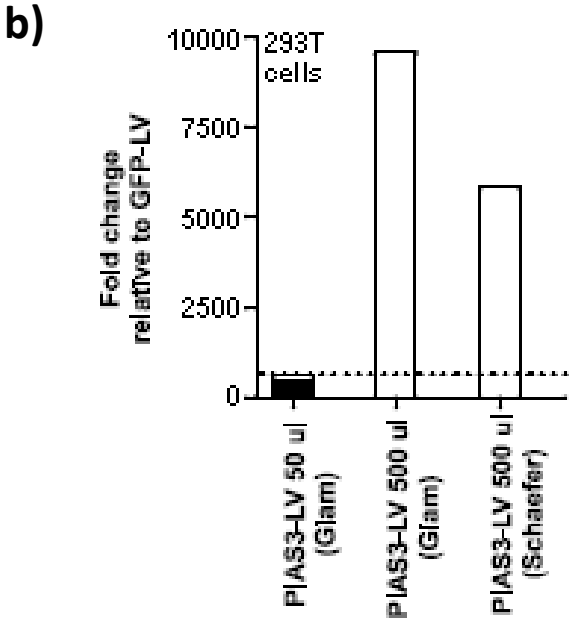
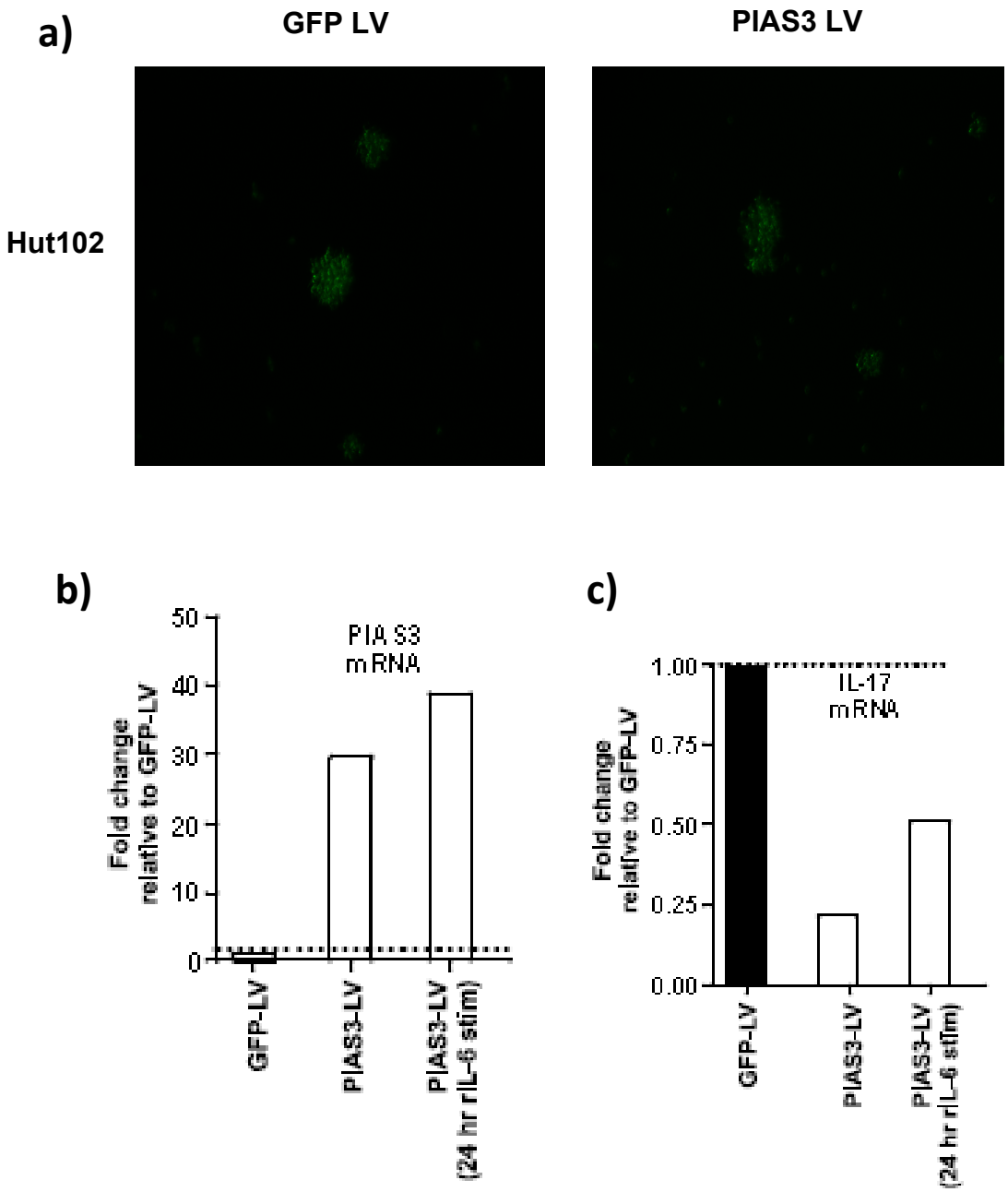

b)
293T cells
Fold change relative to GFP-LV
10000
7500
5000
2500
0
PIAS3-LV 50 ul (Glam)
PIAS3-LV 500 ul (Glam)
PIAS3-LV 500 ul (Schaefer)

Figure 41. Lentiviral transduction of Hut102 cells results in overexpression of PIAS3 and a corresponding suppression of IL-17 responses. Hut102 cells were transduced with GFP or PIAS3 LV for 48 hours. Transduced cells were either left unstimulated or stimulated with rIL-6 which was added 24 hours following transduction. (a) GFP expression in Hut102 cells was assessed 24 hours following infection with either GFP LV or PIAS3 LV. (b) PIAS3 and (c) IL-17 mRNA expression in Hut102 cells following transduction with GFP LV or PIAS3 LV. Total RNA was isolated from cells 48 hours after transduction and used to assess expression of PIAS3 and IL-17 by relative qRT-PR as normalized to β-actin. Fold changes were calculated using the ddCT method (307), with fold change in PIAS3 LV-infected cells shown relative to control GFP LV-infected cells. The dashed line represents baseline expression of GFP LV-infected cells.


a)
GFP LV
PIAS3 LV
Hut102
b)
PIAS3 mRNA
Fold change relative to GFP-LV
50
40
30
20
10
0
GFP-LV
PIAS3-LV
PIAS3-LV (24 hr rIL-6 stim)
c)
IL-17 mRNA
Fold change relative to GFP-LV
1.00
0.75
0.50
0.25
0.00
GFP-LV
PIAS3-LV
PIAS3-LV (24 hr rIL-6 stim)

expression of PIAS3 and IL-17 by PCR following transduction. PIAS3 was highly overexpressed in Hut102 cells after transduction and PIAS3 overexpression was associated with suppression of IL-17 (**Figure 41b-c**), suggesting that the negative regulator PIAS3 is sufficient to mediate defects in IL-17 expression in CD4+ T cells.

DISCUSSION

The transcription factor STAT3 plays a critical role in the expression of IL-17 by Th17 cells (57; 172; 270). The activation of STAT3 is known to be regulated by three proteins: SOCS3, SHP2, and PIAS3. Our results show that the expression of all three genes was elevated during SIV infection and correlated with the loss of IL-17 expression, suggesting that their elevated expression likely contributes to suppression of IL-17 responses.

Interestingly, we did not observe an increase in expression of total STAT3 message following IL-6 stimulation during infection. However, the main mechanism of action for IL-6 is to induce activation and phosphorylation of STAT3, which occurs rapidly in response to short-term stimulation. As CD4+ T cells were only stimulated with rIL-6 for 15 minutes, it likely that this time frame was not sufficient to affect STAT3 mRNA expression. In support of this, other studies have shown that longer periods of stimulation of 1-3 hours were required before an increase in STAT3 expression in response to IL-6 stimulation was observed (10; 168).

Our Western blot data indicated that neither STAT3 expression nor activation appeared to be altered during SIV infection. Interestingly, IL-6-induced phosphorylation not only occurred in SIV-infected animals, but also appeared to be elevated as compared to uninfected animals. In support of this, other studies have shown a high degree of

STAT3 phosphorylation in macrophages and CD3+ T cells in the gastrointestinal tract of SIV-infected animals (250). It is believed that high levels of phosphorylated STAT3 are related to the extensive degree of immune activation that occurs in infection (250). Most importantly, IL-17 expression remained suppressed in spite of elevated STAT3 activation in CD4+ T cells.

The lack of IL-17 expression despite the presence of high level of phosphorylated STAT3 suggested that a defect was occurring in the STAT3 signaling cascade downstream of STAT3 phosphorylation. Of the three proteins that are known to negatively regulate STAT3, only PIAS3 interferes with signaling downstream of phosphorylation (94). While SHP2 and SOCS3 reverse or prevent phosphorylation of STAT3 respectively, PIAS3 binds to phosphorylated STAT3, thereby preventing STAT3 from binding to the IL-17 promoter (61; 94; 354). Interestingly, of the three negative regulators examined, both mRNA expression and protein levels of PIAS3 were elevated in our SIV-infected samples. Taken together, these data suggest that elevated levels of PIAS3 in SIV infection may contribute to Th17 dysregulation by preventing STAT3-mediated expression of IL-17.

While our data indicated that PIAS3 may be upregulated in CD4+ T cells from peripheral blood, it is not known if PIAS3 is differentially expressed in other tissue compartments during SIV infection. Similarly, while we did not observe any change in SOCS3 or SHP2 protein expression in SIV infection, it is possible that there may be changes in their expression patterns in other tissues and at other time points during infection. Additional studies will need to be performed using multiple tissues and longitudinal sampling.

To confirm a role for PIAS3 in suppression of IL-17 responses, we used a lentiviral system to overexpress PIAS3 in the IL-17-producing CD4+ T cell line Hut102. Overexpression of PIAS3 in Hut102 cells resulted in a corresponding suppression of IL-17 expression, suggesting that PIAS3 plays an important role in regulating IL-17 expression. These results, however, need to be confirmed in rhesus macaque primary cells in order to assess an *in vivo* role for PIAS3 in suppression of Th17 cells during SIV infection.

In conclusion, our results indicate SIV infection may lead to disruption of the STAT3 signaling pathway through the negative regulatory factor PIAS3. Although phosphorylation of STAT3 occurs normally during SIV infection, elevated expression of PIAS3 may prevent binding of activated STAT3 to the IL-17 promoter, inhibiting Th17 responses.

CHAPTER 6: Discussion

PREFACE AND SIGNIFICANCE

The hallmarks of HIV and SIV infection include loss of CD4+ T cells from multiple tissue compartments, particularly mucosal sites, high levels of systemic immune activation, and microbial translocation (19; 41; 89; 99; 238; 303; 336). The loss of CD4+ T cells during infection includes the Th17 subset which is thought to be a critical component of barrier maintenance and mucosal homeostasis (40; 52; 67; 256; 288). Several studies in the field have linked the loss of Th17 cells in HIV and SIV infection to the translocation of microbial products into systemic circulation, contributing to chronic immune activation (40; 101; 104; 196; 290; 303).

Th17 cells are likely a critical immune subset in HIV and AIDS for several reasons. Opportunistic infections are AIDS-defining and can be the result of a broad range of microbes, including bacteria, viruses, parasites, fungi, and protozoa. Th17 cells are important in the immune response to infections with fungi and extracellular bacteria, including *Candida*, *M. tuberculosis*, and *Salmonella* (256). Additionally, Th17 cells are enriched at mucosal sites such as the gastrointestinal tract, which are important sites for infection (21; 40; 170). Mucosal sites are not only where the virus gain entry, but also where the most significant depletion of CD4+ T cells occurs (238; 336). Finally, HIV infection is characterized by generalized dysfunction of the gastrointestinal tract, as evidenced by chronic diarrhea and malabsorption in infected patients (80; 204; 303). Th17 cells are thought to contribute to maintenance of barrier integrity in the gastrointestinal tract through IL-17-mediated upregulation of proteins involved in tight junction formation in the epithelial barrier (75; 192). The loss of Th17 cells in infection

may be at least partially responsible for the gastrointestinal dysfunction and associated symptoms observed.

Most importantly, the loss of Th17 cells during HIV and SIV infection has been linked to translocation of microbial products from the mucosa into circulation, leading to high levels of chronic immune activation (40; 196; 290; 303). There is substantial evidence that microbial translocation and immune activation are linked to disease progression and patient prognosis (304).

Although the loss of Th17 cells during HIV and SIV infection has been well documented, it is not clear why these cells remain depleted during infection while other CD4+ T cell populations are capable of recovery. Although several mechanisms have been proposed to explain the failure of Th17 cells to recover, the exact cause of Th17 dysregulation during infection remains unknown. Using the SIV model in rhesus macaques as a surrogate for HIV infection in humans, our studies sought to determine if the failure of Th17 cells to recover during infection was due to defects in the differentiation and induction of these cells.

Th17 cells require a number of cytokines and cytokine receptors on CD4+ T cell precursors to be present in order for them to develop into Th17 cells. It is possible that HIV and SIV infection may alter expression of these factors, thereby preventing differentiation of Th17 cells. Additionally, expression of IL-17 by Th17 cells requires activation of the STAT3 signaling pathway; it is possible that defects in STAT3 activation and activity occur during infection, leading to the failure of CD4+ T cells to produce IL-17.

CONCLUSIONS BASED ON RESULTS

In support of previous studies in HIV and SIV (40; 52; 104; 175; 178), we concluded that Th17 dysregulation likely occurs very early during the course of SIV infection, as IL-17 responses were suppressed during acute infection and suppression persisted into the chronic phase of infection. Acute loss of Th17 cells along with other memory CD4+ T cells likely accounts for the loss of pre-existing Th17 cells. However, the fact that cells failed to produce IL-17 in response to stimulation suggests a potential functional defect in the ability of CD4+ T cells to develop into Th17 cells, as opposed to simply a loss of those cells.

In agreement with other studies (41), we concluded that SIV infection was associated with increased microbial translocation and immune activation during SIV infection. Unlike these studies, however, we observed significant levels of microbial translocation during the acute stage of infection. This was surprising and interesting, as it is believed that microbial translocation does not occur during the acute stage of infection due to the necessity for sufficient time for mucosal barrier damage to occur. However during the first two weeks of infection, the CD4+ T cell population is depleted (238; 336), the mucosa is inflamed, and the integrity of the mucosal barrier is likely damaged, facilitating the translocation of microbial products.

Based on the correlation between the microbial translocation and immune activation, the loss of IL-17 responses in infection likely drives microbial translocation and subsequent immune activation during chronic infection. Interestingly however, while markers of microbial translocation correlated with loss of IL-17 expression in acute infection there was no correlation between levels of immune activation and IL-17 expression. This suggests that the loss of IL-17 responses early in infection may not be a

contributing factor to increased levels of immune activation. It is likely that the high levels of activation observed during acute infection are the result of massive viral replication (93). These phenomena may mask any effect of Th17 loss on immune activation. Increased levels of IL-23 and expression of CD14 on monocytes suggest that translocated microbial products during the early phases of infection could potentially activate monocytes leading to the pro-inflammatory cascade that likely persists into chronic infection.

An analysis of Th17-promoting cytokines and their cognate receptors during SIV infection did not show a deficiency in expression of these factors in either acute or chronic infection, suggesting that a paucity of key signaling molecules required for Th17 cells to develop properly is not responsible for Th17 dysregulation during infection. These results are in keeping with other studies, which suggest that pro-inflammatory cytokines are present in various capacities during HIV infection (34; 96; 320). Collectively, these data suggest that mechanisms other than Th17-promoting cytokines or their receptors are likely involved in dysregulation of Th17 cells during infection.

As extracellular signaling factors did not appear to be affected during SIV infection, we hypothesized that intracellular signaling pathways, namely STAT3, may be dysregulated in Th17 cells, leading to a suppression of IL-17. The three negative regulators of STAT3, PIAS3, SHP2 and SOCS3, were upregulated very early during infection and their increased expression correlated with suppressed levels of IL-17. Therefore, we concluded that negative regulation of the STAT3 pathway likely plays a role in Th17 dysregulation. To our knowledge, these are the first studies that demonstrate

a potential role for negative regulation of STAT3 in Th17 dysregulation during HIV and SIV infection.

Analysis of STAT3 phosphorylation revealed high levels of STAT3 activation in CD4+ T cells during SIV infection, making negative regulation at the level of activation of STAT3 unlikely. This is supported by other studies that have shown that STAT3 phosphorylation is elevated in other cells types during SIV infection, reflecting the generalized pro-inflammatory environment present during infection (250).

Based on the high levels of STAT3 activation observed during infection, we concluded that any defects in the STAT3 signaling pathway during infection were likely downstream of phosphorylation and activation. Although all three negative regulatory factors that affect STAT3 were upregulated during acute infection, only PIAS3 acts downstream of STAT3 phosphorylation by interacting with phosphorylated STAT3 and preventing its binding to the IL-17 promoter (94). As PIAS3 protein levels were significantly elevated during infection, we concluded that increased PIAS3 expression during infection may contribute to the failure of cells to produce IL-17. Overexpression of PIAS3 in the IL-17-producing cell line Hut102 led to a significant decrease in IL-17 expression, demonstrating that high levels of PIAS3 are sufficient to recapitulate the phenotype observed in SIV-infected animals.

Importantly, PIAS3 involvement does not exclude the possibility that other factors may play a role in Th17 dysregulation. It is possible that other mechanisms are involved in dysregulation of Th17 responses during HIV and SIV infection, such as elevated expression of IDO and inhibition by regulatory T cell expansion.

LIMITATIONS

It is not clear from these studies if PIAS3 upregulation during SIV infection can be linked to the lack of Th17 responses at the mucosal barrier. The studies performed herein were restricted to the use of peripheral blood cells for the majority of the experiments, due to the technical challenges of obtaining other tissues at various time points during infection. Therefore, peripheral blood was used as a surrogate for other tissue sites. Additional studies using cells from tissues such as the gastrointestinal tract and lymph nodes are needed to determine if dysregulated signaling observed in peripheral blood also plays a role in suppression of Th17 cells at other sites.

Samples from the jejunum of several SIV-infected macaques were also studied and an analysis of Th17 cells and the expression of Th17-promoting cytokines was performed in both peripheral blood and jejunum samples. Similar trends were observed for both jejunum and peripheral blood. However, mesenteric lymph nodes are a primary source of cells that home to the gastrointestinal tract. As such, analysis of the STAT3 signaling pathway and PIAS3 expression in these tissues is required to confirm their importance in SIV infection.

These studies utilized samples taken at specific time points during the course of infection. A consequence of this is that our data provides only a limited perspective of the changes that occur throughout the course of SIV infection. It is possible that these signaling pathways may be altered in other ways at different points during infection. A longitudinal study of various tissue samples from a large number of animals will provide more detailed information about the role PIAS3 plays in Th17 suppresssion in the body.

FUTURE DIRECTIONS/UNANSWERED QUESTIONS

Future experiments will focus on confirming a role for PIAS3 upregulation in the suppression of Th17 responses during infection using primary cells from healthy rhesus macaques. To determine whether PIAS3 is a contributing factor to IL-17 suppression during infection, PIAS3 lentiviral constructs will be used to infect total PBMC from healthy rhesus macaques. Following lentiviral transduction, GFP+ CD4+ T cells will be sorted by FACS and subsequently stimulated with rIL-6 to induce STAT3 phosphorylation and expression of IL-17. Production of IL-17 in response to PIAS3 overexpression will be assessed by relative qRT-PCR. It is expected that overexpression of PIAS3 in cells from healthy animals will result in suppression of IL-17 responses that mimics the phenotype observed in SIV-infected macaques.

To confirm the role of PIAS3 in suppression of IL-17 during HIV and SIV infection, siRNA knockdown of PIAS3 in cells from SIV-infected animals will be performed to determine if the deficiency in Th17 cells can be reversed. If PIAS3 contributes to suppression of IL-17 during SIV infection, knockdown of PIAS3 will likely restore IL-17 production in cells from SIV-infected animals.

Although PIAS3 upregulation in CD4+ T cells appears to have a role in SIV infection, it is not known if PIAS3 may be involved in Th17 dysregulation in HIV infection in humans. Therefore, future experiments should be aimed at determining whether PIAS3 upregulation is observed in CD4+ T cells from HIV-infected patients.

The potential role for PIAS3 in Th17 differentiation raises the question about the cause of PIAS3 upregulation during SIV infection. While PIAS3 expression is known to be negatively regulated by several microRNAs and Trim factors (257), there is little known about the positive regulation of PIAS3. PIAS3 overexpression has been

implicated in several types of cancers (343), and its expression in prostate cancer has been linked to treatment with compounds such as dihydrotestosterone (174). It is possible that elevated PIAS3 may be due to a direct result of viral infection. HIV and SIV encode several regulatory and accessory proteins that are known to affect expression of other immune factors. Alternatively, it is possible that PIAS3 upregulation may be an indirect effect of viral infection and related to the immune milieu that is present during infection. Future directions should be aimed at assessing the potential causes for elevated PIAS3 expression during infection and how modulation can be used to restore Th17 responses.

CONCLUSION

Our data suggests a model in which SIV infection is associated with increased expression of PIAS3 in CD4+ T cells. Despite the presence of Th17-promoting cytokines that induce activation of the STAT3 signaling pathway, high levels of PIAS3 bind activated STAT3 in these cells and inhibit binding to the IL-17 promoter, ultimately preventing IL-17 production. The loss of IL-17 responses subsequently contributes to breakdown of the intestinal barrier and mucosal immune homeostasis, enabling the translocation of microbial products into the bloodstream. Microbial translocation, along with other factors such as viral replication, contributes to the high levels of immune activation that ultimately influence disease progression and patient mortality.

As Th17 cells are important for mucosal integrity and immune homeostasis, their selective failure to repopulate during infection likely contributes to HIV disease progression. Understanding the mechanisms by which HIV causes dysregulation of the Th17 subset is the first step in assessing how to restore this lost subset and its immune function, including maintenance of gut barrier integrity and prevention of microbial

translocation and subsequent immune activation. Recovery of the Th17 subset during HIV infection through the development of therapeutic options may ultimately contribute to better patient prognosis and disease outcome.

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Appendix

CO-IMMUNOPRECIPITATION OF STAT3 AND PIAS3 IN CD4+ T CELLS

Total PBMC stimulated with rIL-6 for 15 minutes at 37˚C or left unstimulated were lysed in high salt extraction buffer and protease phosphatase inhibitor as previously described in the Materials and Methods. Supernatants were collected following 15 minutes of centrifugation at 14,000 x g at 4˚C. Lysates were pre-cleared with protein G agarose beads for 15 minutes at room temperature. After beads were removed by centrifugation, lysates were incubated with anti-PIAS3 antibody (Cell Signaling Technology) for 1 hour at room temperature with gentle rotation. Beads were added to lysates for 1 hour at room temperature, after which immune complexes were collected by centrifugation and washed in lysis buffer with protease and phosphatase inhibitors. Beads were resuspended in sample buffer and boiled at 95˚C for 5 minutes. Beads were collected by centrifugation and supernatant was recovered for Western blot analysis.

RESULTS

In order for PIAS3 to inhibit STAT3, it must first bind to phosphorylated STAT3, forming a complex. Previous studies have shown that STAT3 and PIAS3 are binding partners in co-immunoprecipitation assays (61). To determine whether STAT3 and PIAS3 were in fact interacting in cells from SIV-infected animals, an experiment was designed to co-immunoprecipitate PIAS3 and STAT3. We first performed a pilot experiment using total PBMC from one uninfected and one SIV-infected animal that were stimulated with rIL-6 for 15 minutes to induce STAT3 phosphorylation. Unstimulated cells were prepared simultaneously as controls. We used a human anti-

PIAS3 antibody for IP and then assessed total STAT3 expression by Western blot to determine if STAT3 and PIAS3 could be co-immunoprecipitated in a complex.

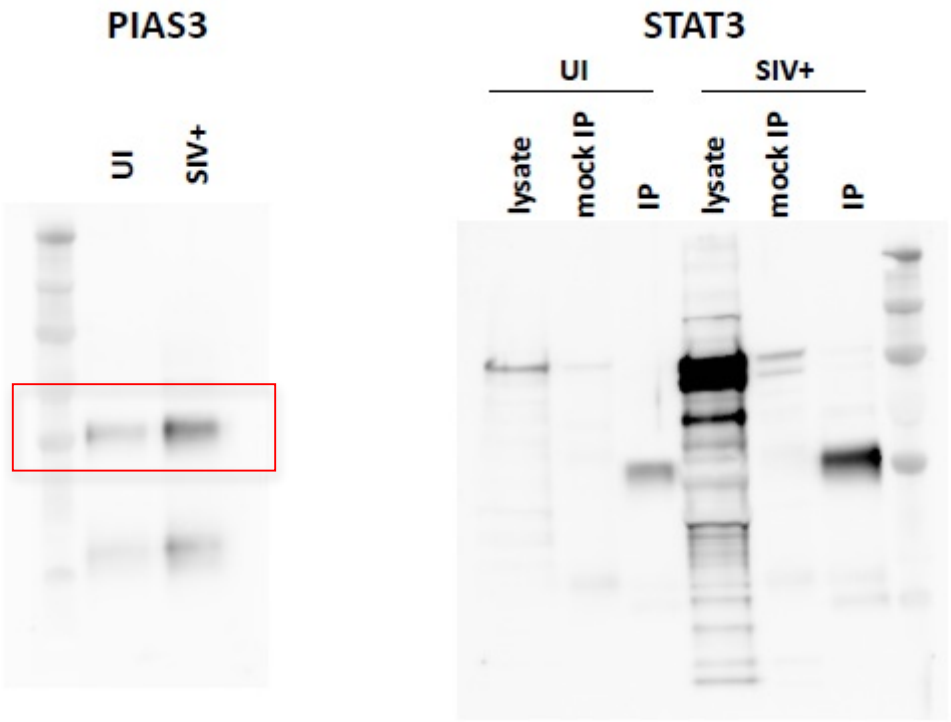
We first assessed PIAS3 expression and confirmed that PIAS3 was in fact present in the lysate from both uninfected and SIV-infected animals; however the expression was very low. When we examined STAT3 expression, we found that STAT3 was present in the total lysate of both uninfected and SIV-infected samples. However, non-specific binding was present to a high degree in the SIV-infected sample. As a control, a mock IP condition with beads alone was analyzed. Surprisingly, bands were present in the mock IP lanes, indicating non-specific binding to the beads themselves. The same bands appeared to be present in the PIAS3 IP lanes as well; however, as binding was observed under the mock IP conditions, it is not known if these bands are due to non-specific binding or specific interactions between PIAS3 and STAT3.

DISCUSSION

There are several potential explanations as to why the co-immunoprecipitation experiments were not successful. The PIAS3 IP antibody used had not been previously validated for use in non-human primates. It is possible that PIAS3 is not being immunoprecipitated at a high efficiency due to incomplete cross-reactivity. Additionally, the high-salt extraction buffer used may be too harsh for the immunoprecipitation protocol, leading to dissociation of the PIAS3 and STAT3 interaction during the wash steps.

The Western blot results indicate that that there are many other proteins present in the mock IP and PIAS3 IP samples. As such, it may be necessary to pre-clear lysate for a longer period of time in order to reduce non-specific binding. The non-specific binding is

Figure 42. Co-immunoprecipitation of STAT3 and PIAS3 in total CD4+ T cells. Lysates of total PBMC were immunoprecipitated with anti-PIAS3 antibody and Western blot was performed for total STAT3. One representative uninfected and SIV-infected animal are shown for total lysates, mock IP, and PIAS3 IP.


PIAS3
UI
SIV+
STAT3
UI
SIV+
lysate
mock IP
IP
lysate
mock IP
IP

likely due to the fact that primary cells were used, in which many other proteins and potential binding partners are present and PIAS3 may be expressed at low levels.

Future studies will be aimed at using an alternative technique to demonstrate STAT3 and PIAS3 interactions. We will use confocal microscopy to assess the relative locations of STAT3 and PIAS3 in CD4+ T cells from uninfected and infected animals. In addition to co-localization, confocal microscopy will also enable us to assess the cellular localization of PIAS3 and p-STAT3 to determine if they are in fact translocating to the nucleus as expected.