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HIV Immunopathogenesis: Inhibition of CD4+ T Cell Activation

Haitao Hu
University of Pennsylvania, haitaohu@mail.med.upenn.edu

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Abstract
Human immunodeficiency virus (HIV) infection causes profound impairment of CD4+ T cell immunity. Anti-retroviral therapy (ART) restores CD4+ T cell responses to common antigens, but HIV-specific responses remain deficient. The immunization of chronically HIV infected, ART treated subjects also leads to poor HIV-specific CD4+ T cell responses. The mechanisms are not fully understood. In this thesis, I demonstrate that HIV envelope glycoprotein (Env), when delivered in the form of a vaccine or when present on free viral particles, suppresses antigen-stimulated CD4+ T cell proliferation. I investigate the potential involvement of human T regulatory cells (Treg) using an in vitro model system. I show that Env exposure neither changes the frequency nor the suppressive activity of Treg cells in human PBMC, and that Env-induced suppression of CD4+ T cell proliferation is independent of Tregs. The studies of HIV-induced inhibition of CD4+ T cell immunity were then moved to an in vivo model to determine physiological significance. A macaque model of HIV-infected individuals treated with ART during chronic infection was used to study the effect of SIV antigen stimulation in lymph nodes early after immunization. CMV seropositive rhesus macaques were infected with pathogenic SIVmac251 and after 4 months, treated with D4T and PMPA for viral control and immune reconstitution. I studied the immune and viral responses to SIV and CMV antigen immunization in draining lymph nodes. Animals were immunized with both SIV gag and CMV pp65 encoding plasmids in both arms and legs, which allowed draining lymph nodes for each antigen to be obtained at the same time, thus allowing direct comparisons of the effect of each antigen stimulation in the same animal. I observed that both SIV and CMV antigen immunizations stimulated antigen-specific T cell responses in draining lymph nodes. The CMV-specific responses were found in the periphery for 50 days post-immunization, while the SIV-specific responses transiently appeared. The SIV antigen stimulation also induced transient SIV viral replication in the draining lymph nodes, suggesting a mechanism for the early loss and poor HIV-specific CD4+ T cell response observed in HIV-infected progressors.

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Haitao Hu

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Supervisor of Dissertation

Signature _____

Drew Weissman, MD, PhD, Associate Professor of Medicine

Graduate Group Chairperson

Signature _____

Daniel Kessler, PhD, Associate Professor of Cell and Developmental Biology

Dissertation Committee

Michael Betts, PhD, Assistant Professor of Microbiology

James Hoxie, MD, Professor of Medicine

Guido Silvestri, MD, Professor of Pathology and Laboratory Medicine

David Weiner, PhD, Professor of Pathology and Laboratory Medicine

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ABSTRACT

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Haitao Hu

Dissertation Supervisor: Drew Weissman, MD, PhD

Human immunodeficiency virus (HIV) infection causes profound impairment of $CD4^+$ T cell immunity. Anti-retroviral therapy (ART) restores $CD4^+$ T cell responses to common antigens, but HIV-specific responses remain deficient. The immunization of chronically HIV infected, ART treated subjects also leads to poor HIV-specific $CD4^+$ T cell responses. The mechanisms are not fully understood. In this thesis, I demonstrate that HIV envelope glycoprotein (Env), when delivered in the form of a vaccine or when present on free viral particles, suppresses antigen-stimulated $CD4^+$ T cell proliferation. I investigate the potential involvement of human T regulatory cells (Treg) using an in vitro model system. I show that Env exposure neither changes the frequency nor the suppressive activity of Treg cells in human PBMC, and that Env-induced suppression of $CD4^+$ T cell proliferation is independent of Tregs. The studies of HIV-induced inhibition of $CD4^+$ T cell immunity were then moved to an in vivo model to determine physiological significance. A macaque model of HIV-infected individuals treated with ART during chronic infection was used to study the effect of SIV antigen stimulation in lymph nodes early after immunization. CMV seropositive rhesus macaques were infected with pathogenic SIVmac251 and after 4 months, treated with D4T and PMPA for viral

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Table of Contents

List of Figures

List of Tables

CHAPTER 1

Introduction

HIV and the global epidemic

Human immunodeficiency virus (HIV) is a lentivirus that causes acquired immunodeficiency syndrome (AIDS) in humans [48, 162]. As of December 2008, between 31.1 million and 35.8 million people were believed to be living with HIV. The number of people newly infected in 2008 was estimated to be 2.7 million and approximately 2 million people died from HIV infection (AIDS epidemic update 2009, UNAIDS).

HIV is composed of two copies of single-stranded genomic RNA that are enclosed by capsid and contain nine genes encoding 19 proteins. The HIV replication cycle is summarized in Figure 1.1[144]. First, the glycoprotein complex present on the surface of virus, known as Env or gp160, enables the virus to attach to target cells to initiate entry and infection [27]. Entry of HIV into the host cell depends on the interaction between the viral envelope and CD4 plus a co-receptor (generally either CCR5 or CXCR4) on the surface of target cells. Binding of gp120 to CD4 leads to a conformational change in Env and the subsequent exposure of the co-receptor binding site, which allows gp120 to interact with a target chemokine receptor to mediate fusion and entry [28, 169].

Figure 1.1

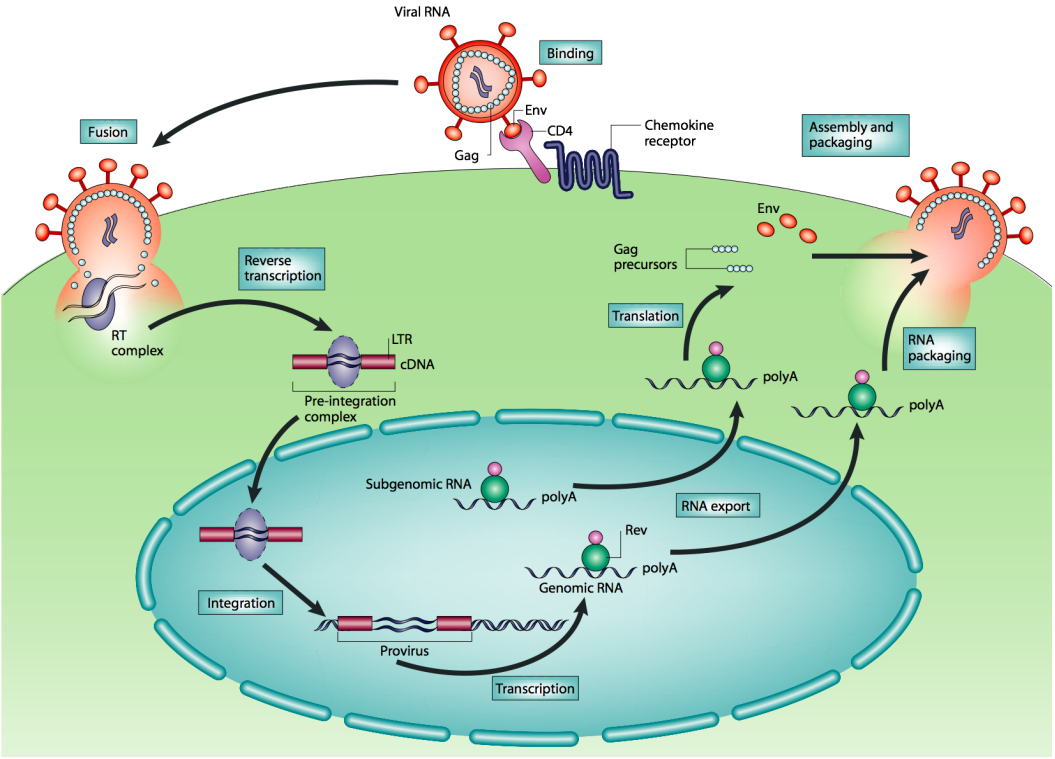


Figure 1.1. Schematic illustration of HIV replication cycle in an infected cell. LTR: long terminal repeat; RT: reverse transcriptase [144]

The viral RNA genome is reverse transcribed shortly after the virus enters the cell and the preintegration complex is transported into the cell nucleus and integrates into host cell's genome. Infection of $CD4^+$ T cells has 2 main outcomes, lytic and latent. In latent infection, the integrated viral DNA may lie "dormant" in resting memory cells. When such cells become activated, the integrated DNA provirus starts viral gene expression and viral replication. The HIV genome contains multiple splice sites, resulting in alternatively spliced mRNAs. They can be divided into three groups: unspliced, singly

spliced, and doubly spliced (Figure 1.2). In brief, the proviral DNA is first transcribed into a primary viral RNA transcript, which is about 9 kb in length and serves as both genomic RNA for progeny virus and the unspliced viral mRNA encoding viral Gag and Gag-Pol proteins. Alternative retention or splicing of the primary transcript and nuclear export of the unspliced transcript are well regulated. During the early phase of viral replication, two doubly spliced viral transcripts are produced and exported into cytoplasm, where they are translated into the regulatory and accessory proteins Tat, Rev and Nef. These viral proteins regulate subsequent viral gene expression and cellular function. As the newly generated Rev proteins accumulate in the nucleus, they bind to the transcribed viral mRNA and facilitate the export of singly spliced and unspliced mRNAs, which encode the viral structural proteins Env and Gag. The full-length unspliced viral genomic RNA binds to Gag polyproteins which traffic to the cell membrane for viral assembly. Among the numerous cellular factors reported to be involved in HIV assembly and budding, the ESCRT cellular machinery (Endosomal Sorting Complex Required for Transport), which was initially discovered to be important for the biogenesis of multivesicular bodies (MVB), recruits the Gag p6 domain and plays a key role in formation and release of new particles. It is currently thought that the resulting late-budding structure is released by the cellular ESCRT machinery severing the membrane tether connecting it to the producer cell [13, 104].

Figure 1.2

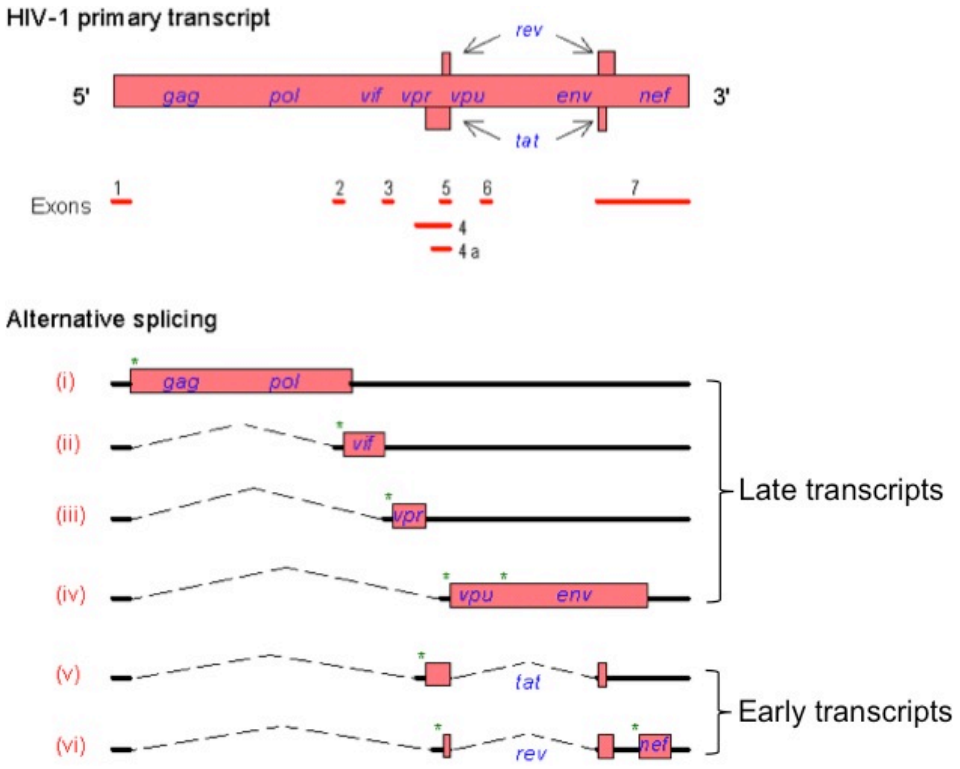


Figure 1.2. Schematic illustration of alternative splicing of the HIV-1 primary transcript. (i) is unspliced, (ii) to (iv) are singly spliced, (v) and (vi) are doubly spliced. The resulting mRNA (i), (iv) and (vi) are bicistronic. The star "*" indicates the location of the initiation codon (AUG). (From Web-book: http://www.web-books.com/)

Clinical course of HIV infection

The clinical course of HIV infection generally includes three phases or stages: 1) acute infection; 2) chronic or latent infection; and 3) AIDS-defining illness. Such a course of infection is characteristic of the so-called typical progressor who represents the majority of HIV-infected individuals (Figure 1.3). Three major clinical parameters are

associated with HIV disease progression: viral load, immune activation, and peripheral $CD4^+$ T cell number. In acute infection, rapid viral replication occurs leading to acute viremia, followed by a massive depletion of $CD4^+$ T cells [33, 124]. Activation of $CD8^+$ T cells is then detected and believed to significantly control acute viral replication [116]. The disease transition from primary infection to the clinical latent stage is characterized by downregulation of viremia and resolution of symptoms, if present. A set point for viral load is usually formed in this stage. HIV disease is still active with persistent replication of virus and progressive loss of $CD4^+$ T cells. Finally, when $CD4^+$ T cell numbers decline below a critical level cellular immunity drops to a point leading to infections with a variety of opportunistic pathogens defined as AIDS [51, 117].

Figure 1.3

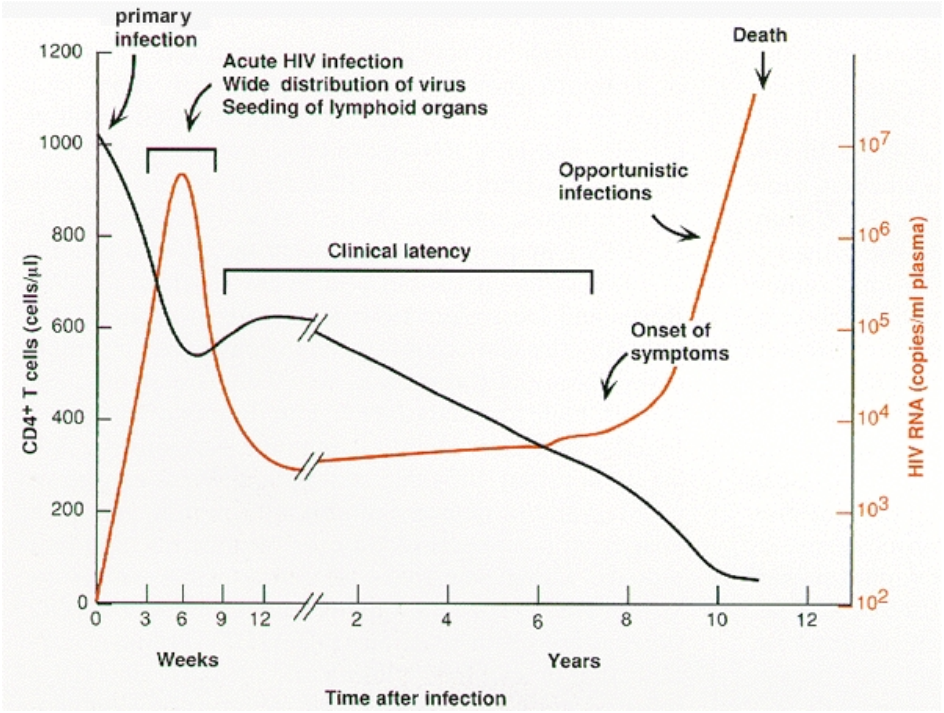


Figure 1.3. Natural course of HIV infection. The diagram demonstrates the relationship between viral load and peripheral $CD4^+$ T cell count.

Impairment of $CD4^+$ T cell immunity in HIV

$CD4^+$ T cells play a central role in maintaining effective cellular and humoral immune responses by providing help to $CD8^+$ T cells, B cells and innate effectors [22]. In HIV infection, the number of circulating $CD4^+$ T cells is commonly used as a measure of global "immune competence" and provides a predictor for the immediate risk of opportunistic infections [101]. The importance of cellular immunity in HIV infection has been identified in many studies, even though the specific protective immune correlates have not been fully elucidated. Enhanced cellular immunity towards HIV has been linked with better control of viral replication in patients who show slow or delayed disease progression [67, 106, 118].

However, one of the key features for HIV infection is the profound impairment of $CD4^+$ T cell immunity, attributed both to the massive loss of $CD4^+$ T cells mediated by direct viral infection during early infection and the subsequent progressive loss of $CD4^+$ T cells during the chronic stage [47, 125]. In addition to cell depletion, functional abnormalities of $CD4^+$ T cells also contribute significantly to the impaired $CD4^+$ T cell immunity in HIV. T cells from HIV-infected individuals showed reduced proliferation following stimulation with antigens or mitogens in vitro[34]. Diminished expression of IL-2

is readily demonstrated in these cells and may be related to the proliferation defects [139]. Furthermore, a sequential loss of immune responsiveness to recall antigens, followed by alloantigens and then mitogens has been described for $CD4^+$ T cells from HIV-infected individuals [34]. Further studies using TCR stimulation to characterize the proliferation defects in $CD4^+$ T cells indicate that such defects are associated with early-phase cell-cycle arrest [140] and are commonly observed in patients with sustained $CD4^+$ T cell loss [141]. Since $CD4^+$ T cells play a key role in regulating antibody responses, CTL responses and other innate effectors, the loss of these cells constitutes a major impairment in immune capacity in HIV infection.

HIV Envelope protein and inhibition of $CD4^+$ T cell response

As described above, HIV infection causes profound $CD4^+$ T cell impairment leading to immune suppression and the development of AIDS (49). Mechanisms have been suggested for this including direct cytopathic effects of HIV, accumulation of unintegrated viral DNA or toxic viral proteins, virus-induced changes in target cell membrane permeability, and apoptosis of HIV-infected $CD4^+$ T cells (50). Other proposed mechanisms include lysis of $CD4^+$ T cells by cytolytic T cells and tryptophan depletion by indolamine 2,3-dioxygenase (51, 52). However, in many patients only a small fraction of $CD4^+$ T cells appear to be infected and the degree of cell loss greatly exceeds the number of infected cells, indicating that virus-induced bystander dysfunction plays a role in $CD4^+$ T cell deficiency.

Among the HIV proteins, Env, expressed on the surface of free virions and infected cells, is a potent immune inhibitory molecule and has been demonstrated to play a predominant role in suppressing $CD4^+$ T cells by multiple mechanisms (61, 62, 63). CD4 is a transmembrane glycoprotein that plays an important role in T helper cell function by interacting with MHC class II during T cell activation [73]. However, it has been shown that cross-ligation of CD4 molecules prior to TCR stimulation primes the cells for apoptosis or a non-responsiveness state upon TCR activation [146,63]. Env is a trimeric protein with each of its monomers possessing a CD4 and co-receptor binding site, and it has been demonstrated to produce a similar effect on CD4 ligation. Env multimers created in vitro with antibodies against monomeric gp120 subunits can mediate cross-linking of CD4 and/or co-receptor leading to signaling that causes aberrant activation of $CD4^+$ T cells [10, 132]. These observations from in vitro culture systems are consistent with the studies of HIV-infected patients demonstrating that similar interactions between Env on infected cells or virions can cause apoptosis of uninfected bystander $CD4^+$ T cells, which occurs primarily in lymphoid tissues where antigen presentation and T cell activation occur [55]. A proposed hypothesis is that when bystander, uninfected HIV-specific $CD4^+$ T cells have CD4 pre-ligated by Env, they undergo apoptosis after they are activated via TCR stimulation by the cognate antigen presenting cells (APCs) [37]. For Env-induced apoptosis of bystander $CD4^+$ T cells, multiple apoptotic pathways have been proposed. Ligation of CD4 receptor by Env prior to TCR stimulation leads to upregulation of Fas in $CD4^+$ T cells, and the subsequent expression of Fas-L upon TCR activation thereby renders the T cells susceptible to

apoptosis by Fas-FasL pathway [146]. Fas independent apoptosis and other death pathways, including tumor necrosis factor (TNF)-TRAIL pathway, have also been reported as mechanisms for Env-induced apoptosis [15, 58, 77, 113, 127]. In addition to apoptosis, lack of proliferation [132] and induction of IL-2 reversible anergy[99] have also been suggested as forms of Env-induced inhibition of $CD4^+$ T cells.

Although inhibition of $CD4^+$ T cells by Env has been well established, the underlying mechanisms in terms of cellular signaling defects and the receptors utilization are not fully understood. CD4 binding has been shown to play a major role in mediating suppression in most studies [3, 19, 82]. Treatment of CCR5Delta32 homozygote PBMCs with a CCR5-specific envelope induced apoptosis in T cells, demonstrating that CD4 could mediate suppression in the absence of co-receptor [7]. In contrast, involvement of co-receptor binding in Env-induced inhibition of $CD4^+$ T cells was also reported. Stimulation of CCR5 by R5 Env triggers apoptosis of $CD4^+$ T cells [4]. Another study showed that bystander apoptosis of $CD4^+$ T cells required direct contact with virions and Env/CXCR4 binding [71].

As previously mentioned, Env could deliver aberrant signals to $CD4^+$ T cells through interaction with CD4 and/or coreceptors. Therefore, it is likely that T cell signaling cascades (TCR signaling and cytokine signaling) could be affected by Env as the molecular mechanisms for $CD4^+$ T cell inhibition. Env-induced defects in JAK/STAT signaling pathways have been reported in multiple studies [89, 135]. It was demonstrated that

exposure of $CD4^+$ T cells to Env caused a failure of normal JAK-1 and JAK-3 activation and the unresponsiveness of STAT5a and STAT5b to IL-2 [89]. In addition, many studies have demonstrated defects in TCR signaling induced by Env. Binding activities of important transcription factors to target promoters, including NFAT, NFκB and AP-1, were observed to be significantly decreased in T cells with exposure to Env [75]. Pre-exposure of a T cell line to gp120 led to the inhibition of Lck activation in response to TCR/CD3 stimulation [109]. In a study with primary human $CD4^+$ T cells, pre-treatment of these cells with gp160 resulted in marked inhibition of tyrosine phosphorylation of p59 (Fyn), PLC-gamma1 and ras activation, providing insight into the mechanism by which the Env may alter TCR/CD3-activation-induced signal transduction [145].

Collectively, the interference of TCR or cytokine signaling by Env is complex and the biochemical mechanisms are not fully understood. Env protein is a key component of an effective vaccine because it exclusively induces neutralizing antibodies [72]. Accordingly, understanding the mechanism for Env-induced inhibition is critical for designing effective Env-based HIV vaccine candidates and approaches.

T regulatory cells (Treg) and HIV

The concept of suppressor T cells was first described by Gerson et al when they suggested that a population of T cells could suppress immune responses [61]. Later on CD25 was identified as a surface marker for a population of suppressor T cells that could control autoimmune responses in mice, and these cells were termed regulatory T cells

(Treg). The discovery of the transcription factor FoxP3, which is highly expressed in both mouse and human Treg cells and is required for Treg cell function and development, has led to intensive studies of Treg biology in mouse models as well as for more reliable identification of Tregs in human [21, 29, 56, 95, 166].

Several subclasses of Treg cells have been identified. Tregs expressing FoxP3, CD4 and high levels of CD25 are termed natural regulatory T cells and are thought to primarily originate in the thymus, although studies have shown that these cells can be generated in the periphery from non-Treg cells under certain conditions [18]. The other type of Treg cell is known as T regulatory 1 cells (Tr1) that express little or no FoxP3 but preferentially produce large amounts of IL-10 [74]. The TGF-ß-producing intraepithelial T cells in gut associated lymphoid tissues (GALT) are known as T helper 3 (Th3) with suppressive function [160]. The current knowledge on Treg classification, phenotype and development is still limited and more research is needed. Treg cells can exert suppression by multiple mechanisms, although much remains to be elucidated [156, 172]. Treg cells could induce suppression by direct cell-cell contact or soluble factors like IL-10 and/or TGF- ß [31, 137]. Whether Treg cells release IL-10 and thus exert contact-independent suppression is not clear, but it should be noted that IL-10 is primarily found in adaptive regulatory T cells, such as Tr1 cells, and it is indicated to play an important role in mediating suppression for these cells [156].

Treg cells are traditionally believed to protect the host from autoimmune reactions by down-regulating autoimmune responses. However, there is accumulating evidence supporting an active role for Tregs in modulating immune responses directed to infectious agents, including HIV [2]. Treg cells isolated from the peripheral blood and lymphoid tissues of HIV-infected patients have been demonstrated to maintain a suppressive activity on HIV-specific $CD4^+$ and $CD8^+$ T cell response [85, 86, 161]. Both increased and decreased numbers of Treg cells have been reported in HIV infection, and a number of factors could influence the result, such as the tissues studied, the stage of infection, and the institution of ART. In addition, it becomes more complicated by the use of different Treg detection methods by different groups [5, 6, 49, 52, 85-87, 108, 112, 115, 149, 161]. In studies using peripheral blood, both increased [1, 85, 161] and reduced [49, 115] frequencies of $CD4^+CD25^+$ Treg cells were reported, leading to two converse hypotheses that HIV drives expansion of Treg cells or HIV induces a selective loss of Treg cells. Subsequent studies using LN or gastrointestinal mucosal tissue demonstrated an increased frequency of suppressive Treg cells in HIV-infected patients, as detected by FoxP3 expression, supporting the hypothesis that Treg cells, rather than undergoing a selective loss induced by virus, accumulate at sites of viral expression, where they can exert suppression on Ag-specific T cell activation [6, 52, 112]. How HIV regulates the frequency and function of Treg cells during infection and how this regulation relates to HIV pathogenesis and host immunity, remains highly controversial. There is limited evidence, so far, demonstrating the direct interaction of Treg cells and HIV or its gene products. Human Treg isolated from healthy donors express the HIV coreceptor CCR5 and were reported to be the target

of HIV infection with high susceptibility [115]. In contrast, it has been demonstrated that HIV increases Treg viability through Env-CD4 interaction, resulting in accumulation of Treg cells [112]. Collectively, the regulation of Treg frequency and function by HIV and the physiological relevance in the context of HIV infection or vaccination are not fully understood.

Immunization and immune dysfunction in chronic HIV/SIV infection during ART

The introduction of anti-retroviral therapy (ART) results in delayed disease progression and a remarkable decrease in AIDS related deaths. However, the current regimens for HIV treatment have major limitations. First, it fails to eradicate the virus. It has also been demonstrated that even during the most potent regimens of retroviral suppression, viral production continues, as detected by some ultrasensitive assays [43, 70, 122]. In addition, immune recovery subsequent to ART often appears to be partial and limited to certain T cell responses, and does not include HIV-specific $CD4^+$ T cell responses [8, 83, 129].

Cell-mediated immunity is involved in controlling HIV replication and correlates with better control of disease progression, which provides a rationale for active immunization of chronically infected individuals to augment the existing T cell responses. A number of approaches attempting to stimulate cell-mediated responses by immunization have been studied in SIV-infected rhesus macaques during ART with mixed results reported regarding immunologic and virologic benefits [42, 57, 69, 96, 150, 157, 173].

Rhesus macaques with long-standing SIVmac251 infection were immunized three times with a poxvirus vector expressing the SIVmac structural and regulatory genes during ART. Following antiretroviral treatment interruption, the viral set point was significantly lower in immunized macaques than in control macaques [148]. A strategy using autologous dendritic cells (DC) pulsed either with viral peptides or chemically inactivated virus, which could elicit strong immune control of the virus, has been tested in the SIV macaque model and demonstrated promising result [42, 96]. Immunizations with DNA plasmids encoding multiple SIV antigens also offered long lasting immunologic and virologic benefits to SIVmac251 infected and ART treated macaques [157]. In contrast to these promising results, very limited immunologic and virologic benefits by immunization in ART-treated, SIV infected macaques have also been reported. A study using the SIVmac239 model demonstrated that immunization with SIVmac239-derived Env, Gag/Pol, and Tat/Rev/Nef plasmids by in vivo electroporation elicited T-cell immune responses, but no control of SIVmac293 viremia [173]. Collectively, using various immunization strategies including DNA, poxvirus vectors, and antigen-pulsed DCs, SIV-specific immune responses were evoked in SIV-infected rhesus macaques during ART. The virologic benefits of these immunizations after release of macaques from ART were variable from no control to temporal control or long-lasting control.

However, similar immunization (either vaccine or treatment interruption) for chronically HIV-infected, ART-treated humans has been disappointing, as no significant HIV-specific T cell responses were induced by immunization in this setting [66], which is

consistent with the observation that treatment with effective ART results in regained T cell responses to common antigens, but poor responses to HIV-specific antigens, suggesting the long-lived defects in the generation of HIV-specific $CD4^+$ T cell immunity in HIV [8, 83, 129]. The mechanisms are not fully characterized or understood. Compared to peripheral blood, which has been commonly used to study the antigen-specific T cell responses to HIV/SIV infection or immunization, the secondary lymphoid tissues represent an important site where antigen presenation, antigen-specific T cell response and the viral replication occur. APCs, primarily DCs, function by obtaining antigens in the periphery, migrating to draining lymph nodes (LNs), and interacting with antigen-specific T cells, leading to the T cell activation and antigen-specific responses. However, in the context of HIV infection, the interaction between viral antigen-expressing APC and HIV-specific T cells could blunt the immune response through multiple mechanisms [65, 168]. It has been suggested that DC-T cell interaction can stimulate viral replication in DC, $CD4^+$ T cells and the cell conjugates, which represent an explosive site of HIV infection that could escape from ART [128]. Multiple mechanisms have been proposed for this suppressed T cell activation induced by the inappropriate deleterious DC-T interactions in LN during HIV infection, including T cell death secondary to activation of latent infection, aberrant signals from DCs expressing inhibitory HIV proteins, and $CD4^+$ T cell fusion with infected DCs or DCs expressing Env. Collectively, the mechanisms for the impaired antigen-specific T cell response in chronic HIV are complex and not fully understood.

Aims of thesis

In this thesis, I have examined the mechanisms for the inhibition of $CD4^+$ T cell immunity in HIV. An in vitro system modeling the interaction between HIV Env and human primary $CD4^+$ T cells will be used to study the inhibitory effect of Env protein, when delivered as a vaccine on APC and non-APC, or as free virions, on antigen-stimulated bystander $CD4^+$ T cell proliferation. I investigate the mechanisms by studying various forms of T cell inhibition, including anergy, apoptosis and cell death. Some important TCR and cytokine signaling pathways are also investigated in $CD4^+$ cells with Env exposure. Considering the suppressive effect of human Treg cells on autologous $CD4^+$ and $CD8^+$ T cell proliferation and its ability to interact with HIV Env, I study the regulation of Treg frequency, activation phenotype, and suppressive activity in human peripheral blood by Env, and the functional involvment of Treg cells in Env-induced inhibition.

In the third chapter of this thesis, I move the studies to an in vivo model. Using chronically SIV and CMV co-infected, ART-treated rhesus macaques, I study the early immunologic and virologic consequences of immunization with SIV and CMV antigen in draining lymph nodes, where antigen presentation, antigen-specific T cell responses and viral replication occur during infection. The systemic response in peripheral blood after immunization is also studied to track the fate of immunization-induced antigen-specific T cells.

CHAPTER 2

HIV Envelope Suppresses $CD4^+$ T Cell Activation Independent of T Regulatory Cells

Introduction

One of the hallmarks of HIV-associated defects in cell-mediated immunity is the progressive loss of $CD4^+$ T cell responses, even before a significant decrease in peripheral blood $CD4^+$ T cell numbers occurs [105]. The Env protein of the virus is a key component of an effective vaccine because it exclusively induces neutralizing antibodies, which are protective in the macaque model [100]. However, Env has been demonstrated to suppress $CD4^+$ T cells by multiple mechanisms. Inhibition has been characterized by a lack of proliferation[82, 132], the induction of anergy [93, 99], and various apoptotic mechanisms[10, 165]. The role of Env interactions with CD4 and coreceptors in mediating the various forms of suppression is under debate with studies demonstrating a role for each [7, 15, 154]. R5 Env caused apoptosis of $CD4^+$ T cells from a CCR5Δ32 homozygous donor, whereas, in another study, Env interaction with CXCR4 and a nonsignaling CD4 induced $CD4^+$ T cell apoptosis[130]. The mechanism of suppression observed and the receptors involved varied according to the nature of Env used and the cell culture system studied. Env is a trimeric protein with each of its monomers possessing a CD4 binding site. Thus, an Env trimer [132] or aggregates created by antibodies against monomeric gp120 subunits [10] can mediate cross-linking of CD4 and/or coreceptor leading to signaling. In patients infected with HIV, trimeric Env capable of suppression is found on

the surface of infected cells as well as on the virus itself. The use of Env in HIV vaccines, especially in commonly used DNA and viral vectors, could be envisioned to deliver similar $CD4^+$ T cell suppressive signals.

Although APCs such as DCs are prime targets for vaccines, other cell types at the site of vaccine delivery, and potentially the major vaccine antigen expressing cell types, are non-APCs such as myocytes, fibroblasts, subepithelial, and epithelial cells for muscle, dermal, subcutaneous, and mucosal delivery, respectively, as well as cells in the draining lymph nodes [30, 38]. $CD4^+$ T cells that are recruited to the site of vaccine delivery as well as to the draining lymph node in response to vaccine-induced inflammation may then interact with and be suppressed by interaction with Env expressed on the surface of all of these cells.

Naturally occurring regulatory T cells (Treg) are a subset of $CD4^+$ T cells co-expressing CD25 and FoxP3 and have the ability to suppress autologous $CD4^+$ and $CD8^+$ T cell activation and proliferation. The role of Treg cells in HIV has been intensively studied and there are accumulating evidence supporting the active engagement of Treg cells in modulating immune response towards HIV infection [1, 86, 112, 161]. Issues with regard to whether and how HIV regulates the frequency and function of Treg cells during infection, and how this regulation relates to HIV pathogenesis and host immunity, remains highly controversial. There is limited evidence, so far, demonstrating the direct interaction of Treg cells and HIV or its gene products. One study showed that HIV

interacts directly with Treg cells through Env-CD4 signaling and increases Treg frequency in PBMC through selectively enhancing Treg viability and the subsequent accumulation of Treg cells [112].

In this chapter, using an in vitro system that models the in vivo interaction between $CD4^+$ T cells and Env, we investigated the mechanisms for how Env suppresses $CD4^+$ T cells concentrating on signaling pathways and the involvement of Tregs.

Materials and Methods

Cells and HIV-1

Human embryonic kidney 293T cells (American Type Culture Collection) were maintained in DMEM (Invitrogen Life Technologies) supplemented with 10% FCS (HyClone) and glutamine (Invitrogen Life Technologies). Monocyte-derived dendritic cells were obtained from purified monocytes (Center for AIDS Research (CFAR) Immunology core) and plated in 6-well plates at 3 x 10^6 cells in 3 mls of AIM V serum-free media (Life Technologies, Rockville, MD), supplemented with GM-CSF (50 ng/ml) and IL-4 (100 ng/ml). After 3 days, cells were fed by adding 1.5 mls of Aim V supplemented with GM-CSF and IL-4. Peripherable blood mononuclear cells (PBMC) from HIV-negative donors were obtained through an IRB-approved protocol and maintained in complete RPMI 1640 (Invitrogen Life Technologies) containing 10% FCS and glutamine. Aldrothiol-2 (AT-2) inactivated [131] HIV-1 NL4–3 virions were obtained from University of Pennsylvania CFAR virology core.

Plasmid constructs and RNA synthesis

HIV Envs IIIB, YU2, and 89.6 were cloned into the RNA expression plasmid pT7TS that contains a T7 promoter, 5' and 3' UTR sequences of Xenopus-globin, and a 30-nuc long poly (A) tail [80]. Plasmids were linearized with restriction enzymes and in vitro transcribed with T7 polymerase (mMessage T7 Ultra Kits (Ambion)), extended poly-A tail wer added. RNA was maintained in nuclease-free water at a concentration of 1 μg/μl and stored at -20°C.

DC infection with vaccinia virus

DCs were suspended at a concentration of 2 million/mL and infected with vaccinia-encoding IIIB, MN, or 92UG037.8 Env (NIH AIDS Research and Reference Reagent Program), T7 Polymerase (VTF7.3), or wild-type WR strain for 2 hours, washed, and incubated for 4 hours. Infected DCs were cocultured with 5-and 6-carboxyfluorescein diacetate, succinimidyl ester (CFSE)-labeled PBMCs at a ratio of 1:10 and activated with 0.1 μg/mL toxic shock syndrome toxin-1 (TSST-1) superantigen.

Exposure of $CD4^+$ T cells to Env and $CD4^+$ T cell proliferation

Transcribed mRNA encoding HIV Env was complexed to lipofectin for delivery to cells as described previously [80]. 293T cells seeded in 12-well plates, approximately 90% confluent (5×10^5 cells), were transfected with Env (IIIB, YU2, and 89.6) encoding mRNA (5 μg/ml) or control poly(AC) RNA (Sigma-Aldrich), incubated for 4 h to allow

protein expression, and cocultured with 2 x 10^6 CFSE loaded PBMC in the presence of stimulation with soluble anti-CD3 mAb (1 μg/ml; Ortho Biotech) or 0.1 μg/mL superantigen TSST-1 (Sigma) and IL-2 (20 U/ml; AIDS Reference and Reagent Program) with or without soluble CD4 (sCD4) (10 μg/ml; AIDS Reference and Reagent Program). Increasing or decreasing the ratio of Env-expressing 293T cells to PBMC did not affect suppression of $CD4^+$ T cell proliferation. For exposure to free virions, 2 x 10^6 CFSE-labeled PBMC were incubated with AT-2-inactivated HIV-1 NL4-3 virions at concentrations of 1, 0.3, or 0.1 μg HIV p24 Gag equivalents/ml, which corresponds to a gp120 concentration of 1, 0.3, and 0.1 nM, respectively [112]. Four days after Env exposure, PBMC were collected, washed with PBS containing 1% FCS, and stained with anti-CD4-allophycocyanin (RPA-T4; BD Pharmingen). Cells were analyzed on a FACS-Calibur flow cytometer, and proliferation of $CD4^+$ T cells was analyzed by CFSE dilution using Cellquest software (BD Pharmingen). A minimum of 10,000 events were analyzed. The percent of stimulated $CD4^+$ T cells that proliferated was calculated as described [164].

Detection of apoptosis and activation in $CD4^+$ T cells exposed to Env

PBMCs were cocultured with Env-expressing 293T cells or 700 μM cycloheximide (Sigma) and activated with 0.1 μg/mL TSST-1 or 1 μg/mL anti-CD3 mAb. Apoptosis was examined 6, 17, and 36 hours after coculture and identified as annexin V-positive (BD Pharmingen, San Diego, CA) and 7-AAD–negative (Sigma) cells. Total cell death was measured by positive staining for 7-AAD in ungated cells. For studies measuring up-regulation of activation markers in Env-treated PBMCs, CD69

peridinin chlorophyll protein (day 1), HLA-DR fluorescein isothiocyanate and CD25 phycoerythrin cyanine 7 (BD Immunocytometry Systems, San Jose, CA) (day 2) were used.

Deetection of phosphorylation of signaling molecules in $CD4^+$ T cells

Purified $CD4^+$ T cells were exposed to IIIB Env-expressing 293T cells for 1 day, stimulated with 1 μg/mL anti-CD3 antibody for 10 minutes for p38 or 2 days followed by 20 U/mL IL-2 for 2 to 20 minutes for STAT5a, fixed, and analyzed by flow cytometry using anti–phospho-MAPK (pT180/ pY182) Alexa Fluor 647 or anti-phospho-STAT5 (pY694) Alexa Fluor 647 (BD Pharmingen), respectively. For AKT phosphorylation, PBMCs were cocultured with IIIB Env-expressing 293T cells for 2 hours in complete DMEM and then for 18 hours in DMEM with 0.5% serum, stimulated with 1 μg/mL anti-CD3 antibody for 10 minutes, fixed, and stained with anti–phospho-AKT (ser 473) Alexa Fluor 488 (Cell Signaling Technologies, Danvers, MA) and anti-CD4 v4 (clone L120) phycoerythrin (BD Immunocytometry Systems).

$CD25^+$ cell depletion and T cell proliferation assay

$CD25^+$ cells were depleted from PBMC using magnetic beads (Dynabeads CD25; Invitrogen Life Technologies). Briefly, PBMC and magnetic beads were washed twice with PBS containing 1% FBS. Then 1 x 10^7 cells/ml were mixed with a 40:1 ratio of beads to $CD25^+$ cells assuming 5% of cells expressed CD25, and rotated at 4°C for 45 minutes. A second round of depletion was performed with a decreased volume of beads

(half of that used in the first round) to achieve near complete depletion of $CD25^+$ cells. Whole and CD25-depleted PBMC were CFSE labeled, and cultured with Env-expressing and control 293T cells in presence of TCR stimulation.

Treg sorting and suppression culture

Whole PBMC or CD25-depleted PBMCs were cultured with Env-expressing or control 293T cells. During Env exposure, whole PBMC were unstimulated, whereas CD25-depleted PBMC were stimulated with anti-CD3 and IL-2 to expand induced Tregs. A total of 48 h after Env exposure, PBMC were removed from 293T monolayers and stained with anti-CD4-FITC, anti-CD25-PE-Cy5, and anti-CD127-PE (BD PharMingen). $CD4^+$, $CD127^-$, $CD25^+$ cell sorting was performed on a FACS/Aria (BDbioscience) to obtain Tregs. A total of 2 x 10^5 CFSE labeled CD25-depleted autologous PBMC responders were mixed with increasing amounts of FACS sorted $CD4^+$, $CD127^-$, $CD25^+$ Tregs (Suppressor to Responder ratios of 1:3 and 1:6) in the presence of stimulation with anti-CD3 and IL-2. Four days after coculture, the proliferation of responder cells was determined by flow cytometry.

Treg analyses

PBMC were exposed to Env (either on 293T as AT-2-treated virions) as described above. For exposure to 293T cells, PBMC were either resting or stimulated by anti-CD3 and IL-2. For incubation with AT-2 inactivated virions, resting PBMCs were used. At various time points after Env exposure (days 1, 3, and 5), PBMC were collected, washed

with PBS containing 1% FCS, and stained with anti-human CD4-FITC (SK3; BD Pharmingen) and anti-CD25-PE-Cy5 (M-A251; BD Pharmingen). Cells were then washed, fixed with 2% paraformaldehyde (eBiosicence), permeabilized with PBS 1% FCS containing 0.1% saponin (eBioscience), incubated with normal rat serum at 4°C for 15 min, and stained with anti-human FoxP3-allophycocyanin (PCH101; eBioscience) on ice for 30 min according to the manufacturer's instructions. The appropriate isotype-matched Abs were used to define background staining. Expression of CD4, CD25, and FoxP3 in cells were determined by flow cytometry. A minimum of 10,000 events were analyzed.

IL-10 and TGF-β ELISA

IL-10 and TGF-β concentrations in culture supernatants were determined by commercially available ELISA kits (BD OptEIA) according to the manufacturer's instructions.

Statistics

Mean, standard error of the mean (SEM), and student's t-test were performed using Microsoft Excel software.

Results

Env expressed on 293T cells induces suppression of $CD4^+$ T cell proliferation

Following vaccine delivery, a number of cell types other than APCs take up and express vaccine-derived antigens. Expression by these cells presents Env in the absence of costimulatory molecules. 293T cells were transfected with Env-encoding mRNA as consistent, and low-level protein expression is obtained in most cells [163], allowing uniform exposure of $CD4^+$ T cells to Env. PBMCs were used as a source of $CD4^+$ T cells and cocultured with 293T cells expressing X4-tropic IIIB, dual tropic 89.6, and M-tropic JR-Fl, YU-2, and SF162 Envs. Cells were stimulated with a concentration of anti-CD3 mAb that typically stimulates one quarter of the $CD4^+$ T cells. To monitor proliferation, PBMCs were stained with CFSE[97]. $CD4^+$ T cells that encountered any of the Envs had lower proliferation levels compared with cultures exposed to control RNA-transfected 293T cells (Figure 2.1A). Similar inhibition was observed when cells were activated by the superantigen TSST-1, a model antigen for activation via the T-cell receptor (TCR), which requires the same costimulatory and adhesion molecules as conventional antigens [138]. This suggested that suppression of proliferation was not dependent on a particular mode of activation. In addition, negatively selected purified $CD4^+$ T cells exposed to Env also failed to proliferate, arguing against a role for $CD8^+$ T cells and natural killer cells in mediating suppression (Figure 2.1B).

To characterize the signal delivered by Env, we examined whether suppression could be overcome by blocking Env interaction with CD4. sCD4 almost completely

abrogated Env-induced suppression (Figure 2.1A). P-values were calculated using results from 3 independent experiments. A paired Student t-test with 2 tails was performed using the percentage of change from control across experiments (no Env) or sCD4 treatment and is shown in Table 1.

Expression of functional trimeric Env on 293T cells was confirmed in fusion assays demonstrating that mRNA delivery resulted in surface expression of fusion-competent Env (Figure 2.1C-D). Delivery of Env-containing vaccines in vivo results in a variety of expression levels that are governed by the cellular vaccine target and the vaccine delivery method. In our experimental system that mimics vaccination with Env, we chose delivery of Env by encoding mRNA to 293T cells, which results in expression of Env in nearly 100% of cells at lower levels compared with other methods of delivery, such as DNA. This was important as lymphocyte trafficking does not occur in our system. Thus, if only 25% of the cells expressed Env, then only about 25% of the $CD4^+$ T cells added would be exposed. This low level of expression was sufficient to mediate suppression, and, in fact, a 5-fold reduction in the quantity of Env encoding mRNA delivered, which results in a 5-fold lower level of protein expressed, resulted in similar levels of suppression (data not shown). Thus, at the range of expression levels seen in our culture system, large variations in the amount of Env expressed did not alter the degree of suppression exerted. This could explain the lack of correlation observed between the degree of suppression mediated by an Env strain (Figure 2.1A) and the amount of fusion mediated (Figure 2.1C-D).

Several reports have found that trimeric or aggregated forms of Env are required to mediate suppression [10, 93]. However, others have found that monomeric gp120 could exert suppressive effects [82 165]. In our system, $CD4^+$ T cells were exposed to trimeric cell-surface expressed Env as well as monomeric gp120 released into the supernatant, likely from dissociation of cell-surface gp160. PBMCs exposed exclusively to exogenous monomeric gp120 derived from IIIB or SF162 failed to suppress $CD4^+$ T cell proliferation (Table 2). The added gp120 retained the ability to block binding of CD4 mAb (leu-3a) demonstrating functional binding to CD4. This confirmed that trimeric surface expressed Env-mediated suppression in our system. Thus, transfection of 293T cells with encoding mRNA resulted in surface expression of functional Env, which mediated inhibition of antigen-specific $CD4^+$ T cell proliferation in a CD4-dependent manner.

Figure 2.1

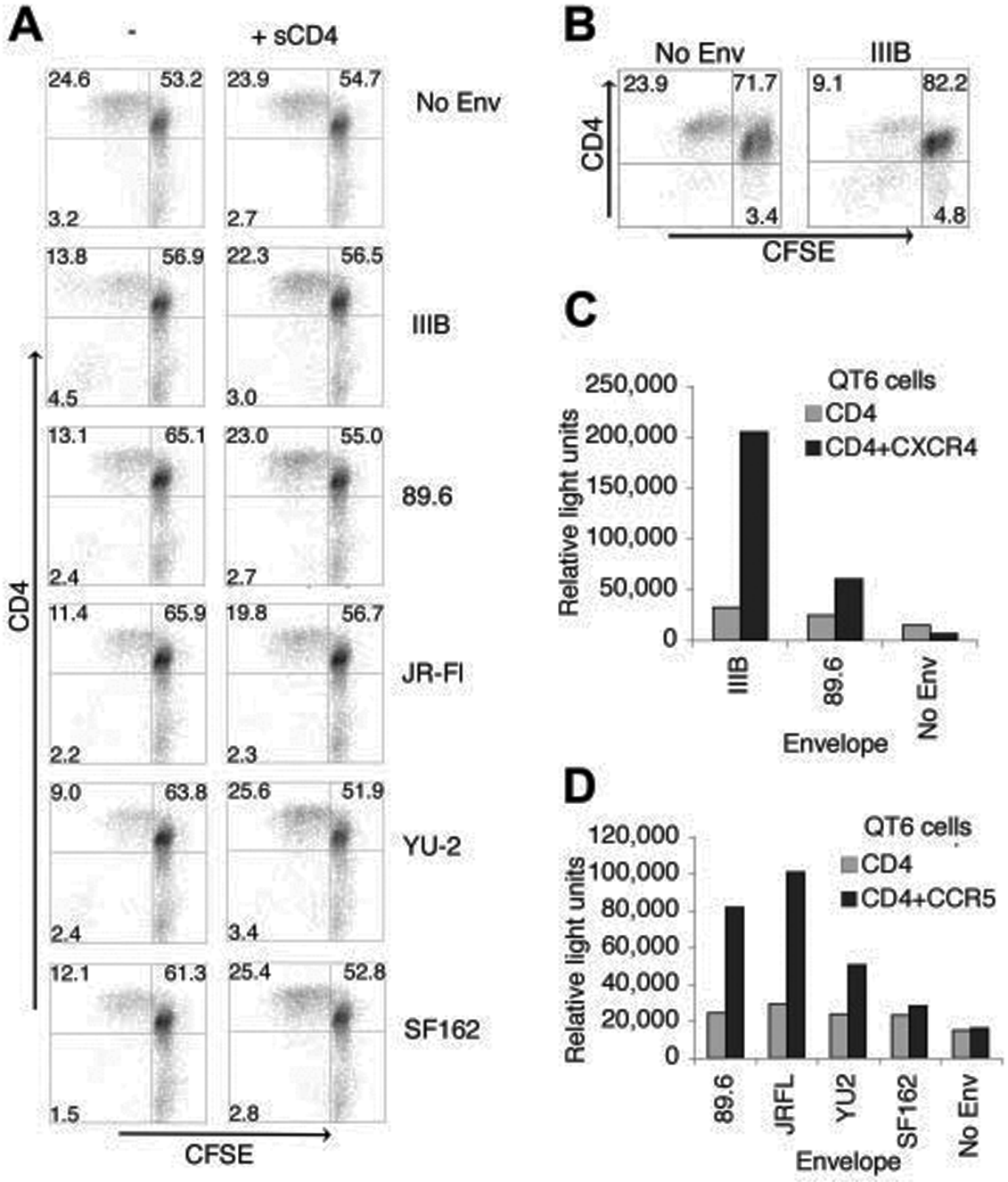

A
-
+ sCD4
24.6
53.2
3.2
23.9
54.7
2.7
No Env
13.8
56.9
4.5
22.3
56.5
3.0
IIIB
13.1
65.1
2.4
23.0
55.0
2.7
89.6
11.4
65.9
2.2
19.8
56.7
2.3
JR-FI
9.0
63.8
2.4
25.6
51.9
3.4
YU-2
12.1
61.3
1.5
25.4
52.8
2.8
SF162
CD4
CFSE
B
No Env
IIIB
23.9
71.7
3.4
9.1
82.2
4.8
CD4
CFSE
C
QT6 cells
CD4
CD4+CXCR4
Relative light units
250,000
200,000
150,000
100,000
50,000
0
IIIB
89.6
No Env
Envelope
D
QT6 cells
CD4
CD4+CCR5
Relative light units
120,000
100,000
80,000
60,000
40,000
20,000
0
89.6
JRFL
YU2
SF162
No Env
Envelope

Figure 2.1. HIV Env suppresses anti-CD3-activated CD4+ T cell proliferation. (A) 293T cells expressing IIIB, 89.6, YU-2, SF162, and JR-Fl Envs or no Env poly (A,C) RNA transfected) were cocultured with CFSE-labeled PBMCs with or without sCD4 (10 μg/ml). T cells were activated with anti-CD3 mAb (0.5 μg/mL), and monitored for proliferation 4 days after coculture by flow cytometry with staining with anti-CD4 mAb. (B) Negatively selected purified CD4+ T cells were cocultured with 293T cells expressing IIIB Env or no Env and activated using anti-CD3 mAb (0.5 μg/mL). Quadrants are labeled with the percent of events in each. Fusion assays demonstrated surface expression of functional Env. Encoding mRNA-transfected 293T cells were mixed with QT6 cells expressing CD4 and CXCR4 (C) or CCR5 (D), and fusion of the 2 cell populations was measured by luciferase production. Experiments shown are representative of 3 independent experiments.

Table 1. Statistical analysis of suppression of $CD4^+$ T cells proliferation by Env

Env strain	*P* of suppression compared with no Env control	*P* of suppression compared with sCD4 treatment
IIIB	< .005	< .005
89.6	.01	.02
JR-F1	.02	.04
Yu-2	.01	.02

P values were calculated using results from 3 independent experiments. Percentage of change from control (no Env) or sCD4 treatment was used in a paired Student *t* test with 2 tails.

Table 2. Effect of monomeric gp120 on $CD4^+$ T-cell proliferation

	No Env	IIIB gp120	SF162 gp120
$CD4^+$ cells undergoing proliferation, %	15.3	14.4	12.5
$CD4^-$ cells undergoing proliferation, %	7.7	7.4	7.3

Anti-CD3 mAb (0.5 μg/mL) stimulated CFSE-labeled PBMCs were exposed to 2 μg/mL IIIB or SF162 gp120 or left untreated. $CD4^+$ T cells were monitored for proliferation 4 days after coculture by flow cytometry and expressed as percentage of total $CD4^+$ T cells.

DCs expressing Env suppress $CD4^+$ T cell proliferation

Presentation of vaccine-derived antigens by APCs activates immune responses and is the basis of an effective vaccine. APCs, such as DCs in their mature form, express a variety of costimulatory molecules on their surface and provide a strong activation signal for T and B cells. Certain studies have shown that activation, including potent costimulation, could overcome suppression induced by CD4 cross-linking [146]. Other studies have not supported this, demonstrating that HIV-infected DCs were able to suppress $CD4^+$ T cells in an Env-dependent fashion. Currently, viral vector or nucleic

acid delivery of encoding antigen to DCs (either directly in vivo or after in vitro culture and loading of DCs) is in human trials and represents a potent vaccine approach [62, 111]. We infected DCs with vaccinia virus encoding various Envs and tested their ability to affect $CD4^+$ T cell proliferation in response to superantigen. DCs expressing the X4 Envs IIIB and MN and the R5 Env 92UG037.8 (a Clade A primary isolate) were found to inhibit $CD4^+$ T cell proliferation to antigen, which was overcome by sCD4 (Figure 2.2). The p-values comparing IIIB Env to no Env or sCD4 treatment were less than 0.005. Expression of Env by these types of vectors in vitro has been previously demonstrated[59, 76, 121]. Very low multiplicities of infection (MOIs) of vaccinia were used as T-cell toxicity was observed at MOIs typically used in fusion assays. However, even at low MOIs (0.3-0.6) a minority of the suppression could not be overcome with sCD4. This suppression of T-cell proliferation was also observed with 2 control viruses, vTF7-3 encoding T7 Polymerase and WR, the strain used to create all of the Env-expressing and control viruses, suggesting a vaccinia mediated suppression was also present. We concluded that vaccinia viruses had a small but significant level of toxicity in our system, which accounts for the inability of sCD4 to completely overcome the suppression of proliferation. In addition, the ability of Env to interact with CD4 molecules on DCs and suppress their maturation and function cannot be ruled out in our system, as it has been observed in other systems [54, 167]. Expression of functional Env was confirmed in fusion assays between DCs and Quail cells (data not shown). Thus, mature DCs expressing Env mediate suppression of antigen-specific $CD4^+$ T cell proliferation despite expressing multiple costimulatory molecules.

Figure 2.2

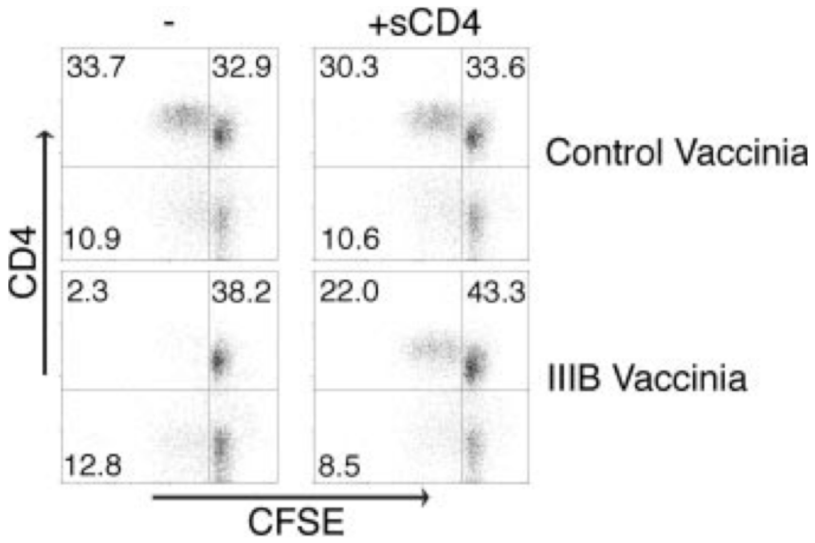


Figure 2.2. DCs expressing Env and presenting antigen suppress CD4$^+$ T cell proliferation. DCs were infected with vaccinia virus encoding IIIB Env or control vaccinia encoding T7 polymerase and cocultured with CFSE-labeled PBMCs with 0.1 μg/mL TSST-1 with or without sCD4 (10 μg/ml). This concentration of superantigen requires presentation by DCs as no proliferation is observed in their absence. Proliferation of CD4$^+$ T cells was measured 5 days later by flow cytometry. Quadrants are labeled with the percent of events in each. Experiments shown are representative of 3 independent experiments.

Neither apoptosis nor IL-2-dependent anergy mediate CD4$^+$ T cell suppression

Env-induced CD4$^+$ T cell dysfunction has been described as anergy, apoptosis, cell death, or a failure to proliferate. We observed that Env suppresses CD4$^+$ T cells in our system by hindering proliferation. We next investigated whether anergy or apoptosis

occurred. PBMCs were cocultured with Env-expressing 293T cells and were activated using TSST-1 superantigen. 6, 18, and 36 hours later, $CD4^+$ T cells were analyzed for early apoptosis using annexin V staining of 7-AAD–negative cells. Cyclohexamide is a protein synthesis inhibitor that induces apoptosis of PBMCs and was used as a positive control. Env failed to induce apoptosis of $CD4^+$ T cells above the level seen in cells unexposed to Env (Figure 2.3A). In addition, an increase in cell death (7-AAD uptake in ungated cells) in Env-exposed $CD4^+$ T cells was not observed (Figure 2.3B). Thus, apoptosis and cell death are not major contributors to the suppression of proliferation induced by Env in our system. T-cell anergy is defined as a state of nonresponsiveness to activation with antigen. The addition of exogenous IL-2 overcomes most but not all types of anergy [133]. Selliah and Finkel observed that $CD4^+$ T cell suppression induced by Env could be overcome with exogenous IL-2 [134]. To investigate this mechanism, CFSE-stained PBMCs were cultured with control or Env-expressing 293T cells and activated with TSST-1 in the presence of increasing amounts of exogenous IL-2 (20, 40, or 80 U/mL). IL-2 was unable to prevent suppression of proliferation of $CD4^+$ T cells by Env (Figure 2.3C, and data not shown), arguing against this form of anergy as the mechanism of suppression.

Figure 2.3

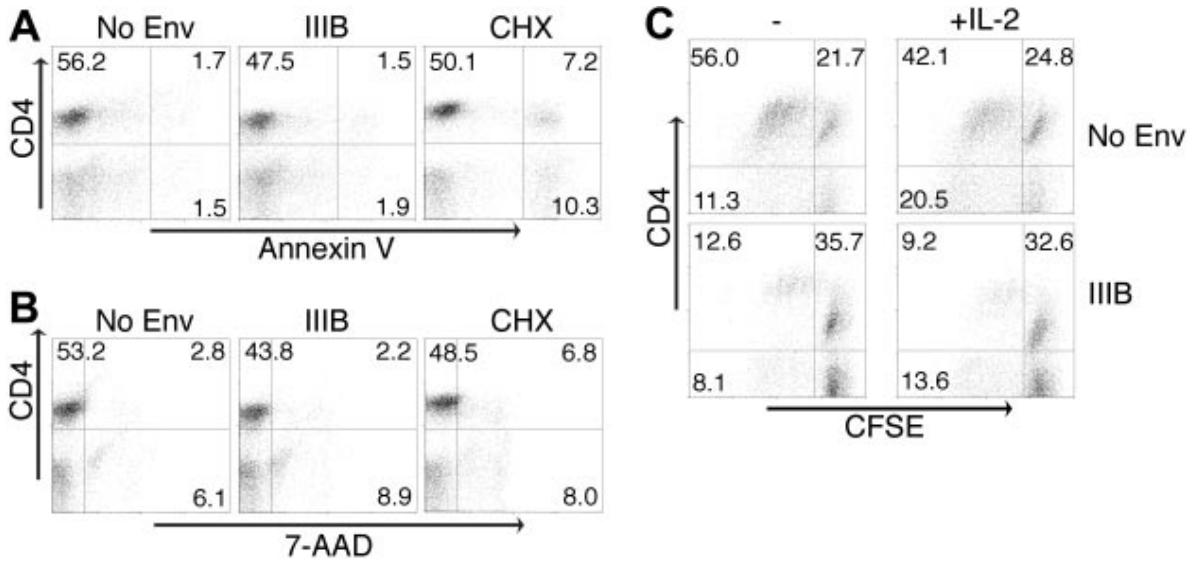


Figure 2.3. Neither apoptosis nor IL-2-dependent anergy mediate Env-induced CD4$^+$ T cell suppression. PBMCs were cocultured with IIIB Env or luciferase-expressing 293T cells or 700 μM cycloheximide (CHX) and activated with 0.1 μg/mL TSST-1. Thirty-six hours later, apoptotic CD4$^+$ T cells were analyzed by flow cytometry and defined as Annexin V positive and 7-AAD negative, early apoptotic cells (A) and 7-AAD positive for dead cells (B). (C) CFSE-labeled PBMCs were cocultured with IIIB Env-expressing 293T cells in the presence of 80 U/mL IL-2 and activated with 0.1 μg/mL TSST-1. CD4$^+$ T cell proliferation was measured 5 days later by flow cytometry. Quadrants labeled with percent of events in each. Experiments shown are representative of 3 independent experiments.

$CD4^+$ T cells activated in the presence of Env phosphorylate AKT, MAPK, and STAT5a but fail to completely up-regulate activation markers

$CD4^+$ T cells exposed to Env failed to proliferate in response to antigen but did not apoptose, anergize, or die. We next sought to determine the location of the block in the activation cascade. In response to T cell activation, phosphorylation of key signaling molecules p38 MAPK and AKT relay the TCR signal to downstream mediators [44, 133] $CD4^+$ T cells exposed to Env and activated by anti-CD3 phosphorylated AKT and MAPK similar to control treated cells (Figure 2.4). IL-2 is an important cytokine involved in T-cell proliferation, and its binding to both the low and high affinity IL-2R leads to the phosphorylation of STAT5a [78]. Exogenous IL-2 was unable to circumvent the suppression of proliferation induced by Env (Figure 2.3C), but cells exposed to and suppressed by Env were able to phosphorylate STAT5a in response to IL-2 (Figure 2.4B), suggesting that early signaling mediators in the IL-2 cascade also were not impaired. On activation, $CD4^+$ T cells go through a series of events prior to proliferation, including up-regulation of markers of activation such as CD69, HLA-DR, and CD25 [39]. PBMCs were exposed to IIIB Env-expressing 293T cells and activated using anti-CD3 or superantigen. Up-regulation of CD69 (day 1) and HLA-DR and CD25 (day 2) was monitored using flow cytometry. $CD4^+$ T cells exposed to Env were unable to completely up-regulate their activation markers (Table 3). Thus, although exposure of $CD4^+$ T cells to Env did not suppress the phosphorylation of immediate signaling mediators, it suppressed the upregulation of early and late cell-surface markers of activation.

Figure 2.4

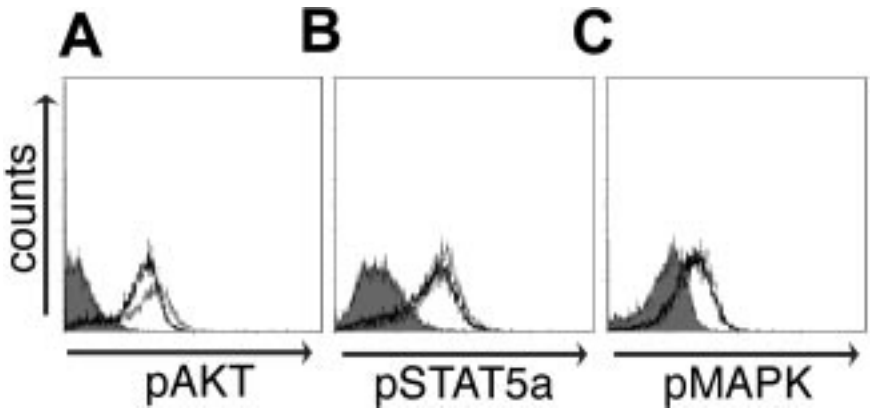


Figure 2.4. $CD4^+$ T cells activated in the presence of Env phosphorylate AKT, p38 MAPK, and STAT5a. PBMCs were cocultured with Env or control RNA-expressing 293T cells for 24 hours. Phosphorylation of AKT or MAPK 10 minutes after anti-CD3 (1.0 μg/mL) stimulation was measured using flow cytometry with gating on $CD4^+$ T cells. Purified $CD4^+$ T cells were exposed to control RNA or Env-expressing 293T cells and activated using anti-CD3 in the absence of IL-2. Two days later, cells were stimulated with 20 U/mL IL-2 and analyzed for STAT5a phosphorylation 7 minutes later. $CD4^+$ T cells exposed to IIIB Env-transfected 293T cells and stained with isotype control mAb (shaded gray) or specific phosphoprotein mAb (thin line) or specific phosphoprotein mAb-stained control-transfected 293T cells (thick line) are shown for each signaling molecule. Experiments shown are representative of 3 independent experiments.

Table 3. Effect of HIV Env on activation marker expression

	No Env	IIIB Env
CD4⁺ cells expressing, %		
CD69	78.9	56.6
HLA-DR	23.5	12.9
CD25 (IL2R-α)	60.4	31.6
CD4⁻ cells expressing, %		
CD69	59.2	61.8
HLA-DR	43.7	41.6
CD25 (IL2R-α)	33.3	22.2

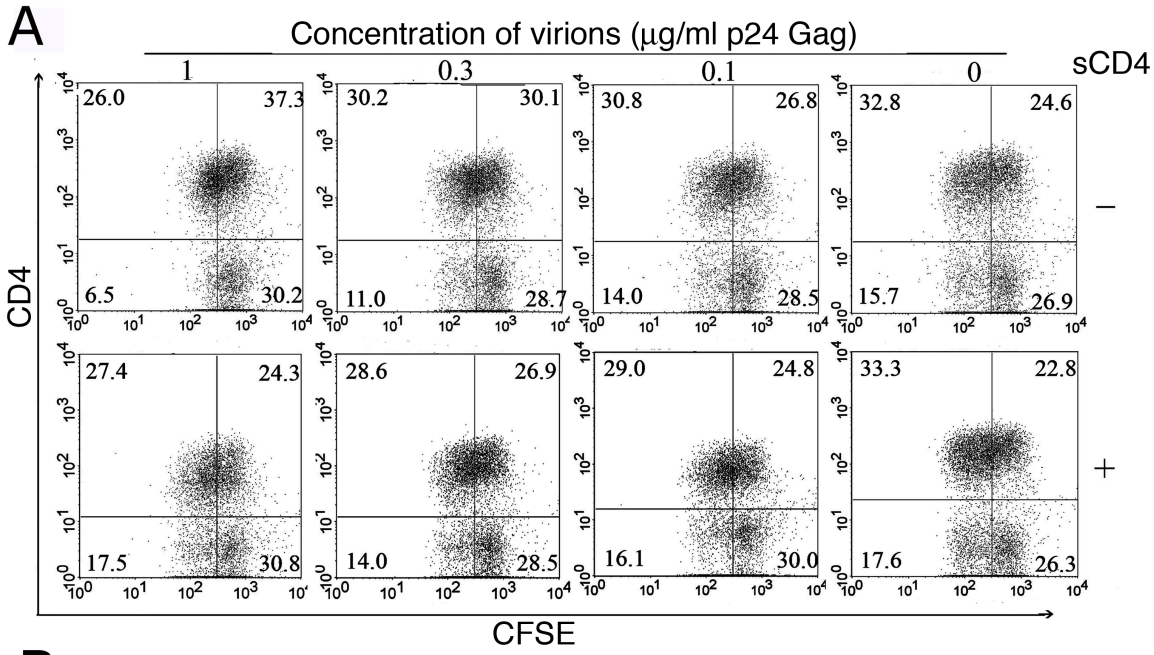
PBMCs were exposed to control or IIIB Env expressing 293T cells and activated with anti-CD3. Expression of CD69 on day 1 and HLA-DR and CD25 on day 2 on $CD4^+$ and $CD4^-$ T cells was determined using flow cytometry.

Env expressed on free virions suppresses $CD4^+$ T cell proliferation

In HIV infection, Env present on infected cells and free virions can contact $CD4^+$ T cells and induce dysfunction [55]. Above, we demonstrate that Env expression on cells as occurs during vaccination with encoding vectors such as DNA or viruses induces an inhibition to antigen-specific $CD4^+$ T cell proliferation in a CD4 dependent manner. This system used human PBMC as a source of $CD4^+$ T cells that were cultured with Env expressing mRNA transfected 293T cells or vaccinia infected DC in the presence of either anti-CD3 or superantigen (TSST-1) stimulation. To test whether Env on virions is also able to induce suppression of anti-CD3 stimulated $CD4^+$ T cell proliferation, we incubated PBMC with increasing amounts of AT-2 inactivated HIV-1 (NL4-3), which is infection deficient, but retains a functional (able to bind CD4) Env, modeling the noninfectious interactions between circulating free virions and CD4 in HIV infection [112]. We found that inactivated virus could inhibit anti-CD3 stimulated $CD4^+$ T cell proliferation in a dose dependent manner, which was overcome by sCD4 (Figure 2.5A-

B), similar to that observation for Env expressed on cells. Variability in the amount of suppression was observed for different preparations of AT-2 virus ranging from 29% (Figure 2.5A-B) to greater than 60% (data not shown). Decreased fluorescence intensity of anti-CD4 SK3 mAb staining for virion-incubated PBMC [14] verified a functional interaction between CD4 and Env (data not shown). Interestingly, we found that proliferation of CD4 negative cells was also abrogated after incubation with 1 μg/ml virions (Figure 2.5A), which might be due to impairment of $CD4^+$ T cell helper functions by Env, leading to decreased growth cytokine production (IL-2 and others) and less activated APCs in PBMC that, in turn, cause a decrease in antigen-stimulated $CD8^+$ T cell proliferation.

Figure 2.5



B

Soluble CD4	Concentration of virions (μg/ml p24 Gag)			
	1	0.3	0.1	0
-	16[1]	21	24	24
+	24	24	24	25

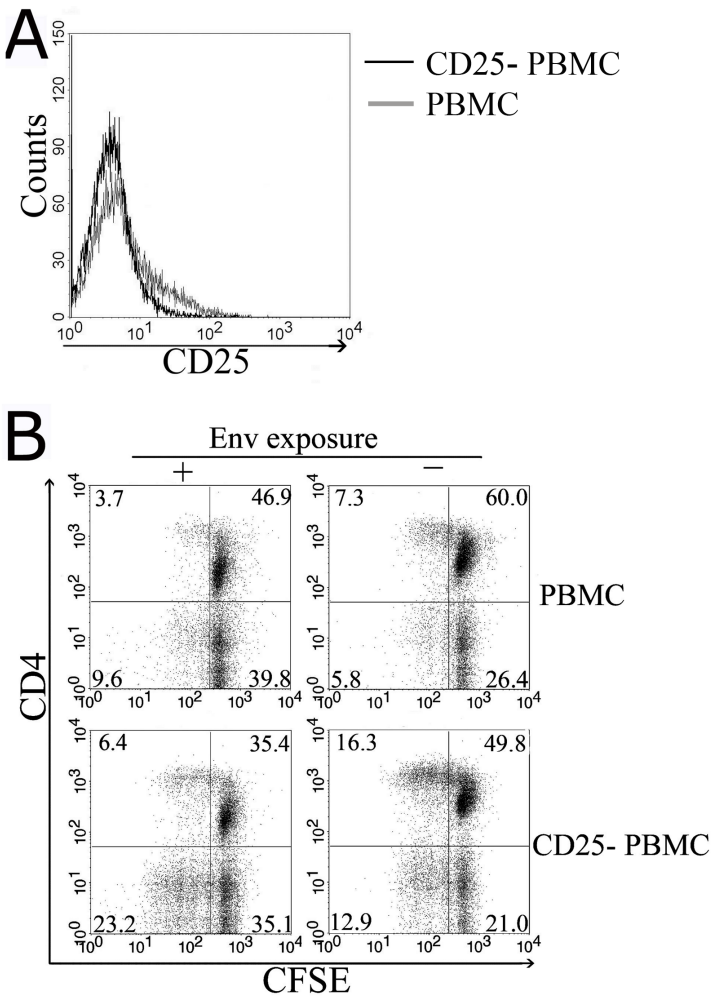
Figure 2.5. HIV Env present on free virions induces suppression of anti-CD3 stimulated CD4$^+$ T cell proliferation through CD4 binding. Freshly isolated human PBMC were CFSE labeled and exposed to AT-2-treated HIV-1 virions (A and B) in the presence or absence of sCD4 (10 μg/ ml). The cultures were stimulated with anti-CD3 mAb and IL-2. Four days after Env exposure and stimulation, PBMC were stained for CD4 and the proliferation of CD4$^+$T cells was monitored by flow cytometry (A). The percent of input CD4$^+$ T cells that proliferated (B) was calculated as described [164]. Data are representative of three experiments. B, [1]=$p < 0.05$. p-values calculated using 2-tailed

homoscedastic student's t-test comparing AT-2 virus-treated $CD4^+$ T cell proliferation to no virus treated.

$CD25^+$ Treg cells are not required for Env induced suppression

We then investigated whether Treg cells are involved in Env induced suppression in our system. CFSE stained total or CD25 depleted PBMC were cultured with Env-expressing or control 293T cells in the presence of anti-CD3 and IL-2 stimulation. CD25 staining verified that more than 95% of $CD25^+$ cells were successfully removed from PBMC (Figure 2.6A). As expected, CD25 depletion significantly enhanced the proportion of proliferating $CD4^+$ T cells in both Env exposed (From 8.1% to 18%) and control cultures (From 13% to 32.7%, Figure 2.6B). However, exposure to Env caused almost a 50% decrease in the proportion of proliferating $CD4^+$ T cells in CD25 depleted PBMC (From 18% to 32.7%, Figure 2.6B), suggesting that pre-existing $CD25^+$ Treg cells were not required for Env induced suppression of $CD4^+$ T cell proliferation.

Figure 2.6



C

	IIIB Env	No Env
PBMC	2.8[1]	4.6
CD25- PBMC	5.2[1]	10.4

Figure 2.6. CD25$^+$ Treg cells are not required for Env-induced suppression of proliferation. CD25$^+$ cells were depleted from human PBMC using two rounds of CD25 magnetic beads. Flow cytometric analysis demonstrated depletion of greater than 95% of CD25$^+$ cells (A). CFSE labeled whole (top, B) or CD25-depleted (bottom, B) PBMC

were exposed or not to IIIB Env-expressing 293T cells in the presence of anti-CD3 and IL-2 stimulation. $CD4^+$ T cell proliferation 4 days after Env exposure is shown. (C) The percent of input $CD4^+$ T cells that proliferated is shown for each condition in B. [1]=$p<0.01$. p-values were calculated using a 2-tailed homoscedastic student's t test comparing Env-treated $CD4^+$ T cell proliferation to no virus-treated. Data are representative of at least four repetitions.

Effect of Env on frequency of $CD4^+$, $CD25^+$, $FoxP3^+$ cells in PBMC

We show that $CD25^+$ Treg cells are not required for Env to induce suppression of $CD4^+$ T cell proliferation. One important question that remains highly controversial is whether and how HIV, or its associated gene products, regulates Treg cells. Both increased and reduced frequency of Treg cells have been reported in HIV^+ patients compared to uninfected people [1, 149, 161]. To test whether Env, as the major surface antigen of HIV interacting with $CD4^+$ T cells, directly induces or activates Treg cells, we first determined and compared the percentage of $CD4^+$, $CD25^+$, $FoxP3^+$ cells in both stimulated and resting human PBMC with or without exposure to Env expressed on 293T cells. Compared to resting PBMC, stimulation caused a one- to two-fold increase in the amount of $CD4^+$, $CD25^+$, $FoxP3^+$ T cells in PBMC at all time points studied (Figure 2.7A), which is consistent with a study demonstrating activation induced FoxP3 expression in human T cells [60]. Of importance, for both resting and stimulated PBMC, Env exposure did not induce significant changes in the percentage of $CD4^+$, $CD25^+$, $FoxP3^+$ T cells (Figure 2.7A). Analyses of T cell proliferation in the activated cells

demonstrated a significant reduction of $CD4^+$ T cell division when cultured with Env mRNA transfected 293T cells, verifying Env expression on 293T cells and its inhibitory effect.

We then investigated whether Env present on free virions affected the percentage of $FoxP3^+$ Treg cells in PBMC, as we observed inhibition of $CD4^+$ T cell proliferation by free virions (Figure 2.5A-B). PBMC were incubated with AT-2 inactivated HIV-1 virions at 1 μg/ml p24 gag protein equivalent for various period of time. Surface and intracellular staining demonstrated no significant difference in the number of $CD4^+$, $CD25^+$, $FoxP3^+$ cells between virion incubated and control PBMC at all studied time points (days 1, 3 and 5) (Figure 2.7B), which is in contrast with the observation that exposure of purified $CD4^+$ T cells to AT-2 treated HIV resulted in significantly increased FoxP3 expression at both mRNA and protein levels, resulting from Env induced enhancement in cell viability and consequent accumulation of $FoxP3^+$ cells [112]. Taken together, our data suggested that neither Env delivered on cells as would be found during infection or after encoding vector delivery as part of a vaccine nor Env present on free virions could directly induce or activate T regulatory cells in resting or stimulated PBMC.

Figure 2.7

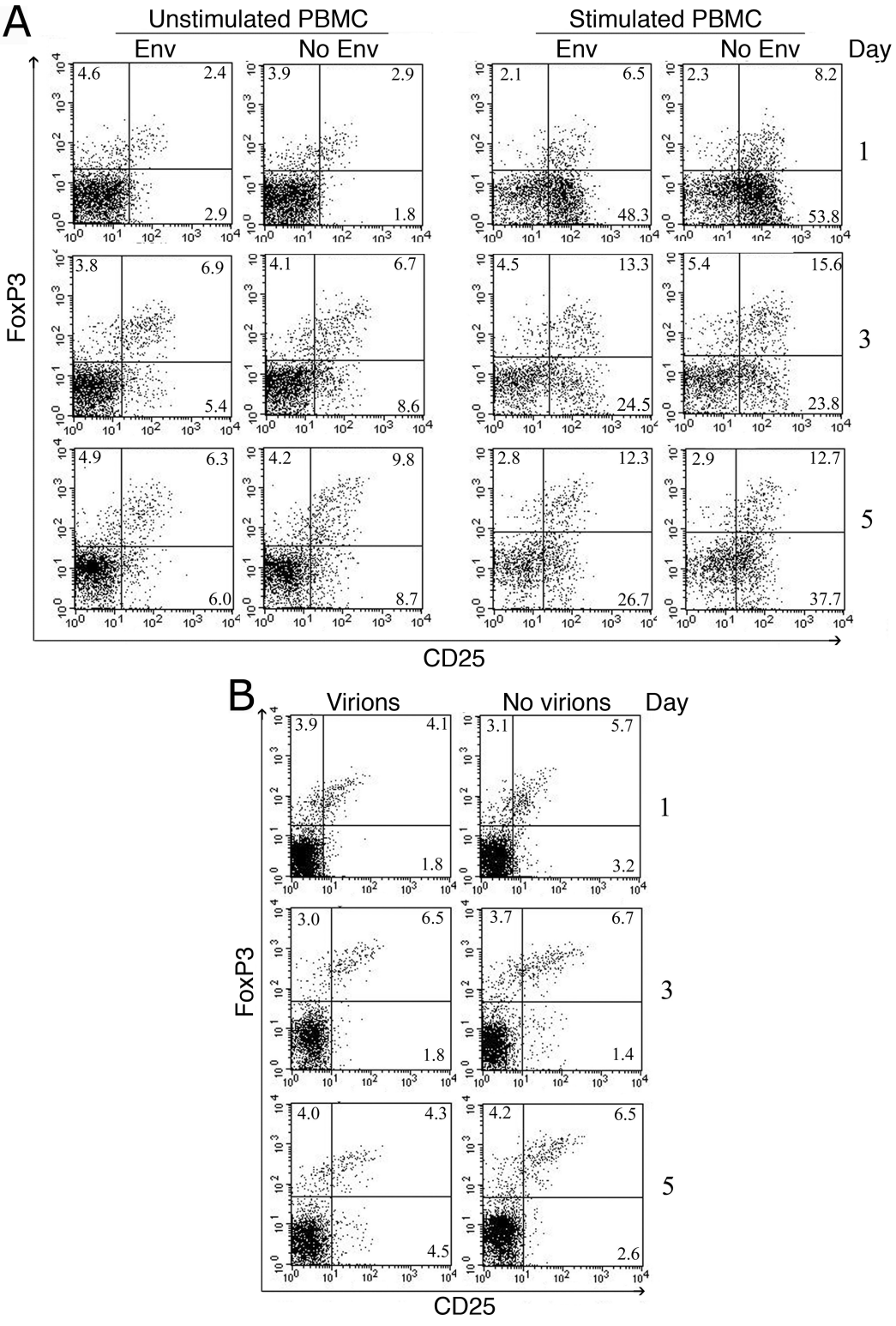

A
Unstimulated PBMC
Stimulated PBMC
Env
No Env
Env
No Env
Day
1
3
5
FoxP3
CD25
4.6
2.4
2.9
3.9
2.9
1.8
2.1
6.5
48.3
2.3
8.2
53.8
3.8
6.9
5.4
4.1
6.7
8.6
4.5
13.3
24.5
5.4
15.6
23.8
4.9
6.3
6.0
4.2
9.8
8.7
2.8
12.3
26.7
2.9
12.7
37.7
B
Virions
No virions
Day
1
3
5
FoxP3
CD25
3.9
4.1
1.8
3.1
5.7
3.2
3.0
6.5
1.8
3.7
6.7
1.4
4.0
4.3
4.5
4.2
6.5
2.6

Figure 2.7. Effect of Env delivered by cell surface expression or on virions on FoxP3$^+$, CD25$^+$, Treg cells in unstimulated and stimulated PBMC. Unstimulated or anti-CD3 stimulated PBMC were analyzed by flow cytometry for CD25 and FoxP3 expression on CD4$^+$ T cells at days 1, 3, and 5 after exposure to HIV Env expressed by 293T cells (A) or AT-2-treated virions (B). Histograms shown are gated on live CD4$^+$cells. Data are representative of at least four repetitions.

IL-10 and TGF-β production in Env exposed PBMC

Immune suppression mediated by Treg cells can occur through either cell contact or suppressive cytokines, particularly IL-10 and TGF-β [31, 156]. To test whether Env exposure, rather than inducing an increased number of FoxP3$^+$ cells, stimulates an enhanced production of IL-10 and/or TGF-β by Tregs, supernatants of cultures with and without Env exposure were collected at various time points (days 1, 3, and 5) after initiation of co-culture, followed by quantitation of IL-10 and TGF-β by ELISA. TGF-β was persistently undetectable at any of the studied time points in both Env exposed and control supernatants. Significant amounts of IL-10 were produced and accumulated in the supernatants starting at 12 hours after stimulation. However, no difference was observed in the amount of IL-10 production between Env-exposed and control cultures (Figure 2.8). Considered together, these data suggest that Env does not induce a more activated phenotype of Treg cells leading to increased suppressive cytokine production.

Figure 2.8

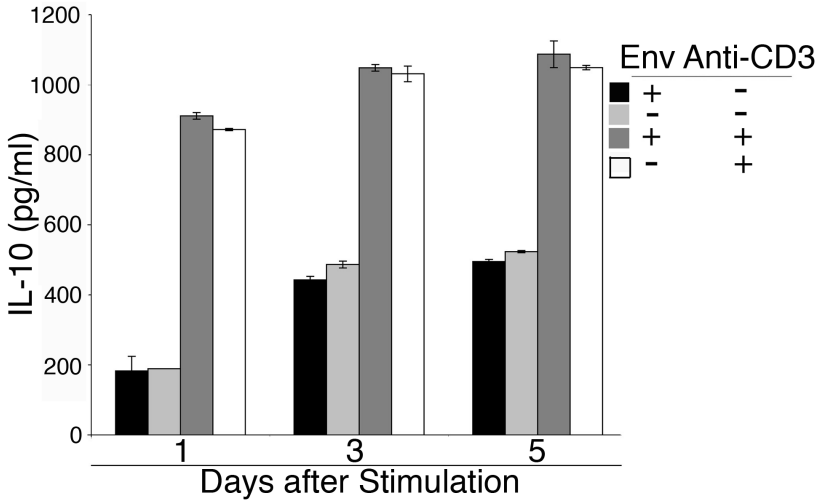


Figure 2.8. Effect of Env exposure on IL-10 production. 293T cells expressing IIIB Env or control transfected were cultured with PBMC in the presence or absence of anti-CD3 and IL-2 stimulation. Supernatants were collected at the indicated time points, and supernatant associated IL-10 was determined by ELISA. Data are representative of at least five experiments. Error bars are the SEM for triplicate measurements.

Env does not enhance suppressive activity of $CD4^+$, $CD25^+$, $CD127^-$ T cells

We demonstrate that Env induces neither an increased number of $CD4^+$, $D25^+$, $FoxP3^+$ cells nor an enhanced IL-10 and TGF-β production in PBMC and their depletion does not negate Envs ability to suppress $CD4^+$ T cell proliferation. To test whether Env activates Treg cells, resulting in an enhanced suppressive activity independent of IL-10 or

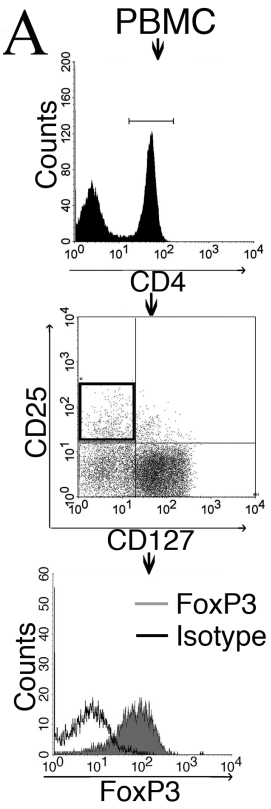
TGF-β, an in vitro suppression assay was developed in which Treg cells isolated from Env exposed and control PBMC were cultured with autologous responder cells in the presence of anti-CD3 stimulation. The suppressive activity of Treg cells, on a per-cell basis, was evaluated by their capacity to inhibit anti-CD3 stimulated responder cell division. In addition to CD25, CD127, a subunit of the IL-7R, has also been shown to be a useful cell surface marker for defining Tregs. We sorted Tregs from control RNA or Env RNA transfected 293T cell exposed PBMC based on $CD4^+$, $CD25^+$, and $CD127^-$ expression[94]. Greater than 90% of the sorted cells expressed FoxP3 (Figure 2.9A). In addition to natively expressed FoxP3 in a subset of $CD4^+$ T cells in PBMC, an additional population of $FoxP3^+$ cells appears after activation. These cells are derived from $FoxP3^-$, $CD4^+$ cells and their ability to suppress immune responses is controversial [60]. We attempted to use this same sorting strategy to isolate Tregs after Env exposure and anti-CD3 stimulation, but it should be noted that activation induces up-regulation of CD25 as well as down-regulation of CD127 on non-Treg $CD4^+$ T cells, making the isolation of $FoxP3^+$ cells less accurate. However, approximate 30-50% of the sorted cells from stimulated PBMCs expressed FoxP3 (data not shown).

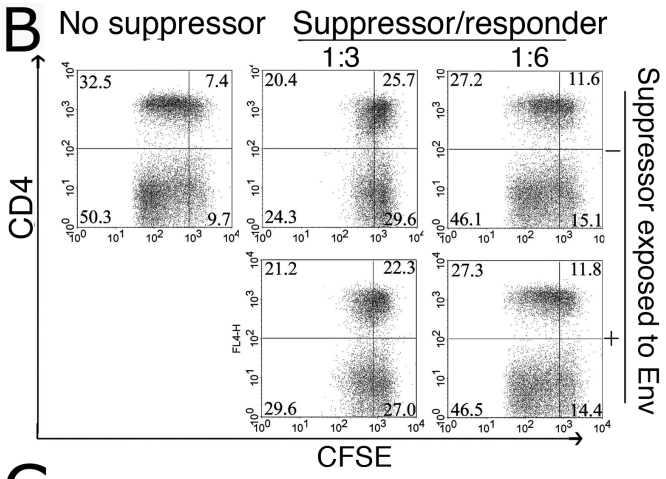
Sorted $CD4^+$, $CD25^+$, $CD127^-$ suppressor cells inhibited responder $CD4^+$ T cell divisions in a dose dependent manner (Percentages of proliferating $CD4^+$ T cells as the proportion of $CD4^+$ T cells are 48% and 74% for Suppressor: Responder ratio of 1:3 and 1:6, respectively) (Figure 2.9B-C). However, Env-exposed and control $CD4^+$, $CD25^+$,

$CD127^-$ T cells were equally suppressive, since no significant difference in the proportion of dividing responder cells was found between them (Figure 2.9B-C).

We then determined whether Env induces a more suppressive activity of activation induced $FoxP3^+$ cells by sorting $CD4^+$, $CD25^+$, $CD127^-$ T cells from PBMC, which were first depleted of $CD25^+$ cells and then co-cultured with 293T cells and activated by anti-CD3. Activation induced $CD4^+$, $CD25^+$, $CD127^-$T cells (approximately 30-50% FoxP3+), although less suppressive than naturally $FoxP3^+$ cells isolated from unstimulated total PBMC, also inhibited $CD4^+$ T cell proliferation in a dose dependent manner (Figure 2.9D-E). Of importance, no difference in suppressive ability between Env exposed and control activation-induced Treg cells was observed (Figure 2.9D-E). Taken together, these data indicate that Env exposure does not enhance the functional suppressive activity of either pre-existing Treg or activation induced $FoxP3^+$ cells.

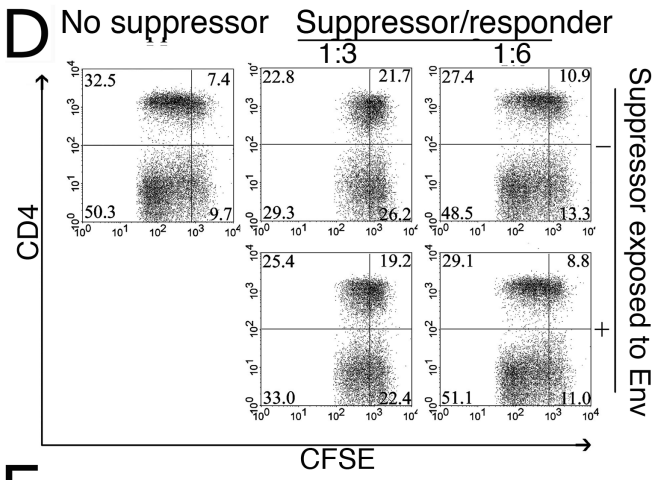
Figure 2.9





C

Env	Ratio of CD25+ T regs per responder		
	none	1:3	1:6
-	56.2	29.1[1]	46.4
+		34.1[1]	47.3



E

Env	Ratio of CD25+ T regs per responder		
	none	1:3	1:6
-	56.2	33.5[1]	49.1
+		37.1[1]	51.8

Figure 2.9. Endogenously present or activation induced (plus FoxP3, CD25) FoxP3 Treg cell suppressive activity is not modulated by Env. (A) Isolation of $FoxP3^+$ Treg cells from PBMC using CD4, CD25, and CD127. $CD4^+$ live lymphocyte sized cells (gate in left panel of A) were sorted for high CD25 and no CD127 expression (gate in middle panel of A). The sorted cells were stained for FoxP3 (A, right panel) and demonstrated greater than 90% positivity. (B) As a source of suppressors, PBMC were cultured with Env expressing or control 293T cells, followed by isolation of $CD4^+$, $CD25^+$, $CD127^-$ T cells by flow cytometric sorting (detailed in A) 48 h after Env exposure. CFSE-labeled responder cells (autologous PBMC depleted of $CD25^+$ cells) were cocultured with graded numbers of sorted $CD4^+$, $CD25^+$, $CD127^-$ suppressor cells in the presence of anti-CD3 and IL-2 stimulation. CFSE intensity of responder cells with CD4 staining was monitored by flow cytometry at day 4 to quantify responder cell proliferation. (C) Percent of input $CD4^+$ T cells that proliferated was calculated for each condition. [1]=$p < 0.007$. p-values calculated using 2-tailed homoscedastic student's t-test comparing 293T Env-treated $CD4^+$ T cell proliferation to no Env-treated. (D) As a source of suppressors, CD25-depleted PBMC were cultured with Env expressing or control 293T cells in the presence of anti-CD3 and IL-2 stimulation, followed by isolation of $CD4^+$, $CD25^+$, $CD127^-$ T cells 48 h after stimulation, as described in A. CFSE-labeled responder cells (autologous PBMC depleted of $CD25^+$cells) were cocultured with graded numbers of sorted $CD4^+$, $CD25^+$, $CD127^-$ suppressor cells in the presence of anti-CD3 and IL-2 stimulation. CFSE intensity of responder cells with CD4 staining was monitored by flow cytometry at day 4 to quantify responder cell proliferation. (E) Percent of input $CD4^+$ T cells that

proliferated was calculated for each condition. [1]=$p < 0.009$. p-values calculated using 2-tailed homoscedastic student's t-test comparing 293T-Env-treated $CD4^+$ T cell proliferation to no Env-treated. Data are representative of at least three repetitions.

Discussion

The system studied here models the encounter of $CD4^+$ T cells with both APCs and non-APCs expressing Env delivered as a vaccine, the interaction between $CD4^+$ T cells and infected cells in an HIV-infected individual, and the interaction between Env expressed on a virion and $CD4^+$ T cells. Both X4 and R5 Envs suppressed the activation and proliferation of $CD4^+$ T cells via specific interaction with CD4. Suppression was characterized by a lack of proliferation despite normal activation of the main signaling pathways involved in T-cell activation and proliferation, AKT, MAPK, and STAT5a but a deficient upregulation of early and late cell-surface activation markers. The suppression of antigen-induced proliferation was not due to IL-2-dependent anergy, apoptosis, or cell death.

The role of CD4 versus the coreceptors CXCR4 and CCR5 in mediating Env-induced suppression is controversial [7, 15, 71, 154]. Some reports have suggested a role for coreceptors in mediating suppression in conjunction with CD4 [15, 154]. Others have suggested that Env can mediate suppression solely via CD4 or coreceptors[7, 71{Arthos, 2002 #110], In this study, we show that Env delivered as a vaccine, as a component of an infected cell, or as part of a virion mediates a suppression of activation and proliferation

regardless of its coreceptor usage. Although CD4 interaction is required for this suppression, both coreceptors could have played a role. To test this, we examined suppression of X4 Envs in the presence of AMD3100, which blocks Env interaction with CXCR4 [45]. AMD3100 caused a non-specific increase in proliferation in both Env and control transfected 293T-exposed $CD4^+$ T cells, which may be attributed to blocking inhibitory chemokine signaling through CXCR4[155]. Similar studies with TAK-779 [9], which blocks R5 Env interaction with CCR5, did not overcome suppression. This suggests that in our system studying Env in the absence of HIV infection, inhibition of $CD4^+$ T cell proliferation occurred in a CD4-dependent, chemokine receptor-independent manner.

Previous studies demonstrating the suppressive effects of Env have focused on a single aspect of $CD4^+$ T cell suppression such as apoptosis or anergy and used systems to model interactions that occur during HIV infection. In this chapter, I present a comprehensive study examining multiple facets of Env-induced suppression when it is used as an immunogen, present on a virion, or expressed in a manner that models HIV-infection of cells. DCs that acquire a vaccine containing HIV Env will express protein from all the delivered antigens. When the protein is processed and presented to antigen-specific $CD4^+$ T cells by the APCs, suppression by surface-expressed Env would occur, thus hindering immune responses to all antigens in the cocktail. Because Env is an essential component in an HIV vaccine that aims to generate neutralizing antibodies, inclusion of nonsuppressive forms of Env will enhance immune responses.

Accumulating studies have implicated the involvement of $CD25^+$ Treg cells in the inhibition of anti-HIV $CD4^+$ and $CD8^+$ T cell responses in HIV infection [1, 5, 52, 85, 86, 112, 161]. The part of thesis was also designed to address three questions: 1) whether HIV virion expressed Env, like that expressed on the cell surface, induces inhibition of polyclonally stimulated $CD4^+$ T cell proliferation; 2) whether Treg cells are responsible, at least in part, for Env induced suppression; and 3) whether Env is able to directly induce, or activate, Treg cells. The answers to these questions are critical to determine whether the frequency and function of Treg cells are regulated through direct interaction with virus, and how this regulation of Treg cells relates to dysfunction of T cell immunity in HIV infection. Here, I demonstrate that Env expressed on either virions or cells induces suppression of $CD4^+$ T cell activation, and that Env does not induce or activate Treg cells as a mediator for this inhibition.

In HIV infection, in addition to infected cells, circulating free virions also bear functional trimeric Env. Although protease-defective, gp120-containing HIV-1 particles were reported to induce apoptosis in human peripheral blood T cells, whether such virions use Env, like its counterpart expressed on cells, to induce inhibition to $CD4^+$ T cell proliferative responses remains unclear [79]. Using AT-2 inactivated HIV-1 viral particles that are non-infectious but maintain integrity of functional Env [112, 131]. I demonstrate that Env on HIV virions inhibits $CD4^+$ T cell proliferation through gp120-CD4 signaling.

HIV impairs host $CD4^+$ T cell immunity through multiple mechanisms. Although the involvement of Treg cells in suppressing anti-HIV specific T cell responses has been documented in a growing body of literature, there is controversy as to whether HIV, or its gene products, inhibits host $CD4^+$ T cell activation through the direct induction or activation of Treg cells. An interaction between Treg cells and HIV through gp120-CD4 signaling has been proposed [112], which provides a basis for our in vitro system where human PBMC, as source of $CD4^+$ T cells, were exposed to Env expressing 293T cells or AT-2 inactivated virions, modeling the in vivo interaction between Env and $CD4^+$ effector as well as $CD4^+$ Treg cells, during an immune response. In this thesis, I show that depletion of pre-existing $CD4^+$, $CD25^+$ Treg cells from PBMC increases the proliferation of polyclonal TCR stimulated $CD4^+$ T cells, which is consistent with observations in HIV infected patients that virus exposed Treg cells maintain suppressive activity. However, Env-induced inhibition remained in the absence of $CD25^+$ Treg cells, strongly suggesting that Treg cells are not functionally involved in Env-induced suppression. This result implies that the suppressive effect of Treg cells on anti-HIV T cell responses, which has been observed in HIV infected patients, may be a general feature of Treg cells.

The regulation of Treg cells in HIV remains highly controversial. Studies using peripheral blood reported increased [1, 149, 161] as well as reduced [49, 115] numbers of Treg cells in HIV infection. In contrast, later studies using LN or gastrointestinal mucosa of

infected patients demonstrated up-regulation of FoxP3 and other markers of Treg cells, supporting Treg accumulation in the tissue at sites of viral replication [5, 6, 52, 112]. Factors including markers used to identify Treg cells, disease stage in selected patients, and ART treatment, might contribute to the various findings from different studies. Our results demonstrate that regardless of the form of Env delivery (Env-expressing cells as would be observed during infection or after encoding vaccine delivery or free virions) and stimulation status of T cells, the percentage of $FoxP3^+$, $CD25^+$, $CD4^+$ T cells was not affected by Env, which is in contrast to the observation that an increase in $FoxP3^+$ cells was detected in $CD4^+$ T cells after exposure to AT-2 inactivated virions, resulting from Env induced enhancement in Treg cell viability [112]. The difference in cells used (PBMC vs purified $CD4^+$ T cells) could have contributed to the different results in these two studies. The purification of $CD4^+$ T cells could have increased their level of apoptosis, which was blunted by Env acting on Tregs or increased by Env acting on non-Treg $CD4^+$ T cells. We observed a low level of apoptosis in our unstimulated PBMC with no difference between Env exposed and control (data not shown). Although, if this were the case, we would have expected to see a difference after anti-CD3 stimulation (Figure 2.7A), which increases apoptosis.

It has been reported that, unlike murine $Foxp3^-$ T cells, a subset of human $FoxP3^-$ T cells could be induced to transiently express FoxP3 upon activation [60, 98]. In agreement with these studies, we found that anti-CD3 stimulation induced increased FoxP3 expression in $CD4^+$ T cells, peaking approximately 48 hours after activation (data

not shown). Furthermore, we measured FoxP3 expression in CD25-depleted PBMC, and found that activation induced FoxP3 expression, which is in agreement with a published report [53]. These activation induced $FoxP3^+$, $CD4^+$ T cells have suppressive activity on T cell proliferation, but this activity was not modulated by Env exposure.

One of the functional characteristics of Treg cells is its capacity to suppress antigen specific $CD4^+$ and $CD8^+$ T cell proliferation in a dose-dependent manner [156]. Since FoxP3 is expressed intracellulary, several surface antigens in addition to CD25 have been identified as alternative markers for isolation of Treg cells, one of which is CD127, a subunit of the IL-7R[94]. Analysis of sorted $CD4^+$, $CD25^+$, $CD127^-$ T cells, which contained 90% $FoxP3^+$ cells, demonstrated that Env exposure did not alter the suppressive activity of peripheral blood Treg cells, which is consistent with a previous finding that Treg cells exposed to AT-2 treated HIV virions are equally suppressive compared with untreated Treg cells [112].

In summary, I used models of the encounter of $CD4^+$ T cells with both APCs and non-APCs expressing Env and virion expressed Env to study Env mediated suppression of $CD4^+$ T cell proliferation. Both X4 and R5 Envs suppressed the activation and proliferation of $CD4^+$ T cells via specific interaction with CD4. Suppression was characterized by a lack of proliferation despite normal activation of the main signaling pathways involved in T cell activation and proliferation, AKT, MAPK, and STAT5a but a deficient upregulation of early and late cell surface activation markers. The suppression

of antigen-induced proliferation was not due to IL-2 dependent anergy, apoptosis, or cell death. My data also demonstrates that HIV Env neither induced nor activated Treg cells, and that $CD25^+$ Treg cells are not responsible for Env induced suppression of $CD4^+$ T cell proliferation. Our findings do not demonstrate that HIV does not regulate Treg number or function, only that Env binding does not, in model in vitro assays. Treg cells could still be depleted by direct infection or bystander effects or their number increased by other gene products or downstream effects of infection, such as chronic immune stimulation. Further work is needed to elucidate the in vivo regulation of Treg cells and their physiological relevance in the context of HIV infection and vaccination.

CHAPTER 3

In vivo analysis of immune and viral responses in retroviral suppressed SIV infected rhesus macaques

Introduction

$CD4^+$ T cells play a central role in maintaining effective cellular and humoral immune responses by providing help to $CD8^+$ T cells, B cells and innate effectors. One of the hallmarks of HIV infection is the progressive loss of $CD4^+$ T cells and their associated antigen-specific responses. This is characterized as both a decline in the number of $CD4^+$ T cells and an early loss of the functional activity of cells with certain antigenic specificities [41, 90, 105]. In particular, HIV antigen-specific $CD4^+$ T cell responses are dysregulated to a greater extent than responses to other common antigens [88, 91, 158]. It has been demonstrated that chronically HIV-1 infected progressing patients fail to mount a potent HIV-specific $CD4^+$ T cell response even in the setting of viral suppression by ART. Immunization of chronically infected ART treated patients with HIV antigens leads to poor HIV-specific responses[50]. The mechanisms for the inability to generate HIV-specific $CD4^+$ T cell responses in chronic infection are not fully characterized or understood.

To initiate an immune response, DC bearing antigens interact with antigen-specific $CD4^+$ T cells that migrate through lymphoid tissue, leading to $CD4^+$ T cell activation and expansion. Signals from the DC induced by the pathogen instruct the type

of T cell response, characterized as Th1, Th2, Th17, Tr1, and Th3/Tr2 [12, 36, 64]. However, in the presence of HIV, the interaction between DC and $CD4^+$ T cells enhances viral replication by multiple mechanisms [168]. It has been demonstrated that even in the presence of potent regimens of viral suppression, a very low level of viral production could still be detected [43, 70, 122]. It is generally believed that this viral replication does not represent acute infection of cells, as no sequence evolution is observed [32, 84, 122], but the source of the low level of virus may be derived from DC mediated activation of latent virus in memory T cells or other reservoirs [126].

I use a macaque model of HIV-infected individuals treated with ART during chronic infection to study the effect of SIV antigen stimulation in lymph nodes early after immunization. CMV seropositive rhesus macaques were infected with SIVmac251 and after 4 months, treated with D4T and PMPA to artificially control viral loads. Animals were immunized in both arms and legs with SIV or CMV antigen, which allowed draining lymph nodes for each antigen to be obtained from the same animal at the same time, allowing a direct comparison of the effect of SIV and CMV antigen stimulation (Figure 3.1). Immunologic and virologic responses were measured in draining lymph nodes and peripheral blood to analyze differences in responses induced by an SIV antigen compared to a control immunization.

Materials and Methods

Ethics statement

All animal experiments were performed in strict accordance with good animal practice following guidelines for relevant national and regional animal welfare bodies. The studies were approved by the University of Pennsylvania and Tulane Institutional Animal Care and Use Committees (IACUC).

Immunogens

Plasmid DNA expressing the SIV Gag core protein from Mac239 (pSIVgag) was used. It is a Rev-independent expression vector that has been codon optimized for a high level of expression, as previously described [20]. Plasmid DNA expressing the Rhesus CMV pp65 protein was used [170]. DNA was formulated in 0.15 M citrate buffer and 0.25% bupivicaine at a pH of 6.5.

Infection of rhesus macaques and overview of study

The animal study timeline is shown in Figure 3.1. Three CMV seropositive Mamu-A*01 positive rhesus macaques were intravenously infected with SIVmac251 at time zero. Four months after SIV infection, ART was introduced (SQ PMPA 20 mg/kg qD and PO D4T 10 mg/kg qD) and continued until the end of the experiment. The macaques were followed for 4 months on ART for recovery of periphery $CD4^+$ T cell count and viral load suppression. Eight months after infection and 4 months after the initiation of ART, each monkey received 2 immunizations with SIV gag encoding DNA

i.m. (2 mg per injection), one in the left arm and one in the left leg and 2 immunizations with CMV pp65 encoding plasmid (2 mg/injection) in the right arm and leg. After immunization, draining lymph nodes were sampled at two-time points for each animal by first removing inguinal LNs on each side followed by axillary LN removal from both sides (FH40 - D3 and D9, DD05 - D5 and D11, and CT64 - D7 and D14). We were unable to collect pre-immunization LNs due to technical limitations. D60 LNs were collected as no-immunization control. PBMC and plasma samples were collected every 2 to 4 weeks throughout the experiment.

Measurement of peripheral viral load and $CD4^+$ T cell counts

Viral RNA in plasma was quantified by a bDNA signal amplification assay from Bayer Inc., version 4.0, specific for SIV, which has a threshold detection limit of 125 viral copies per ml plasma [151].

$CD4^+$ T cell counts using whole blood collected in EDTA were analyzed with anti-CD3 (Biosource International, Camarillo, CA), anti-CD4 and anti-CD8 staining (anti-Leu3a and anti-Leu2a, Becton Dickinson, San Jose, CA) as previously described [35].

PBMC isolation and LN biopsy

Peripheral blood lymphocytes were isolated from EDTA-blood using Ficoll-diatrizoate gradient centrifugation and analyzed for CD4's by flow cytometry or cryopreserved in 90% FCS 10% DMSO. LNs were obtained under anesthesia. The LNs

were teased carefully to generate single-cell suspensions. The single-cell suspensions from lymph nodes were divided into two parts; one was cryopreserved and stored in liquid nitrogen and one was frozen as a pellet.

In vitro stimulation

Cryopreserved LN cells or PBMC were thawed and washed twice with pre-warmed complete RPMI 1640 supplemented with 10% heat-inactivated FCS (HyClone) and L-glutamine (Invitrogen Life Technologies). Cells were resuspended in complete RPMI medium and rested for two hours at 37°C before being washed again and used. Unless otherwise noted, cells were prepared at concentration of 1 x 10^6 cells/ml for in vitro stimulation. For characterizing the SIV- and CMV-specific T cell responses, LN cells or PBMC were incubated with either an SIVmac239 gag 15-mer peptide pool with 11-amino acid overlap, 2 μg/ml each peptide (NIH AIDS Research and Reference Reagent Program, Bethesda, MD) or a rhesus CMV pp65 peptide pool (15-mers overlapping by 11 amino acids) at 2 μg/ml for each peptide [170] at 37°C for 6 hours in the presence of Golgi-Stop (0.7 μg/ml) and Golgi-Plug (1 μg/ml) and 1 μg/ml of co-stimulatory antibodies anti-CD28 and anti-CD49d (BD Bioscience, CA). Negative controls with no stimulation and positive controls with PMA/Ionomycin (50 ng/ml, Sigma-Aldrich) were used.

Cell staining

After 6 hours of stimulation, cells were first washed with 2 ml PBS containing 1% FCS and stained with aqua blue dye for cell viability (Invitrogen, Eugene, OR). Cells were then stained with pre-titrated amounts of fluorochrome-conjugated surface staining antibodies (anti-CD4-PerCP Cy5.5, anti-CD8-FITC, anti-CD14-Pac Blue, anti-CD16-Pac Blue, and anti-CD95-PE-Cy5, anti-CD20-Pac Blue (eBioscience), and anti-CD28-ECD (Bechman Coulter)) and incubated at 4°C for 20 minutes. Cells were then washed and fixed in 250 µl BD Fixation/Permeabilization solution (BD Biosciences) for 20 minutes at 4°C. After fixation, cells were permeabilized with 1 x BD Perm/Wash buffer and stained with pre-titrated fluorochrome-conjugated antibodies (anti-CD3-APC-Cy7, anti-IL-2-PE, anti-IFN-γ-APC, anti-TNF-α-PE-Cy7 (BD Biosciences) at 4°C for 45 minutes. Cells were then washed with Perm/Wash buffer and re-suspended in 300 µl PBS 1% FBS.

Flow cytometric analysis

Cells were analyzed by polychromatic flow cytometry on an LSRII (BD Biosciences) equipped for the detection of 18 fluorescence parameters. In this study, for all 12-color flow analyses, 200,000 to 500,000 events were acquired for each sample. Antibody capture beads (BD Biosciences) stained separately with individual mAbs were used to conduct electronic compensation. Flow Jo version 8.8.7. (Tree star, Ashland, OR) was used to analyze the polychromatic flow data with all the analytic gating as described before[16, 25]. Single cells were gated according to Forward Scatter Height (FSC-H) and Forward Scatter Area (FSC-A). Dead cells, B cells, NK cells, and monocytes were

excluded based on staining with aqua blue LIVE/DEAD fixable violet dead cell stain or CD14, CD16 and CD20 staining. Lymphocytes according to FSC-A and SSC-A, $CD3^+$, $CD3^+CD4^-$, and $CD3^+CD4^+$ were sequentially selected for cytokine analysis. Expressions of IL-2, TNF-α and IFN-γ were identified in $CD3^+CD4^-$ and $CD3^+CD4^+$ T cell populations. In $CD3^+$ lymphocyte population, memory and naive cells were identified through CD28 and CD95 staining as previously described [16, 25].

RNA extraction and quantitative PCR

Total RNA from LN mononuclear cells was extracted using Trizol according to manufacturer's instruction (Invitrogen) and subject to real-time PCR on an ABI 7500 (Applied Biosystem, Carlsbad, CA). Doubly spliced, singly spliced, and unspliced SIV viral RNA and the housekeeping gene GAPDH were analyzed**.** The primers and MGB probes specific for these genes were obtained from Applied Biosystem (Table 4). Changes in the expression of individual viral RNAs with GAPDH normalization were calculated utilizing delta cycle threshold (ΔC_T) values, and the comparisons were made between LNs draining SIV and CMV immunizations from the same animal collected at the same time on the indicated days post immunization.

Levels of IFN-γ mRNA were quantitated by RT-PCR against a standard curve derived from serial dilutions of in vitro made transcripts, using primers and probe listed on Table 4. Copies of IFN-γ mRNA were expressed per 1 μg of RNA.

Statistics

Mean, Standard deviation, and student's t-test were performed using Microsoft Excel software.

Table 4. Primers and Probes used in real-time PCR for quantitation of SIV viral RNA and macaque IFN-γ

RNA	Primers and Probe
SIV Doubly Spliced	Forward: 5'- AGGCTAATACATCTTCTGCATCAAAC-3' Reverse: 5'- GGGTCCTGTTGGGTATGAGTCTA-3' Probe: 5' - CCACCCTCTTATTTCC-3'
SIV Singly Spliced	Forward: 5'- AGAGGCCTCCGGTTGCA-3' Reverse: 5'- CCTTCCCCTTTCCACAATAGC-3' Probe: 5'-ACTGTGGAAGGGACC-3'
SIV Unspliced	Forward: 5'- TTGCAGCACCCACAACCA-3' Reverse: 5'-TGATCCTGACGGCTCCCTAA-3' Probe: 5'- CTCCACAACAAGGACA-3'
Macaque IFN-γ	Forward: 5'- GTGTGGAGACCATCAAGGAAGAC-3' Reverse: 5'- CGACAGTTCAGCCATCACTTGGAT-3' Probe: 5'- ACTGACTCGAATGTCCAACGCAAAGC-3'
Macaque GAPDH	Forward: 5'-GGCATCCTGGGCTACACTGA-3' Reverse: 5'-AGGAGTGGGTGTCGCTGTTG-3' Probe: 5'- AGGTGGTCTCCTCTGAC-3'

RESULTS

PART 1

Infection and immunization of rhesus macaques and study overview

Pathogenic SIVmac251 infection of rhesus macaques has been well described for studying viral dynamics, immune responses, and anti-retroviral therapy [102, 103, 107, 114, 153, 159]. All animals were Mamu A*01 positive to reduce MHC variation in disease course and T cell responses and naturally infected with Rhesus CMV. The study was designed to infect animals (1000 $TCID_{50}$ of SIVmac251 by intravenous injection) and allow them to reach steady state viral loads (4 months) followed by ART treatment (PMPA and D4T) for 4 months. Animals were then immunized with expression plasmids encoding non-mutated SIV gag or rhesus CMV pp65 in both arms and legs and lymph nodes draining either SIV gag or CMV pp65 plasmid injections from the same animal at the same time were obtained 3, 5, 7, 9, 11 and 14 days post immunization (Figure 3.1). Serum and PBMCs were obtained every 2 to 4 weeks throughout the experiment.

Figure 3.1

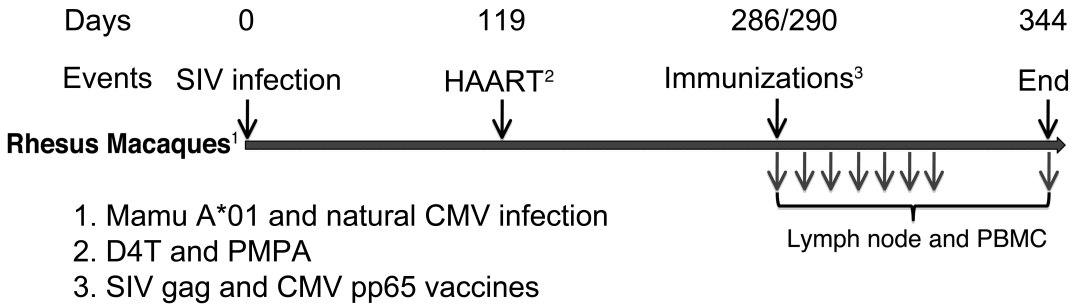


Figure 3.1. Experimental protocol for infection and immunization of rhesus macaques. Mamu A*01 positive rhesus macaques naturally infected with rhesus CMV were intravenously inoculated with SIVmac251, followed by ART treatment (DT4 and PMPA) from days 119 post infection through the end of experiment. On days 286 or 290 post infection, monkeys received 2 immunizations with SIV gag encoding DNA i.m. (2 mg per injection), in the left arm and left leg, and 2 immunizations with CMV pp65 encoding DNA i.m. (2 mg per injection) in the right arm and leg. LN and PBMC were collected from the immunized animals.

PART 2

Establishment of chronically SIV-infected rhesus macaque with retroviral suppression and $CD4^+$ T cell recovery by ART

ART suppresses viral replication leading to recovery of peripheral $CD4^+$ T cell counts

SIV infection was established in all three animals with kinetics typical of SIV replication during primary infection in naïve rhesus macaques (Figure 3.2A) [68, 143]. Introduction of ART 4 months post infection, when set point viral loads had been established, efficiently suppressed viral replication to undetectable levels. One macaque demonstrated occasional blips in viral load but returned to undetectable levels after each without any change in therapy (Figure 3.2A).

Absolute $CD4^+$ T cell counts in the peripheral blood demonstrated increases after introduction of ART in all animals with sustained levels of more than 500 cells/µl (Figure

3.2B). These findings demonstrate that chronic SIV infection was achieved and ART successfully suppressed viral replication leading to recovery of peripheral $CD4^+$ T cell counts.

Figure 3.2

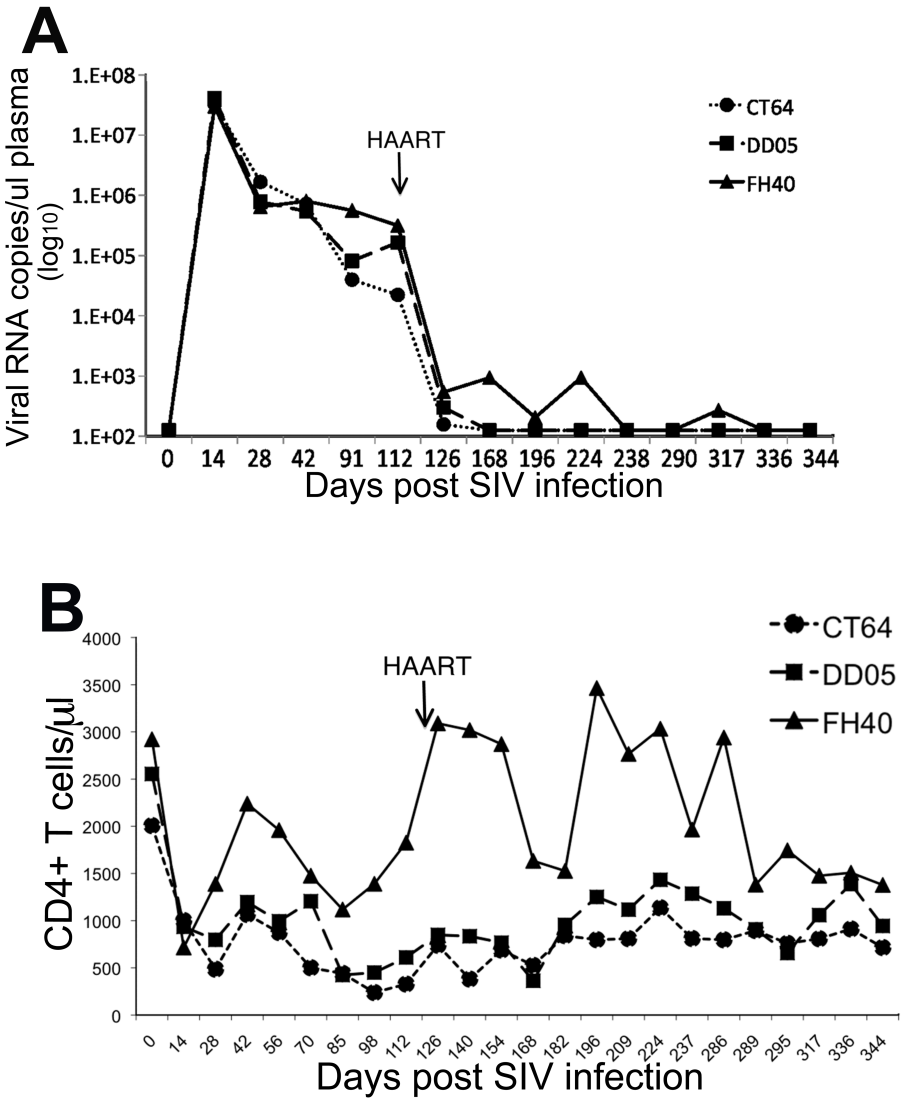


Figure 3.2. Summary of viral loads and peripheral $CD4^+$ T cell counts in rhesus macaques. Viral loads and CD4 counts were measured every 2 to 4 weeks throughout the

experiment. (A) Viral RNA in plasma was quantified by a bDNA signal amplification assay and expressed as RNA copies per ml plasma. ART treatment controlled the viral loads in all three animals. (B) Peripheral $CD4^+$ T cell counts increased after the initiation of ART. Macaque blood samples were stained for anti-CD3, anti-CD4, and anti-CD8 antibodies and the number of $CD3^+$, $CD4^+$ T lymphocytes were determined by flow cytometry. $CD4^+$ T cell counts were expressed as $CD4^+$ T cells per μl blood.

PART 3

Immunization induced immunologic responses in draining lymph nodes

3.1. Immunization-induced immune activation in draining LNs

Animals were immunized with SIV gag encoding DNA in the arm and leg on one side and CMV pp65 encoding DNA on the other side at the same time. LNs, one draining an SIV immunization and one draining a CMV immunization, were excised from each animal at 2 time points post immunization. All comparisons were made between LNs from the same animal obtained at the same time that differed only in whether they drained an SIV or a CMV immunization. I was unable to obtain LNs prior to immunization due to limitations on the number of surgeries in a short time period. I obtained LNs from both the left and right sides of all animals 60 days post immunization. The measurement of immune responses did not demonstrate any difference between LNs draining HIV gag or CMV pp65 and these LNs were assumed to represent preimmune conditions.

LN mononuclear cells were assessed for the amount of IFN-γ mRNA, a major effector cytokine for adaptive immunity, as a measurement of total immune activation induced by immunization. The expression of IFN-γ was maximal 3 days post immunization in LNs draining both SIV gag and CMV pp65, which was followed by a decline at day 5 and a decrease in IFN-γ expression to the levels observed in 60 day post immunization LNs (Figure 3.3.1).

Figure 3.3.1

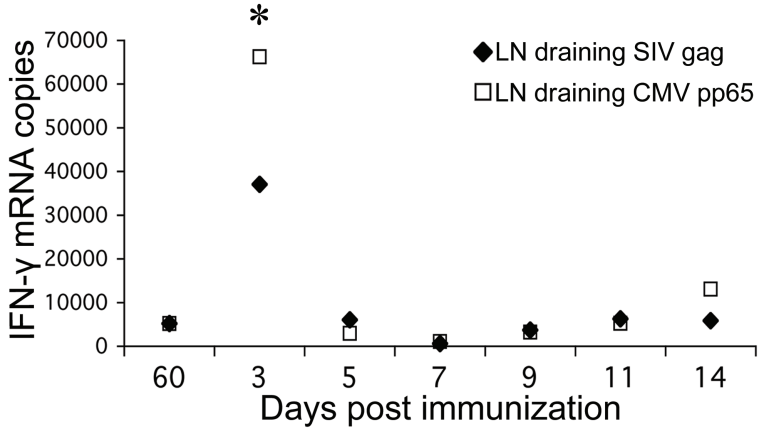


Figure 3.3.1. Total IFN-γ mRNA expression in LNs. Total RNA from LN mononuclear cells draining either SIV-gag or CMV-pp65, collected on indicated days post immunization were subject to real-time PCR with IFN-γ specific primers and probes for absolute quantitation of macaque IFN-γ RNA expression. The results were normalized and expressed as the RNA copy number per μg total RNA. The comparisons between SIV- and CMV-immunization were made in LNs collected from the same

animal at the same time on indicated days. The difference on Day 3 was significant with a p=0.036 noted by a *.

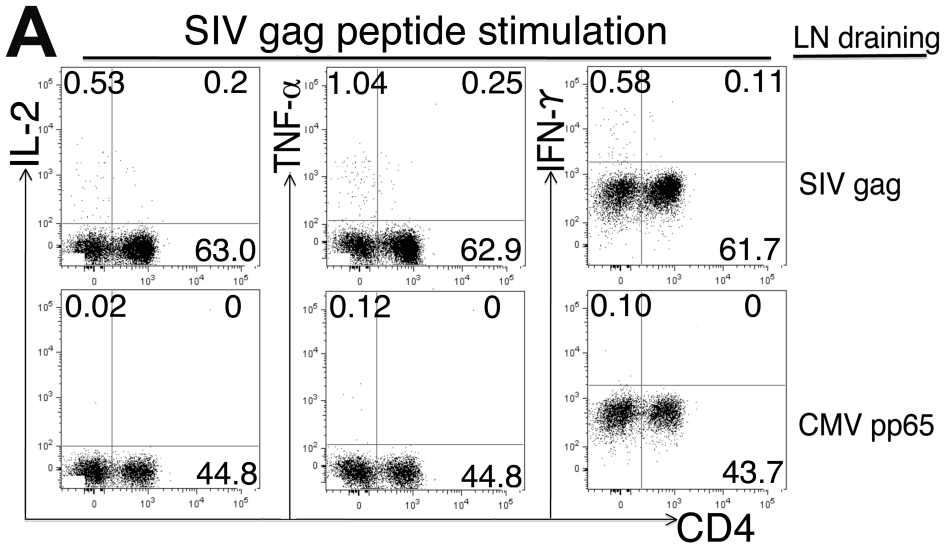
3.2. SIV immunization induced SIV gag-specific T cell responses in LN

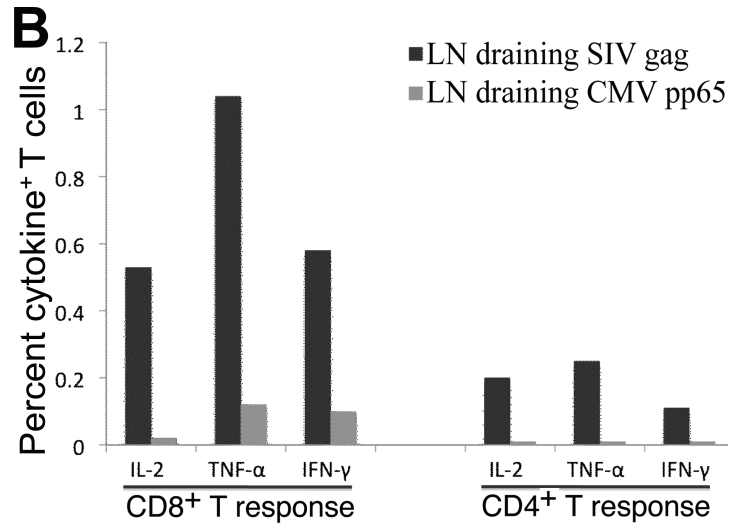
We next evaluated antigen-specific T cell responses by stimulation and intracellular cytokine staining. LN cells were stimulated in vitro with either SIV gag or CMV pp65 peptide pools and the percents of IL-2, TNF-α and IFN-γ-producing T cells were determined by polychromatic flow cytometry. Data of the LN draining SIV gag immunization on Day 3, when the immune activation, as measured by IFN-γ mRNA was the highest, demonstrated a potent response to gag peptide stimulation according to IL-2, TNF-α and IFN-γ productions (Figure 3.3.2A and B). In contrast, the LN draining the CMV pp65 immunization on day 3, when stimulated by gag peptides, demonstrated no significant response (Figure 3.3.2A and B).

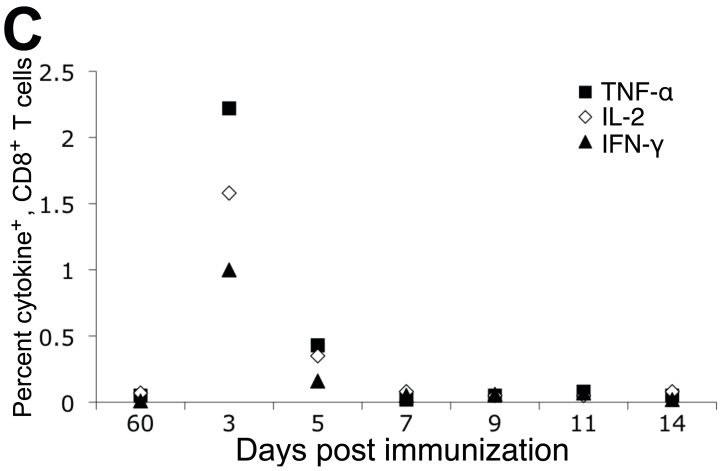
SIV immunization-induced gag-specific $CD8^+$ (Figure 3.3.2C) and $CD4^+$ (Figure 3.3.2D) T cell responses in LNs for all time points post immunization are summarized. As shown, the overall patterns for $CD8^+$ and $CD4^+$ T cell responses are similar in that an immediate and potent memory T cell response is induced on Day 3 post immunization, which is followed by a decline on Day 5 and a further decrease to the levels observed in 60 day post immunization LNs (Figure 3.3.2C and D), consistent with the data obtained by measuring IFN-γ mRNA (Figure 3.3.1).

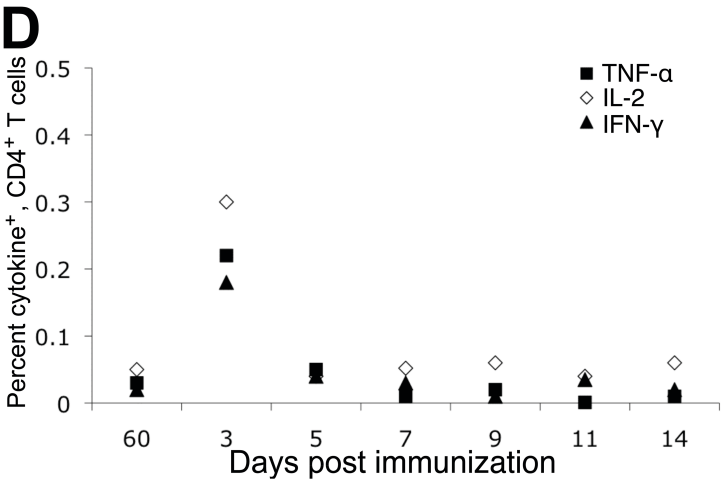
We next assessed the T cell subsets that mediated immunization-induced SIV gag-specific T-cell responses in Day 3 LN mononuclear cells. According to previous studies, CD28 and CD95 could be used to separate macaque T cells into naïve, central memory (CM) and effector memory (EM) populations. As shown in Figure 3.3.2E, the SIV-specific T cell responses were primarily mediated by central memory T cells in Day 3 LN. We then analyzed the polyfunctionality of SIV immunization-induced gag-specific T cells and found that both $CD8^+$ and $CD4^+$ SIV-specific T cell responses demonstrated a polyfunctional profile with the ability to produce 2 (IL-2/TNF-α, TNF-α/IFN-γ and IL-2/IFN-γ) and 3 cytokines (IL-2/TNF-α/IFN-γ), simultaneously (Figure 3.3.2F).

Figure 3.3.2




B
Percent cytokine+ T cells
1.2
1
0.8
0.6
0.4
0.2
0
LN draining SIV gag
LN draining CMV pp65
IL-2
TNF-α
IFN-γ
CD8+ T response
IL-2
TNF-α
IFN-γ
CD4+ T response


C
Percent cytokine+, CD8+ T cells
2.5
2
1.5
1
0.5
0
TNF-α
IL-2
IFN-γ
60
3
5
7
9
11
14
Days post immunization


D
Percent cytokine+, CD4+ T cells
0.5
0.4
0.3
0.2
0.1
0
TNF-α
IL-2
IFN-γ
60
3
5
7
9
11
14
Days post immunization

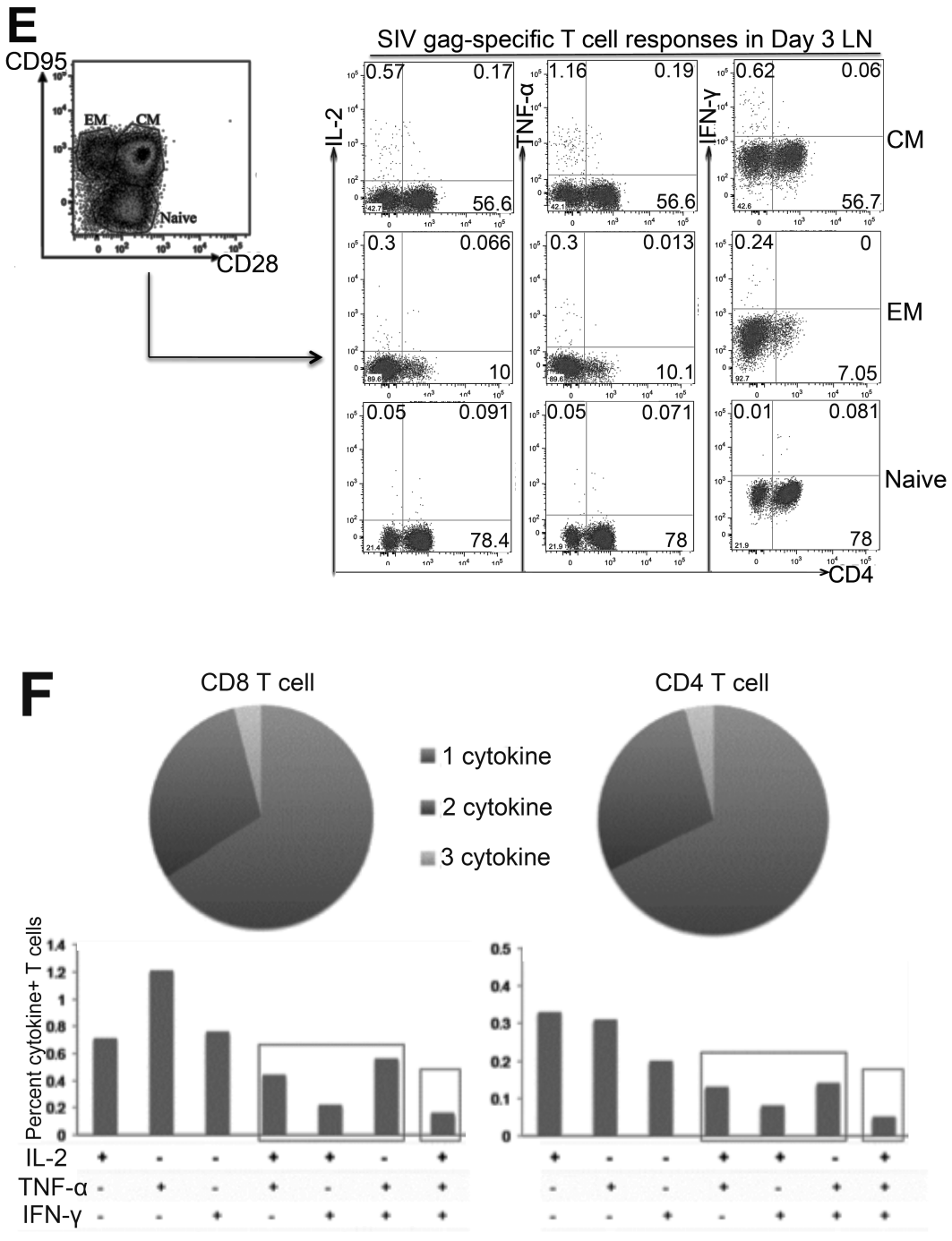


Figure 3.3.2. SIV immunization induced SIV gag-specific T cell responses in LNs.

(A) Representative multi-color flow cytometry data for SIV gag-specific T cell responses in LN draining SIV-gag DNA (Top) or CMV-pp65 (Bottom) on Day 3 post

immunizations. LN mononuclear cells were stimulated with SIV-gag 15-mer overlapping peptide pools and stained intracellularly for IL-2, TNF-α and IFN-γ. The SIV-specific cytokine-producing $CD4^+$ and $CD8^+$ T cells were determined by multi-color flow cytometry. (B) The percent of SIV gag-specific cytokine-producing $CD4^+$ and $CD8^+$ T cells in Day3 LN draining SIV gag and CMV pp65 immunization. (C, D) Summary for the percent of SIV gag-specific cytokine-producing $CD8^+$ (C) and $CD4^+$ (D) T cells in LNs on indicated days post immunization. (E) SIV gag-specific responses in LN were primarily mediated by central memory T cells. Macaque LN mononuclear cells were separated into naïve, central memory (CM) and effector memory (EM) cells according to CD28 and CD95 expressions. The individual cytokine expressios in these populations are shown. (F) Polyfunctionality of SIV-specific $CD4^+$ and $CD8^+$ T cell responses. The percent of $CD4^+$ and $CD8^+$ T cells able to produce 1, 2, and 3 cytokines is shown.

3.3. CMV immunization induced CMV pp65-specific T cell responses in LN

We evaluated the CMV-specific T cell responses in a LN draining the CMV-pp65 immunization. Unfortunately, due to cell viability and limited available cells, this was the only day we were able to analyze. LN mononuclear cells on day 5 post immunization were in vitro stimulated with a CMV-pp65 peptide pool, and the pp65-specific cytokine-producing T cells were assessed by multi-color flow cytometry. Compared to the LN draining SIV-gag immunization (Top panels, Figure 3.3.3A), the LN draining CMV-pp65 immunization demonstrated significantly higher frequencies of IL-2-producing $CD4^+$ and $CD8^+$ T cells in response to pp65 peptides (Bottom panels, Figure 3.3.3A). The

comparison for the percent of IL-2, TNF-α and IFN-γ-producing $CD4^+$ and $CD8^+$ T cells in Day 5 LN draining SIV or CMV immunization is shown in Figure 3.3.3B.

Figure 3.3.3

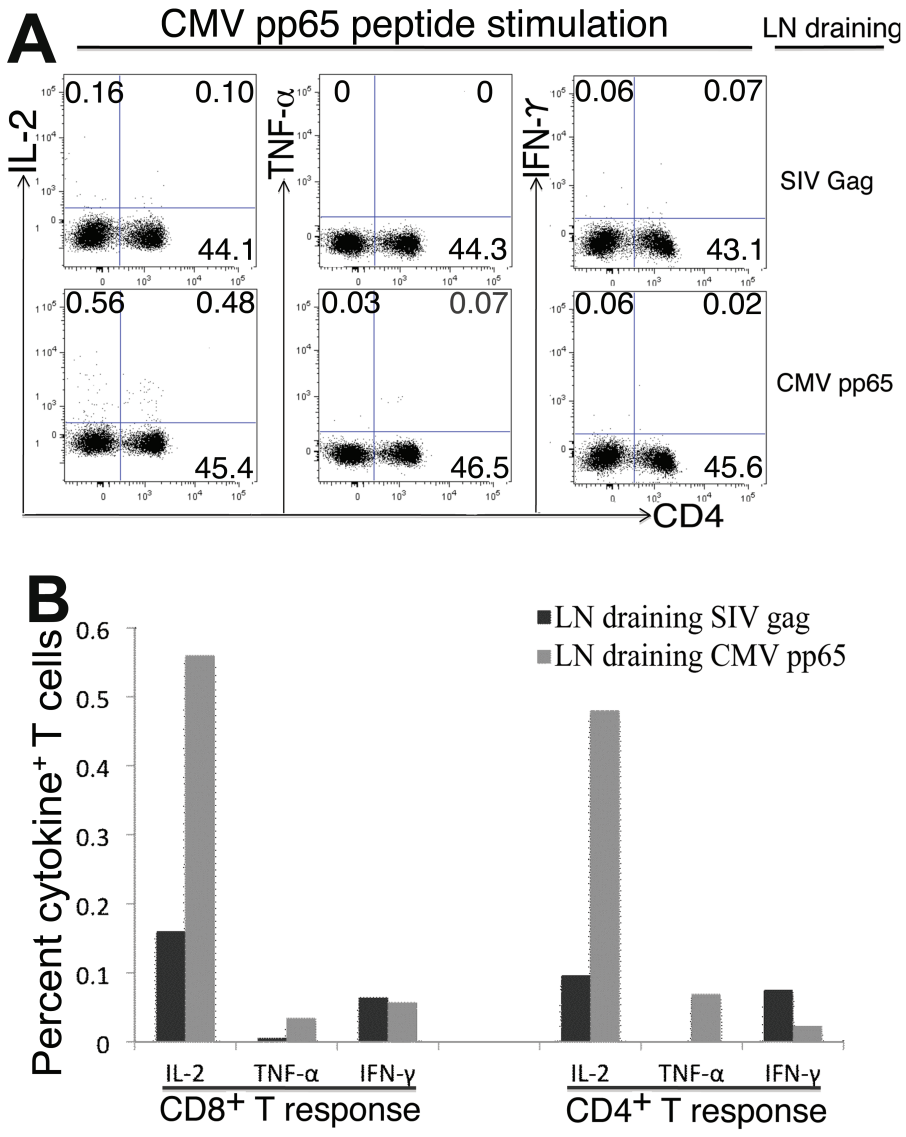


Figure 3.3.3. CMV immunization induced CMV pp65-specific T cell responses in Day 5 LN. (A) Multi-color flow cytometry data for CMV pp65-specific T cell responses in LN draining SIV-gag DNA (Top) or CMV-pp65 (Bottom) on Day 5 post

immunizations. LN mononuclear cells were stimulated with CMV-pp65 15-mer overlapping peptide pools and stained intracellularly for IL-2, TNF-α and IFN-γ. (B) The percent of individual cytokine-producing $CD4^+$ and $CD8^+$ T cells compared between LNs draining SIV gag and CMV pp65 stimulated with CMV pp65 peptide pool.

PART 4

SIV- and CMV-specific T cell responses in peripheral blood

4.1. Staining for the SIV gag-specific tetramer p11C in peripheral blood

After analyzing the immunization-induced local immunologic responses in draining LNs, we next sought to determine the systemic immune response in the peripheral blood of immunized macaques. PBMC were collected from macaques every two to four weeks throughout the experiment. We first determined the percent of circulating SIV-gag tetramer specific $CD8^+$ T cells in PBMCs in all three macaques. PBMC were stained with SIV-gag p11c-MamuA*01 tetramer together with other lineage and viability markers. As shown, $tetramer^+$ T cells were exclusively $CD8^+$, demonstrating the specificity of SIV tetramer (Figure 3.4.1A). The kinetics for the number of $tetramer^+$ SIV-specific $CD8^+$ T cells in three animals are summarized in Figure 3.4.1B. Before SIV infection, no tetramer positive cells were detected. This was followed by potent increases after infection. The introduction of ART led to a dramatic decline of $tetramer^+$ $CD8^+$ T cells to lower levels through the end of experiment. During ART, some transient increases were observed for one animal (FH40), which was consistent with the transient

viral load blips seen in the same animal. After immunization, no significant increases were observed for the number of SIV-specific $CD8^+$ T cells in the periphery of the macaques (Figure 3.4.1B).

Figure 3.4.1

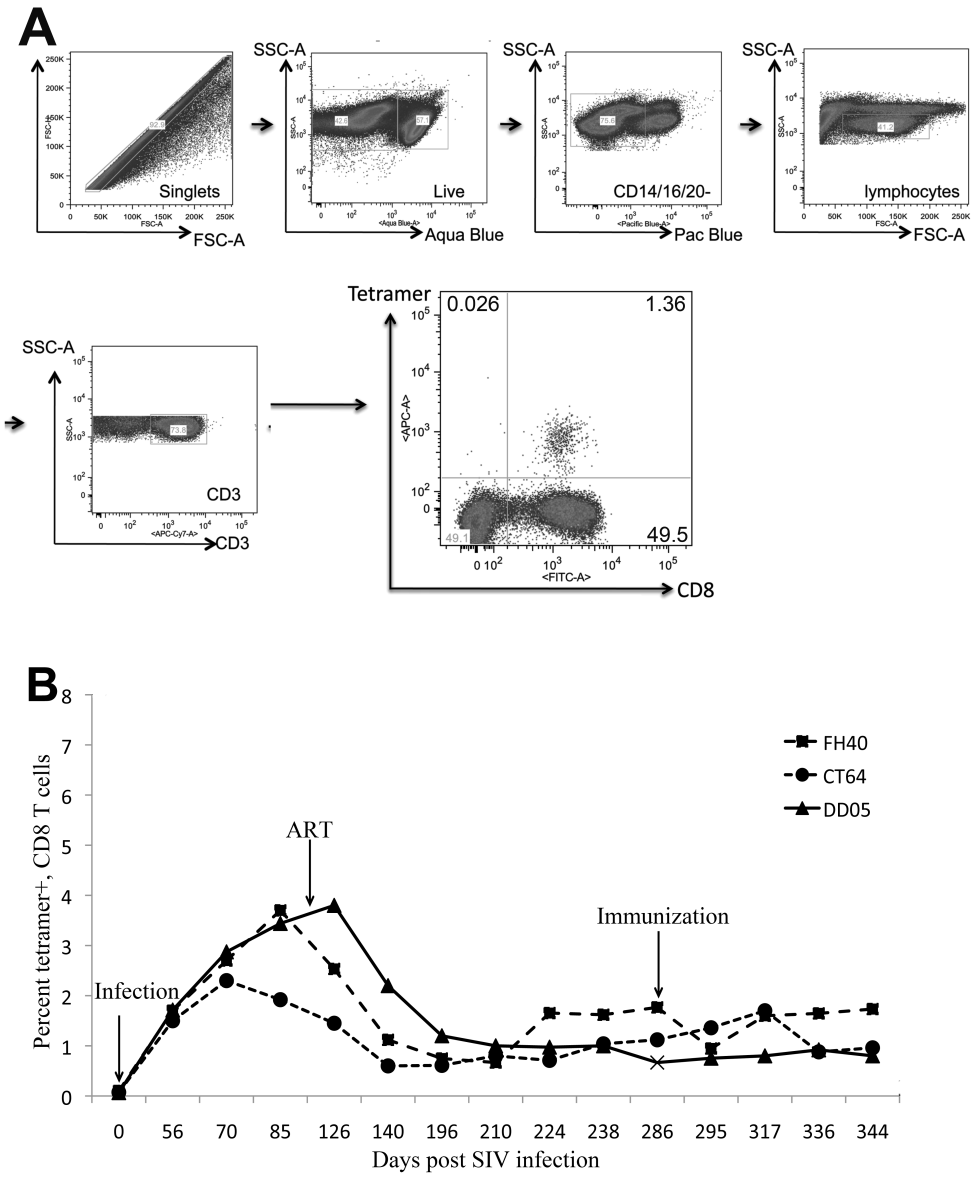


Figure 3.4.1. SIV gag-specific tetramer staining of PBMCs. (A) Representative tetramer staining and analysis for one PBMC sample from one macaque. Single, live and $CD3^+$ T lymphocytes were gated and the staining for tetramer and CD8 are shown. (B) Kinetics of $tetramer^+$ SIV-specific $CD8^+$ T cells in the studied rhesus macaques throughout the experiment. The results are expressed as percent of $tetramer^+$ $CD8^+$ T cells on indicated days post SIV infection for each animal.

4.2. SIV- and CMV-specific T cell responses in PBMCs

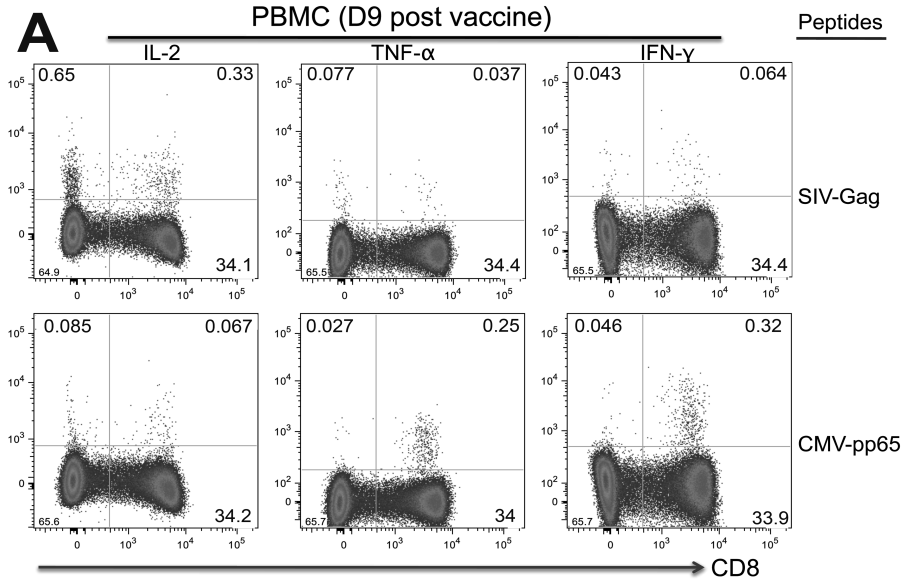
We next determined the systemic response to immunization with SIV gag and CMV pp65 by analyzing peptide stimulated T cell responses. Macaque PBMCs collected before and after immunizations were in vitro stimulated with SIV-gag or CMV-pp65 peptide pools and cytokine-producing T cells were measured by multi-color cytometry. Representative multi-color flow cytometry histograms for SIV- and CMV-specific T cell responses (IL-2, TNF-α and IFN-γ) in PBMC at day 9 post immunization are shown (Figure 3.4.2A). CMV-specific responses were primarily mediated by $CD8^+$ T cells producing the effector cytokines IFN-γ and TNF-α, whereas both $CD4^+$ and $CD8^+$, SIV-specific T cells were induced primarily producing IL-2 with very little IFN-γ and TNF-α on day 9 (Figure 3.4.2A).

The kinetics of immunization-induced SIV- and CMV-specific $CD4^+$ and $CD8^+$ T cell responses are summarized in Figure 3.4.2B. For the $CD4^+$ T cell response, a transient SIV gag-specific, IL-2 producing response was observed at day 9 post immunization, that was not found on day 50 post immunization, whereas no significant CMV-specific $CD4^+$

T cell responses were detected at all studied time points. The gag-specific $CD4^+$ T cells produced very little TNF-α and IFN-γ (Figure 3.4.2 B, Top).

Similar to SIV gag-specific $CD4^+$ T cell responses, the SIV-specific $CD8^+$ T cell response was transient and extinguished quickly with only an increase on day 9 post-immunization. The SIV-specific $CD8^+$ T cells primarily produced IL-2 with very little TNF-α and IFN-γ productions. In contrast, CMV-specific $CD8^+$ T cell responses demonstrated a long-lived, polyfunctional profile. A potent increase in CMV-specific $CD8^+$ T cells was observed at day 3 post immunization with an average of 0.8% of $CD8^+$ T cells able to produce TNF-α or IFN-γ. The TNF-α and IFN-γ-producing CMV-specific response was sustained until day 50 post-immunization. $CD8^+$ and $CD4^+$ T cell CMV-specific T cells produced very little IL-2 (Figure 3.4.2B)

Figure 3.4.2



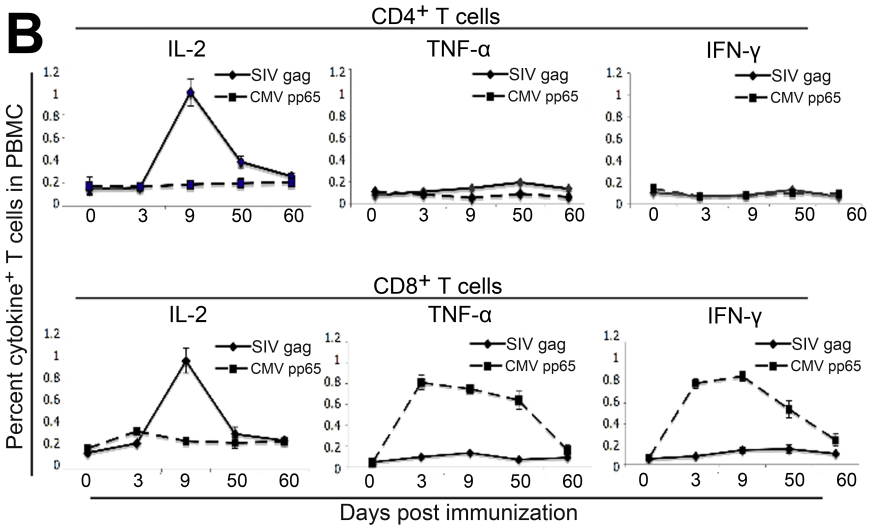


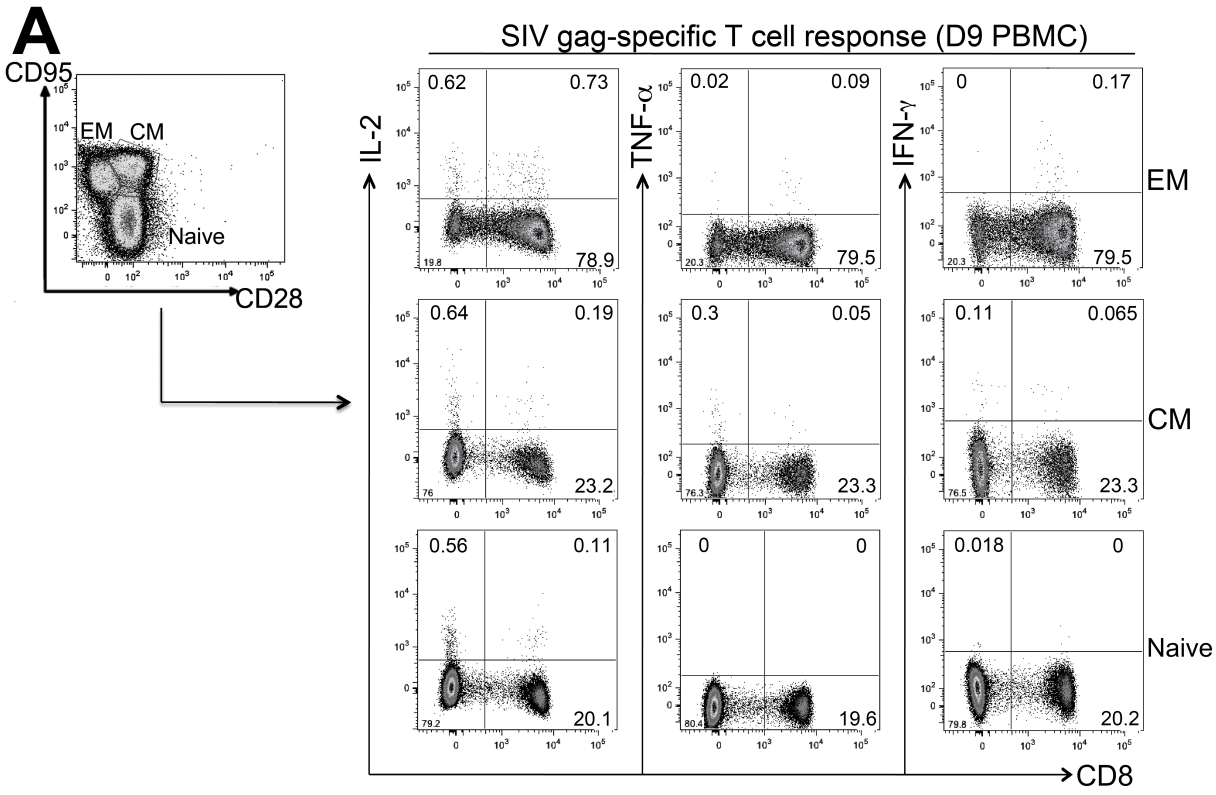
Figure 3.4.2. SIV- and CMV-specific T cell responses in peripheral blood during immunization. (A) Representative multi-color flow cytometry data for SIV- and CMV-specific T cell responses. PBMC before and after immunization were stimulated with SIV gag (Top) or CMV pp65 (Bottom) peptide pools and stained intracellularly for IL-2, TNF-α, and IFN-γ. The percentages of cytokine-producing $CD8^+$ T cells were determined by multi-color flow cytometry. (B) The average of all animals for the percent of $CD4^+$ and $CD8^+$ T cells able to produce individual cytokines (IL-2, TNF-α or IFN-γ) is shown and compared between SIV- and CMV-specific responses.

4.3. T cell subsets that mediate SIV- and CMV-specific T cell responses in PBMC

We also assessed the T cell subsets (naïve, central memory, and effector memory) that mediated the antigen-specific T-cell responses in PBMC post immunization. Using the Day 9 PBMC when both SIV- and CMV-specific T cell responses were highest, we analyzed individual cytokine production in naïve, central memory, and effector memory T cell subsets. The SIV-specific IL-2-producing response was mediated by effector (EM)

and central memory (CM) T cells (Figure 3.4.3A). In contrast, the CMV-specific TNF-α and IFN-γ- responses were primarily mediated by effector memory (EM) T cells (Figure 3.4.3B).

Figure 3.4.3



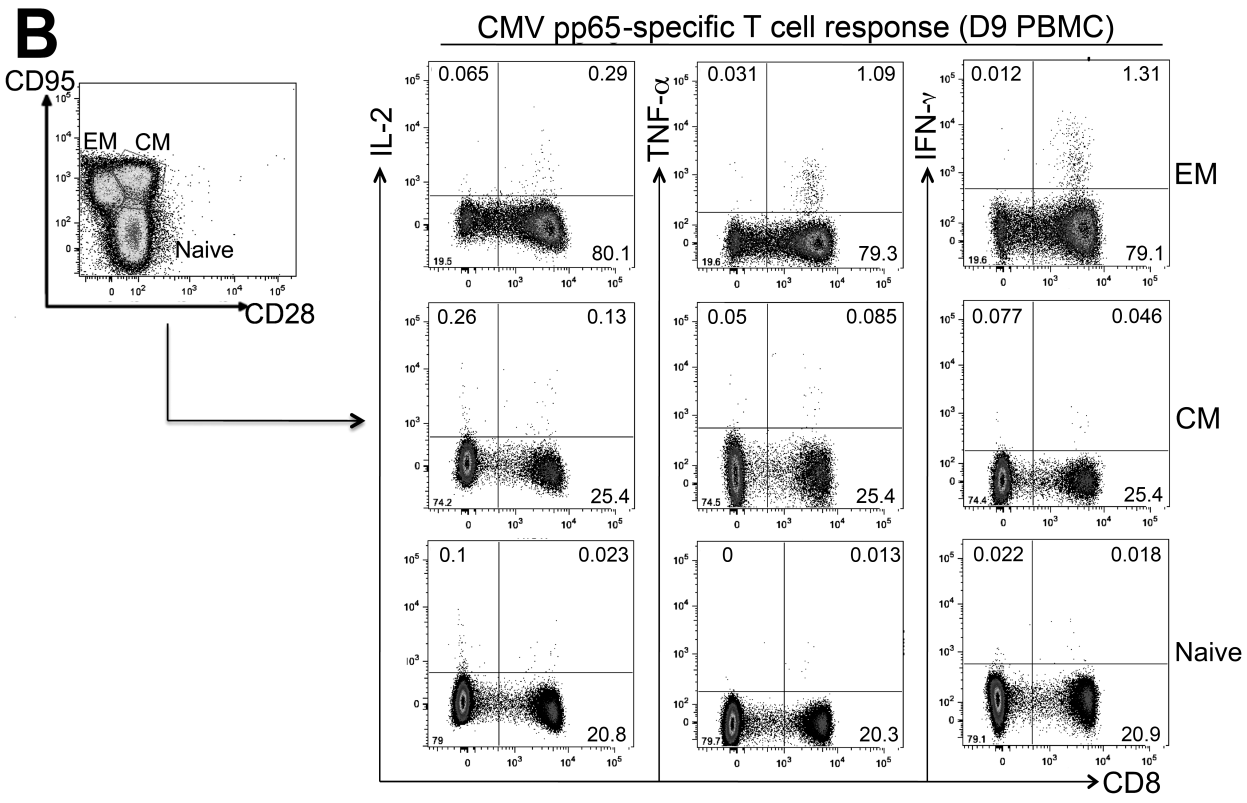


Figure 3.4.3. Analysis of T cell subsets mediating the antigen-specific T cell responses in PBMC. Macaque PBMC were separated into naïve, central memory (CM) and effector memory (EM) cells according to CD28 and CD95 expression. The individual cytokine expression in these populations for SIV-specific (A) and CMV-specific (B) responses are shown.

4.4. Increased PD-1 expression in SIV-specific $CD8^+$ T cells

T cell exhaustion often occurs and prevents optimal viral control during chronic infection [17]. An important role of the PD-1:PD-L1 pathway has been reported for $CD8^+$ T cell exhaustion during chronic viral infection [11]. We co-stained the PBMC for the SIV-

gag tetramer p11c and PD-1 to determine the differential expression of PD-1 on SIV-specific and total $CD8^+$ T cells. As seen in a representative histogram of tetramer-PD-1 staining, PD-1 was significantly upregulated in $tetramer^+$ $CD8^+$ T cells compared to tetramer negative $CD8^+$ T cells (Figure 3.4.4A). The PD1 expression in PBMC during the course of SIV infection and immunization was analyzed, and it was observed that PD1 expression on SIV-specific $CD8^+$ T cells was significantly higher than tetramer negative $CD8^+$ T cells for all time points studied, suggesting exhaustion of SIV-specific $CD8^+$ T cells, which was not reversed by ART and immunization (Figure 3.4.4B).

Figure 3.4.4

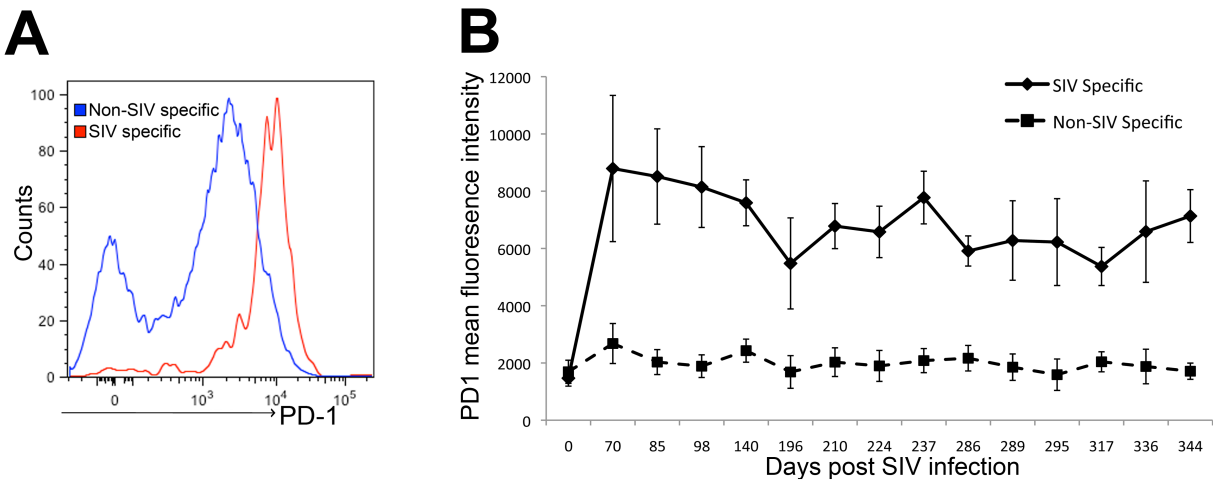


Figure 3.4.4. PD-1 expression on SIV-specific and non-SIV specific $CD8^+$ T cells in macaque PBMC. (A) Representative flow cytometry histogram data for mean fluorescence intensity (MFI) of PD-1 staining on SIV-specific and tetramer negative $CD8^+$ T cells. (B) PD-1 expression on SIV-specific and tetramer negative $CD8^+$ T cells in

PBMC collected on indicated days post SIV infection. The results are shown as the averge of mean fluorescence intensity (MFI) of PD1 staining for two monkeys.

PART 5

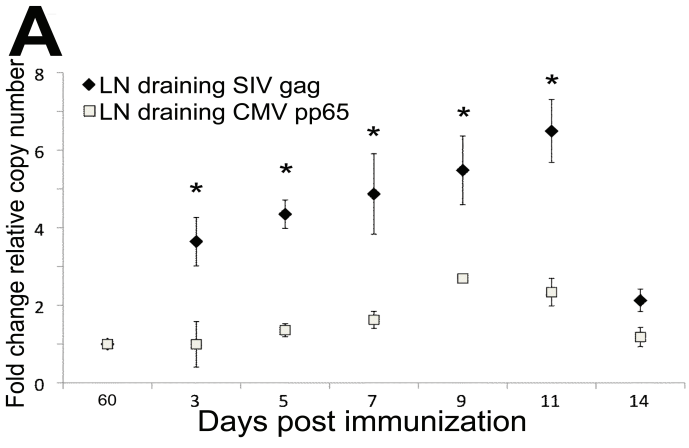
Immunization induced virologic responses in draining LNs

5.1. SIV immunization selectively stimulates transient activation of viral replication in draining LNs

ART can efficiently control viral replication but fails to eradicate virus due to the establishment of long-lived reservoirs [126]. It has been demonstrated that a low level of viral production continues even in the setting of the most potent antiviral regimens [43, 70, 122], but the source of this virus is not believed to be from new infection of cells [32, 84, 122]. Total RNA from LNs draining either SIV gag or CMV pp65 immunizations was analyzed by real-time PCR for multiple SIV viral RNA transcripts, including unspliced, doubly spliced, and singly-spliced RNA[119]. As preimmunization control LNs could not be obtained, LN biopsies from both sides, draining either gag or pp65, from each macaque were collected. No significant differences in levels of unspliced and spliced RNA transcripts were observed comparing right to left side for each animal. Thus, this time point was chosen for comparisons. Copy numbers of SIV RNA were normalized to GAPDH RNA and the results for each time point following immunization are shown as fold change relative to RNA collected at 60 days post immunization (Figure 3.5A, B, C).

The levels of doubly spliced SIV RNA viral transcripts in LNs draining the SIV gag immunization demonstrated a 3.6-fold increase at day 3, which was sustained until day 11 post immunization when a 6.5-fold increase was observed. Minor increases in copies of doubly spliced SIV RNA were observed in LNs draining the CMV pp65 immunization (Figure 3.5A). The levels of singly spliced viral transcripts demonstrated a similar increase in RNA in LNs draining the SIV gag immunization with an 11.8-fold increase at day 11 (Figure 3.5B). Unspliced SIV RNA levels were determined and an 8.3-fold increase was observed at day 7 (Figure 3.5C). The percent of $CD4^+$ T cells in LN mononuclear cells did not differ between LNs draining SIV gag and CMV pp65 immunizations, suggesting that the increase in viral replication was not due to differential $CD4^+$ T cell migration to the LN draining gag antigen, although we cannot rule out that a larger fraction of cells that trafficked to the gag LN were infected and expressing viral RNA transcripts.

Figure 3.5



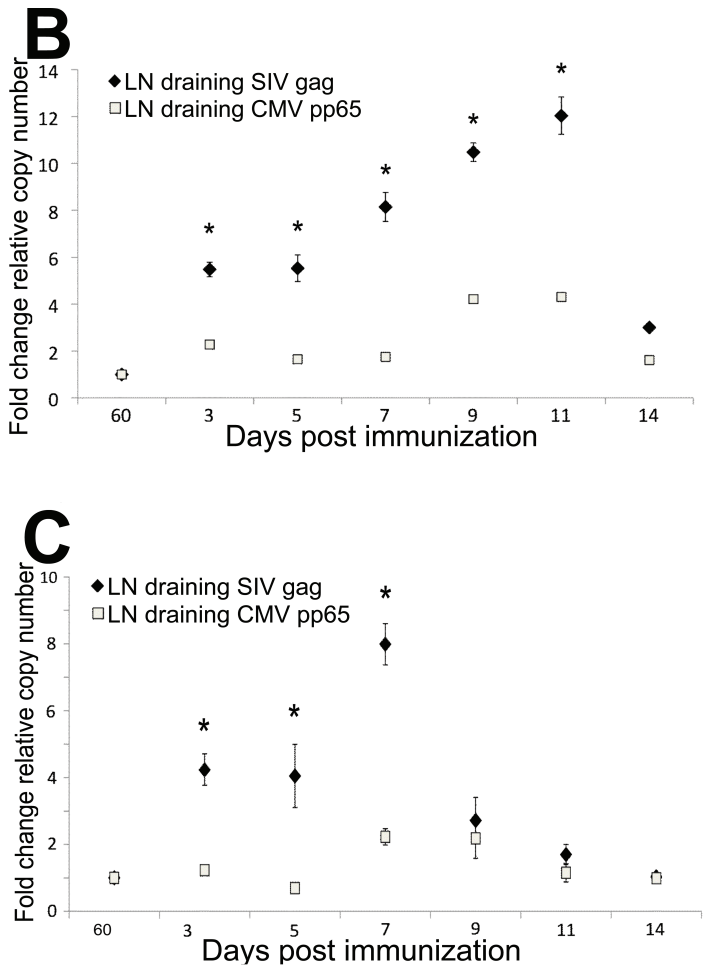


Figure 3.5 Quantitation of multiple SIV viral RNAs in LNs. Total RNA extracted from LN mononuclear cells draining either SIV gag or CMV pp65 were subject to real-time PCR with specific primers and probes for quantiation of SIV doubly spliced RNA (A), singly spliced RNA (B) and unspliced RNA (C). Copy numbers of SIV RNAs were normalized to macaque GAPDH and the results for each time point following immunization were shown as fold change relative to RNA collected on day 60 post immunization as a no vaccine control. The comparisons were made between LNs from the same animal at the same time on indicated days with a * indicating a $p < 0.05$).

5.2. No increases viral load were observed in peripheral blood post immunizations

A transient activation of SIV viral replication in LNs draining the SIV gag immunization was observed beginning at day 3 that returned to baseline at day 14-post immunization (Figure 3.5). We next sought to determine whether this transient viral replication could be seen in peripheral blood. The peripheral viral loads prior to and after antigen immunization in all animals are shown in Table 5. Except for a transient viral blip in one animal at day 31 post-immunization (270 copies/μl) that returned to undetectable 3 weeks later, no increase in viral loads that could be attributed to SIV gag immunization induced viral replication was detected. The data suggests that in the setting of potent anti-viral suppression, SIV antigen immunization activated viral replication was transient and restricted to draining LNs without spread to the periphery.

Table 5. Viral loads in periphery during antigen immunization

Days PI	238	290	317	336	344
Animal					
CT64	<125	<125(D0*)	<125(D27*)	<125	<125
DD05	<125	<125(D0*)	<125(D27*)	<125	<125
FH40	<125	<125(D4*)	270(D31*)	<125	<125

* Days post immunization

Discussion

A biphasic destruction of $CD4^+$ T cells is observed in HIV infection, with a massive loss of $CD4^+$ T cells during early infection and a subsequent progressive loss during the chronic stage of infection [47, 125]. Retroviral suppression by ART results in an increase in peripheral $CD4^+$ T cell counts and functional reconstitution of $CD4^+$ T cell responses to many common antigens [8, 83], but HIV-specific $CD4^+$ T cell responses remain deficient [129]. To identify potential mechanisms for this persistent loss of HIV-specific CD4 immune function, we studied ART-treated, chronic SIV and CMV infected rhesus macaques[136], and observed 1) that both SIV and CMV antigen immunizations could induce immune activation and antigen-specific T cell responses in draining LNs. 2) In peripheral blood, the CMV-specific response induced by immunization was potent and sustained, whereas the SIV-specific response was transient and extinguished quickly. 3) I demonstrate that SIV antigen immunization induces transient activation of SIV viral replication that was not observed with CMV pp65 immunization. The data is consistent with a hypothesis that SIV antigens induce viral replication that leads to depletion or dysfunction of antigen specific cells leading to a reduction in the strength and longevity of the response.

Pathogenic SIVmac 251 infection of rhesus macaques has been well described as one of the preferred experimental models for studying HIV pathogenesis[92]. The macaques in our study were inoculated with SIVmac251 and all established primary infection with typical viral replication dynamics[68, 114, 143]. Similar to the observations for HIV-1 infected

individuals where ART can sustain suppression of plasma viremia to undetectable levels [120], all macaques in our study demonstrated uniform responsiveness to ART (Figure 3.2A). We observed that the macaques also exhibited a sustained increase in peripheral $CD4^+$ T cell numbers above 500 cells/μl after PMPA and D4T treatment, demonstrating the establishment of suppressed viral replication and recovered $CD4^+$ T cell counts observed in HIV-infected subjects treated with ART during chronic infection.

Expression of particular MHC class I alleles are associated with different rates of disease progression in humans and macaques [23, 24, 81, 110, 171]. In our study, all three rhesus macaques expressed Mamu A*01 to control for an MHC effect on viral immune responses and disease progression as well as to aid in the measurement of immune responses. The advantage of selecting one haplotype is that the animals studied will have similar responses to infections and immunizations, which enhances our ability to make conclusions with a small number of animals. Indeed, limited variation in viral replication dynamics were observed across monkeys after primary infection and prior to initiation of ART (Figure 3.2A).

The ability of therapeutic immunization to elicit SIV-specific immune responses in SIV-infected rhesus macaques during ART and its virologic benefits have been investigated primarily in peripheral blood using DNA immunization [57, 150, 157, 173] and other systems [42, 69, 96]. After release from ART, variable immunologic and virologic benefits were reported from no control [173], temporal control [69], to long-lasting control [96].

In this study, using CMV infection as a control, we investigated the immunologic and virologic consequences of immunization with SIV antigen in chronic SIV and CMV co-infected, ART treated rhesus macaques. All the comparisons between LNs at each time point were from the same animal collected at the same time, thus allowing me to use each animal as its own control and reach a statistically significant result. Also, my study focused on the local responses in draining LN where antigen-specific T cell responses and viral replication occurs, rather than systemic responses in peripheral blood. In addition, I chose a range of days post-immunization covering the early activation of memory T cells and generation of effector responses. My data show that both SIV and CMV antigen immunizations induced significant immune activation and antigen-specific T cell responses in draining LNs and peripheral blood. The CMV-specific response was sustained in PBMC with a rapid onset of cytokine producing $CD8^+$ T cells 3 days post immunization, which was maintained at day 50-post immunization. However, the immunization induced SIV-specific T cell response was transient and extinguished quickly in blood. Of interest, a study that repeatedly immunized macaques with long-standing ART-treated SIVmac251 infection induced stronger SIV-specific $CD4^+$ and $CD8^+$ T cell responses in blood [148]. This study used the MVA viral vector and delivered three immunizations, whereas in our study, only single gag DNA immunization was used.

In addition, SIV- and CMV-specific T cell responses in PBMC demonstrated different functional profiles. Both $CD4^+$ and $CD8^+$ SIV-specific T cells were transiently detected and produced mainly IL-2 with very little TNF-α and IFN-γ, whereas the CMV-

specific responses, primarily mediated by $CD8^+$ T cells, exhibited high TNF-α and IFN-γ production but very little IL-2. It was previously shown that the response patterns of antigen-specific $CD4^+$ T cells change as they mature. More mature cells infrequently produced IL-2 and produced effector cytokines and chemokines [25]. My results demonstrate that the SIV-specific IL-2-producing T cell responses were central and effector memory T cells, whereas the CMV-specific TNF-α and IFN-γ-producing responses were almost exclusively mediated by the more mature effector memory T cells. My results suggests that in peripheral blood, in addition to being present for 50 days, the CMV-specific T cells demonstrated a more polyfunctional profile and more mature phenotype, which is in agreement with a study showing that in HIV and CMV co-infected individuals, the CMV-specific $CD4^+$ T cells were able to produce increased β-chemokines (MIP-1α and MIP-1β), which was associated with a more mature phenotype and decreased susceptibility to HIV infection, compared to HIV-specific $CD4^+$ T cells [26]. The in vivo relevance of such IL-2 producing SIV-specific T cells is not clear in terms of protective cellular immunity and susceptibility to viral infection as potential new targets, but my data suggests that they are rapidly lost.

The progressive loss of effector function in the setting of chronic vial infections has been associated with the upregulation of programmed death 1 (PD1)[11]. In HIV/SIV infection, increased expression of PD1 is shown to correlate with $CD8^+$ T cell exhaustion and failure of immune control, which is reversible both in vitro and in vivo with PD1 blockade [40, 147, 152]. In our study, we monitored PD1 expression on SIV-specific and

tetramer negative $CD8^+$ T cells and found that PD1 expression on SIV-specific $CD8^+$ T cells were significantly higher than on tetramer negative $CD8^+$ T cells throughout the entire experiment, which could not be markedly reversed by ART and immunization (Figure 3.4.4). This strongly indicated the selective exhaustion for SIV-specific $CD8^+$ T cells during acute and chronic SIV infection, consistent with a study by Petrovas C et al [123].

HIV-specific $CD4^+$ memory T cells are preferentially infected by HIV, carrying approximately 2- to 5-fold more viral DNA than total memory cells [46]. It has also been shown that even during the most potent regimens of retroviral suppression, the presence of virus could be measured by some ultrasensitive assays [43, 70, 122]. These findings raise the concern that immunizations that induced HIV/SIV-specific T cells may accelerate viral replication even in the setting of potent ART. In support of this hypothesis, a vaccine that induced poor $CD8^+$ T cell and neutralizing antibody responses led to enhanced SIV replication after challenge that was associated with enhanced $CD4^+$ T cell proliferation [142]. We hypothesized that during an HIV-specific response, activation of HIV-specific $CD4^+$ T cells, which bear higher amounts of latent virus, results in activation of viral replication in the LN leading to suppression of $CD4^+$ T cells and the HIV-specific responses through multiple mechanisms, whereas a non-HIV antigen-specific response activates little viral replication allowing efficient expansion of the response.

We used a naturally CMV infected, SIVmac251 infected and ART treated during chronic infection rhesus macaque model to study the effects of immune stimulation using HIV gag and CMV pp65 antigens. The studies concentrated on the early immune and viral responses in draining lymphoid organs. Both antigen immunizations were able to induce transient immune activation and generated antigen-specific T cell responses in draining LNs, but the SIV gag immunization also induced significant viral replication. The SIV response extinguished quickly in peripheral blood, while the CMV response was sustained. Our data suggests that SIV antigens, as part of a vaccine or potentially as part of the normal immune response to the virus, leads to T cell stimulation with subsequent viral replication that leads to an impairment in the immune response to the virus. It is possible that this mechanism is responsible for the observation that in progressing HIV infected subjects; CD4-specific responses to HIV antigens are lost early in infection and are difficult to restore or induce.

CHAPTER 4

Thesis conclusions and future research directions

Thesis conclusions and implications

In the first part of my thesis, using an in vitro system modeling the interaction between HIV Env and $CD4^+$ T cells, I further characterize that Env protein, when delivered as a vaccine and expressed on APCs or non-APCs, present on free circulating virions, or as expressed by HIV-infected cells could induce inhibition of antigen-stimulated $CD4^+$ T cell activation and proliferation, which is dependent of CD4 binding and independent of coreceptor binding. The inhibition is not characterized as apoptosis, anergy or cell death. These Env exposed $CD4^+$ T cells can phosphorylate key signaling mediators including MAPK, AKT, and STAT5a in a normal fashion but fail to fully up-regulate activation markers such as CD69, HLA-DR, and CD25. Treg cells are not functionally involved in Env-induced inhibition of $CD4^+$ T cell activation and exposure of PBMC to Env neither changes the frequency nor the suppressive activity of Tregs. Using this data, I established a hypothesis, which suggests a mechanism for the generation of poor responses to HIV vaccines that contain Env. This hypothesis has implications for the development of novel HIV Env vaccine candidates with improved immunogenicity.

In the second part of my thesis, I employed an in vivo model system to further analyze HIV suppression of $CD4^+$ T cell antigen-specific immune responses. I demonstrate that in draining lymph nodes of chronically SIV and CMV co-infected

rhesus macaques during ART, both SIV- and CMV-immunization could induce immune activation and antigen-specific memory T cell responses. The SIV-specific T cell responses are polyfunctional with the production of multiple cytokines (IL-2, TNF-α and IFN-γ) and primarily mediated by central memory T cells. I further track the immunization-induced antigen-specific systemic responses in peripheral blood, and demonstrate that SIV-specific T cell responses were transient and extinguished quickly in blood. The SIV-specific responses in blood primarily produced IL-2 and was mediated by multiple T cell subsets, effector and central memory. In contrast, the CMV-specific T cell responses were sustained in PBMC with a rapid onset of $CD8^+$ T cells able to produce high levels of TNF-α and IFN-γ with very little IL-2, and was mainly mediated by more mature effector memory cells. The SIV immunization led to a transient activation of viral replication in draining LNs, without spread to the periphery. My results may have some potentially important implications for HIV infection and immunization in humans. First, the immune activation associated with HIV immunization during ART stimulates transient viral replication in draining LNs. Second, the transient viral replication leads to impairment of the HIV-specific response by multiple mechanisms resulting in an impaired HIV-specific systemic T cell response. In applying these findings to the induction of HIV-specific immune responses in the setting of any viral replication, I propose that the generation of HIV-specific $CD4^+$ T cell responses leads to elevated local viral replication with resulting loss of HIV-specific immune responses. These findings demonstrate a potential mechanism for the failure of progressing subjects to develop an HIV-specific $CD4^+$ T cell response.

Ongoing experiments and future directions

In addition to the mononuclear cells prepared from the LN for immunologic and virologc analysis as described in my thesis, fixed LN tissues were also prepared for in situ analysis. We are currently working with Dr. Ashley Haase's laboratory to study these fixed LN tissues using technologies such as in situ hybridization, in situ immunohistochemitry including tetramer specific staining, and quantitative image analysis to visualize in situ viral replication induced by SIV gag immunization and the host's cellular immune responses in LN tissues draining SIV and CMV immunization. In particular, we are interested in further dissecting the mechanisms for the inhibition of HIV-specific $CD4^+$ T cell immunity, including; 1) SIV- and CMV-induced apoptosis; 2) regulation of Treg cells and other potential inhibitory molecules (i.e. PD-1); and 3) viral replication associated with cell type, activation status, and antigen-specificity.

Future interesting research would include: 1) investigation of TCR or cytokine signaling cascades by Env or virus in $CD4^+$ T cells to further dissect the molecular mechanisms involved in inhibition of $CD4^+$ T cell activation; 2) SIV rhesus macaque studies with a larger number of monkeys. Preliminary results obtained in my thesis demonstrate that in chronic SIV infection during ART, immunization-induced systemic SIV-specific T cell responses compared to CMV-specific T cell responses differ in being more transient and have a different profile of cytokine expression, high IL-2, but very little TNF-α and IFN-γ production. Interesting questions could be asked about this difference in a larger SIV macaque study. Are these IL-2 producing $CD4^+$ and $CD8^+$ T

cell responses protective? Or, in contrast, do these IL-2 producing activated T cells serve as targets for viral infection or activation of latent virus? Finally, a number of questions were raised by my pilot macaque study and data worthy of investigation in a larger trial was identified. First, for time points studied, I would have multiple LN samples from different animals draining SIV and CMV immunization. This would give statistical significance for a group instead of significance for the comparison in the same animal. Second, according to our preliminary results, I focus studies on Day 3 and Day 5 when the immunization-induced local responses are highest. I would also add a time point prior to day 3 and reduce the number of time points after day 5. This will allow more LN samples for analysis without greatly increasing the number of animals needed. Moreover, if more cryopreserved LN mononuclear cells are available, cells will be analyzed for infection, apoptosis and exhaustion associated with cell type and antigen-specific immune activation. Cells could also be sorted into different subsets, such as DCs, macrophages, and $CD4^+$ and $CD8^+$ T cells and these cells will be analyzed for the extent of infection and viral integration and effector functions. This may allow for the identification of the cell types that are replicating virus in response to SIV immunization. I would also add studies to determine if the loss of the SIV-specific response is an active process. This could be done by analyzing apoptosis markers on tetramer positive cells. The addition of a CMV specific tetramer would allow a more complete analysis and I would work with Dr. Peter Barry at UC Davis to try and develop one. The analysis of additional LN samples will also allow a more thorough investigation of the T cell responses to antigen.

CHAPTER 5

REFERENCES

1. Aandahl EM, Michaelsson J, Moretto WJ, Hecht FA, Nixon DF. Human CD4(+) CD25(+) regulatory T cells control T-cell responses to human immunodeficiency virus and cytomegalovirus antigens. *Journal of Virology* 2004; 78(5):2454-2459.

2. Aandahl EM, Michaelsson J, Moretto WJ, Hecht FM, Nixon DF. Human CD4+ CD25+ Regulatory T Cells Control T-Cell Responses to Human Immunodeficiency Virus and Cytomegalovirus Antigens. *Journal of Virology* 2004; 78(5):2454-2459.

3. Algeciras A, Dockrell DH, Lynch DH, Paya C. CD4 regulates susceptibility to Fas ligand- and tumor necrosis factor-mediated apoptosis. *Journal of Experimental Medicine* 1998; 187(5):711-720.

4. Algeciras-Schimnich A, Vlahakis SR, Villasis-Keever A, Gomez T, Heppelmann CJ, Bou G, Paya CV. CCR5 mediates Fas- and caspase-8 dependent apoptosis of both uninfected and HIV infected primary human CD4 T cells. *Aids* 2002; 16(11):1467-1478.

5. Andersson J, Boasso A, Nilsson J, Zhang R, Shire NJ, Lindback S, Shearer GM, Chougnet CA. Cutting edge: The prevalence of regulatory T cells lymphoid tissue is correlated with viral load HIV-infected patients. *Journal of Immunology* 2005; 174(6):3143-3147.

6. Apoil PA, Puissant Bnd, Roubinet F, Abbal M, Massip P, Blancher A. FOXP3 mRNA Levels are Decreased in Peripheral Blood CD4+ Lymphocytes From HIV-Positive Patients. *JAIDS Journal of Acquired Immune Deficiency Syndromes* 2005; 39(4):381-385.

7. Arthos J, Cicala C, Selig SM, White AA, Ravindranath HM, Van Ryk D, Steenbeke TD, Machado E, Khazanie P, Hanback MS, Hanback DB, Rabin RL, Fauci AS. The role of the CD4 receptor versus HIV coreceptors in envelope-mediated apoptosis in peripheral blood mononuclear cells. *Virology* 2002; 292(1):98-106.

8. Autran B, Carcelain G, Li TS, Blanc C, Mathez D, Tubiana R, Katlama C, Debre P, Leibowitch J. Positive Effects of Combined Antiretroviral Therapy on CD4+ T Cell Homeostasis and Function in Advanced HIV Disease. *Science* 1997; 277(5322):112-116.

9. Baba M, Takashima K, Miyake H, Kanzaki N, Teshima K, Wang X, Shiraishi M, Iizawa Y. TAK-652 inhibits CCR5-mediated human immunodeficiency virus type 1 infection in vitro and has favorable pharmacokinetics in humans. *Antimicrobial Agents and Chemotherapy* 2005; 49(11):4584-4591.

10. Banda NK, Bernier J, Kurahara DK, Kurrle R, Haigwood N, Sekaly RP, Finkel TH. CROSS-LINKING CD4 BY HUMAN IMMUNODEFICIENCY VIRUS-GP120 PRIMES T-CELLS FOR ACTIVATION-INDUCED APOPTOSIS. *Journal of Experimental Medicine* 1992; 176(4):1099-1106.

11. Barber DL, Wherry EJ, Masopust D, Zhu BG, Allison JP, Sharpe AH, Freeman GJ, Ahmed R. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 2006; 439(7077):682-687.

12. Battaglia M, Gregori S, Bacchetta R, Roncarolo M-G. Tr1 cells: From discovery to their clinical application. *Seminars in Immunology* 2006; 18(2):120-127.

13. Benaroch P, Billard E, Gaudin R, Schindler M, Jouve M. HIV-1 assembly in macrophages. *Retrovirology* 2010; 7.

14. Benkirane M, Hirn M, Carriere D, Devaux C. Functional epitope analysis of the human CD4 molecule - antibodies that inhibit human-immunodeficiency-virus type-1 gene-expression bind to the immunoglobulin CDR3-like region of CD4. *Journal of Virology* 1995; 69(11):6898-6903.

15. Berndt C, Mopps B, Angermuller S, Gierschik P, Krammer PH. CXCR4 and CD4 mediate a rapid CD95-independent cell death in CD4(+) T cells. *Proceedings of the National Academy of Sciences of the United States of America* 1998; 95(21):12556-12561.

16. Betts MR, Nason MC, West SM, De Rosa SC, Migueles SA, Abraham J, Lederman MM, Benito JM, Goepfert PA, Connors M, Roederer M, Koup RA. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8(+) T cells. *Blood* 2006; 107(12):4781-4789.

17. Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, Polley A, Betts MR, Freeman GJ, Vignali DAA, Wherry EJ. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol* 2009; 10(1):29-37.

18. Bluestone JA, Abbas AK. Natural versus adaptive regulatory T cells. *Nature Reviews Immunology* 2003; 3(3):253-257.

19. Bottarel F, Feito MO, Bragardo M, Bonissoni S, Buonfiglio D, DeFranco S, Malavasi F, Bensi T, Ramenghi U, Dianzani U. The cell death-inducing ability of glycoprotein 120 from different HIV strains correlates with their ability to induce CD4 lateral association with CD95 on CD4(+) T cells. *Aids Research and Human Retroviruses* 1999; 15(14):1255-1263.

20. Boyer JD, Robinson TM, Maciag PC, Peng X, Johnson RS, Pavlakis G, Lewis MG, Shen A, Siliciano R, Brown CR, Weiner DB, Paterson Y. DNA prime Listeria boost induces a cellular immune response to SIV antigens in the rhesus macaque model that is capable of limited suppression of SIV239 viral replication. *Virology* 2005; 333(1):88-101.

21. Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA, Wilkinson JE, Galas D, Ziegler SF, Ramsdell F. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nature Genetics* 2001; 27(1):68-73.

22. Cao H, Walker BD. Immunopathogenesis of HIV-1 infection. *Clinics in Dermatology* 2000; 18(4):401-410.

23. Carrington M, Nelson GW, Martin MP, Kissner T, Vlahov D, Goedert JJ, Kaslow R, Buchbinder S, Hoots K, O'Brien SJ. HLA and HIV-1: Heterozygote advantage and B*35-Cw*04 disadvantage. *Science* 1999; 283(5408):1748-1752.

24. Carrington M, O'Brien SJ. The influence of HLA genotype on AIDS. *Annual Review of Medicine-Selected Topics in the Clinical Sciences* 2003; 54:535-551.

25. Casazza JP, Betts MR, Price DA, Precopio ML, Ruff LE, Brenchley JM, Hill BJ, Roederer M, Douek DC, Koup RA. Acquisition of direct antiviral effector functions by CMV-specific CD4(+) T lymphocytes with cellular maturation. *Journal of Experimental Medicine* 2006; 203(13):2865-2877.

26. Casazza JP, Brenchley JM, Hill BJ, Ayana R, Ambrozak D, Roederer M, Douek DC, Betts MR, Koup RA. Autocrine Production of Œ≤-Chemokines Protects CMV-Specific CD4⁺ T Cells from HIV Infection. *PLoS Pathog* 2009; 5(10):e1000646.

27. Chan DC, Fass D, Berger JM, Kim PS. Core Structure of gp41 from the HIV Envelope Glycoprotein. *Cell* 1997; 89(2):263-273.

28. Chan DC, Kim PS. HIV Entry and Its Inhibition. *Cell* 1998; 93(5):681-684.

29. Chatila TA, Blaeser F, Ho N, Lederman HM, Voulgaropoulos C, Helms C, Bowcock AM. JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic disregulation syndrome. *Journal of Clinical Investigation* 2000; 106(12):R75-R81.

30. Chattergoon MA, Robinson TM, Boyer JD, Weiner DB. Specific immune induction following DNA-based immunization through in vivo transfection and activation of macrophages antigen-presenting cells. *Journal of Immunology* 1998; 160(12):5707-5718.

31. Chen ML, Pittet MJ, Gorelik L, Flavell RA, Weissleder R, von Boehmer H, Khazaie K. Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-beta signals in vivoi. *Proceedings of the National Academy of Sciences of the United States of America* 2005; 102(2):419-424.

32. Chun TW, Stuyver L, Mizell SB, Ehler LA, Mican JAM, Baseler M, Lloyd AL, Nowak MA, Fauci AS. Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. *Proceedings of the National Academy of Sciences of the United States of America* 1997; 94(24):13193-13197.

33. Clark SJ, Saag MS, Decker WD, Campbellhill S, Roberson JL, Veldkamp PJ, Kappes JC, Hahn BH, Shaw GM. HIGH TITERS OF CYTOPATHIC VIRUS IN PLASMA OF PATIENTS WITH

SYMPTOMATIC PRIMARY HIV-1 INFECTION. *New England Journal of Medicine* 1991; 324(14):954-960.

34. Clerici M, Stocks NI, Zajac RA, Boswell RN, Lucey DR, Via CS, Shearer GM. Detection of three distinct patterns of T helper cell dysfunction in asymptomatic, human immunodeficiency virus-seropositive patients. Independence of CD4+ cell numbers and clinical staging. *The Journal of Clinical Investigation* 1989; 84(6):1892-1899.

35. Connor RI, Montefiori DC, Binley JM, Moore JP, Bonhoeffer S, Gettie A, Fenamore EA, Sheridan KE, Ho DD, Dailey PJ, Marx PA. Temporal Analyses of Virus Replication, Immune Responses, and Efficacy in Rhesus Macaques Immunized with a Live, Attenuated Simian Immunodeficiency Virus Vaccine. *Journal of Virology* 1998; 72(9):7501-7509.

36. Coquerelle C, Moser M. DC subsets in positive and negative regulation of immunity. *Immunological Reviews* 2010; 234(1):317-334.

37. Cottrez F, Manca F, Dalgleish AG, ArenzanaSeisdedos F, Capron A, Groux H. Priming of human CD4(+) antigen-specific T cells to undergo apoptosis by HIV-infected monocytes - A two-step mechanism involving the gp120 molecule. *Journal of Clinical Investigation* 1997; 99(2):257-266.

38. Danko I, Wolff JA. Direct gene-transfer into muscle. *Vaccine* 1994; 12(16):1499-1502.

39. Dannecker G, Mahlknecht U, Schultz H, Hoffmann MK, Niethammer D. Activation of human t-cells by the superantigen staphylococcus enterotoxin-b - analysis on a cellular-level. *Immunobiology* 1994; 190(1-2):116-126.

40. Day CL, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, Mackey EW, Miller JD, Leslie AJ, DePierres C, Mncube Z, Duraiswamy J, Zhu BG, Eichbaum Q, Altfeld M, Wherry EJ, Coovadia HM, Goulder PJR, Klenerman P, Ahmed R, Freeman GJ, Walker BD. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* 2006; 443(7109):350-354.

41. Day CL, Mkhwanazi N, Reddy S, Mncube Z, van der Stok M, Klenerman P, Walker BD. Detection of Polyfunctional Mycobacterium tuberculosis Specific T Cells and Association with Viral Load in HIV-1-Infected Persons. *The Journal of Infectious Diseases* 2008; 197(7):990-999.

42. De Rose R, Fernandez CS, Smith MZ, Batten CJ, Alcantara S, Peut V, Rollman E, Loh L, Mason RD, Wilson K, Law MG, Handley AJ, Kent SJ. Control of viremia and prevention of AIDS following immunotherapy of SIV-infected macaques with peptide-pulsed blood. *Plos Pathogens* 2008; 4(5).

43. Deeks SG, Wrin T, Liegler T, Hoh R, Hayden M, Barbour JD, Hellmann NS, Petropoulos CJ, McCune JM, Hellerstein MK, Grant RM. Virologic and Immunologic Consequences of

Discontinuing Combination Antiretroviral-Drug Therapy in HIV-Infected Patients with Detectable Viremia. *N Engl J Med* 2001; 344(7):472-480.

44. Dong C, Davis RJ, Flavell RA. MAP kinases in the immune response. *Annual Review of Immunology* 2002; 20:55-72.

45. Donzella GA, Schols D, Lin SW, Este JA, Nagashima KA, Maddon PJ, Allaway GP, Sakmar TP, Henson G, De Clercq E, Moore JP. AMD310, a small molecule inhibitor of HIV-1 entry via the CXCR4 co-receptor. *Nature Medicine* 1998; 4(1):72-77.

46. Douek DC, Brenchley JM, Betts MR, Ambrozak DR, Hill BJ, Okamoto Y, Casazza JP, Kuruppu J, Kuntsman K, Wolinsky S, Grossman Z, Dybul M, Oxenius A, Price DA, Connors M, Koup RA. HIV preferentially infects HIV-specific CD4(+) T cells. *Nature* 2002; 417(6884):95-98.

47. Douek DC, Picker LJ, Koup RA. T cell dynamics in HIV-1 infection. *Annual Review of Immunology* 2003; 21:265-304.

48. Douek DC, Roederer M, Koup RA. Emerging Concepts in the Immunopathogenesis of AIDS*. *Annual Review of Medicine* 2009; 60(1):471-484.

49. Eggena MP, Barugahare B, Jones N, Okello M, Mutalya S, Kityo C, Mugyenyi P, Cao HY. Depletion of regulatory T cells in HIV infection is associated with immune activation. *Journal of Immunology* 2005; 174(7):4407-4414.

50. Elrefaei M, McElroy MD, Preas CP, Hoh R, Deeks S, Martin J, Cao HY. Central memory CD4(+) T cell responses in chronic HIV infection are not restored by antiretroviral therapy. *Journal of Immunology* 2004; 173(3):2184-2189.

51. Embretson J, Zupancic M, Ribas JL, Burke A, Racz P, Tennerracz K, Haase AT. Massive covert infection of helper T-lymphocytes and macrophages by HIV during the incubation period of AIDS. *Nature* 1993; 362(6418):359-362.

52. Epple H-J, Loddenkemper C, Kunkel D, Troger H, Maul J, Moos V, Berg E, Ullrich R, Schulzke J-D, Stein H, Duchmann R, Zeitz M, Schneider T. Mucosal but not peripheral FOXP3+ regulatory T cells are highly increased in untreated HIV infection and normalize after suppressive HAART. *Blood* 2006; 108(9):3072-3078.

53. Fantini MC, Becker C, Monteleone G, Pallone F, Galle PR, Neurath MF. Cutting Edge: TGF-{beta} Induces a Regulatory Phenotype in CD4+CD25- T Cells through Foxp3 Induction and Down-Regulation of Smad7. *J Immunol* 2004; 172(9):5149-5153.

54. Fantuzzi L, Purificato C, Donato K, Belardelli F, Gessani S. Human Immunodeficiency Virus Type 1 gp120 Induces Abnormal Maturation and Functional Alterations of

Dendritic Cells: a Novel Mechanism for AIDS Pathogenesis. *Journal of Virology* 2004; 78(18):9763-9772.

55. Finkel TH, Tudorwilliams G, Banda NK, Cotton MF, Curiel T, Monks C, Baba TW, Ruprecht RM, Kupfer A. Apoptosis occurs predominantly in bystander cells and not in productively infected-cells of HIV-infected and SIV-infected lymph-nodes. *Nature Medicine* 1995; 1(2):129-134.

56. Fontenot JD, Rudensky AY. A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. *Nature Immunology* 2005; 6(4):331-337.

57. Fuller DH, Rajakumar PA, Wu MS, McMahon CW, Shipley T, Fuller JT, Bazmi A, Trichel AM, Allen TM, Mothe B, Haynes JR, Watkins DI, Murphey-Corb M. DNA immunization in combination with effective antiretroviral drug therapy controls viral rebound and prevents simian AIDS after treatment is discontinued. *Virology* 2006; 348(1):200-215.

58. Gandhi RT, Chen BK, Straus SE, Dale JK, Lenardo MJ, Baltimore D. HIV-1 directly kills CD4(+) T cells by a Fas-independent mechanism. *Journal of Experimental Medicine* 1998; 187(7):1113-1122.

59. Gao F, Morrison S, Robertson D, Thornton C, Craig S, Karlsson G, Sodroski J, Morgado M, Galvao-Castro B, von Briesen H. Molecular cloning and analysis of functional envelope

genes from human immunodeficiency virus type 1 sequence subtypes A through G. The WHO and NIAID Networks for HIV Isolation and Characterization. *Journal of Virology* 1996; 70(3):1651-1667.

60. Gavin MA, Torgerson TR, Houston E, deRoos P, Ho WY, Stray-Pedersen A, Ocheltree EL, Greenberg PD, Ochs HD, Rudensky AY. Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. *Proceedings of the National Academy of Sciences of the United States of America* 2006; 103(17):6659-6664.

61. Gershon RK, Kondo K. Infectious immunological tolerance. *Immunology* 1971; 21(6):903.

62. Goepfert PA, Horton H, McElrath MJ, Gurunathan S, Ferrari G, Tomaras GD, Montefiori DC, Allen M, Chiu YL, Spearman P, Fuchs JD, Koblin BA, Blattner WA, Frey S, Keefer MC, Baden LR, Corey L, Network NHVT. High-dose recombinant canarypox vaccine expressing HIV-1 protein, in seronegative human subjects. *Journal of Infectious Diseases* 2005; 192(7):1249-1259.

63. Goldman F, Jensen W, Johnson G, Heasley L, Cambier J. gp120 ligation of CD4 induces p56lck activation and TCR desensitization independent of TCR tyrosine phosphorylation. *J Immunol* 1994; 153(7):2905-2917.

64. Groux H. An overview of regulatory T cells. *Microbes and Infection* 2001; 3(11):883-889.

65. Groux H, Torpier G, Monte D, Mouton Y, Capron A, Ameisen JC. Activation-induced death by apoptosis in CD4+ T-cells from human-immunodeficiency-virus infected asymptomatic individuals. *Journal of Experimental Medicine* 1992; 175(2):331-340.

66. Hardy G, Imami N, Nelson M, Sullivan A, Moss R, Aasa-Chapman M, Gazzard B, Gotch F. A phase I, randomized study of combined IL-2 and therapeutic immunisation with antiretroviral therapy. *Journal of Immune Based Therapies and Vaccines* 2007; 5(1):6.

67. Harrer T, Harrer E, Kalams S, Barbosa P, Trocha A, Johnson R, Elbeik T, Feinberg M, Buchbinder S, Walker B. Cytotoxic T lymphocytes in asymptomatic long-term nonprogressing HIV-1 infection. Breadth and specificity of the response and relation to in vivo viral quasispecies in a person with prolonged infection and low viral load. *J Immunol* 1996; 156(7):2616-2623.

68. Harrington PR, Connell MJ, Meeker RB, Johnson PR, Swanstrom R. Dynamics of Simian Immunodeficiency Virus Populations in Blood and Cerebrospinal Fluid over the Full Course of Infection. *The Journal of Infectious Diseases* 2007; 196(7):1058-1067.

69. Hel Z, Venzon D, Poudyal M, Tsai WP, Giuliani L, Woodward R, Chougnet C, Shearer G, Altman JD, Watkins D, Bischofberger N, Abimiku A, Markham P, Tartaglia J, Franchini G. Viremia control following antiretroviral treatment and therapeutic immunization during primary SIV251 infection of macaques. *Nature Medicine* 2000; 6(10):1140-1146.

70. Hermankova M, Ray SC, Ruff C, Powell-Davis M, Ingersoll R, D'Aquila RT, Quinn TC, Siliciano JD, Siliciano RF, Persaud D. HIV-1 Drug Resistance Profiles in Children and Adults With Viral Load of <50 Copies/mL Receiving Combination Therapy. *JAMA* 2001; 286(2):196-207.

71. Holm GH, Gabuzda D. Distinct Mechanisms of CD4+ and CD8+ T-Cell Activation and Bystander Apoptosis Induced by Human Immunodeficiency Virus Type 1 Virions. *Journal of Virology* 2005; 79(10):6299-6311.

72. Hoxie JA. Toward an Antibody-Based HIV-1 Vaccine. *Annual Review of Medicine* 2010; 61(1):135-152.

73. Irvine DJ, Purbhoo MA, Krogsgaard M, Davis MM. Direct observation of ligand recognition by T cells. *Nature* 2002; 419(6909):845-849.

74. Itoh M, Takahashi T, Sakaguchi N, Kuniyasu Y, Shimizu J, Otsuka F, Sakaguchi S. Thymus and autoimmunity: Production of CD25(+)CD4(+) naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *Journal of Immunology* 1999; 162(9):5317-5326.

75. Jabado N, Ledeist F, Fischer A, Hivroz C. Interaction of HIV gp120 and anti-CD4 antibodies with the CD4 molecule on human CD4(+) T-cells inhibits the binding-activity

of NF-AT, NF-kb and AP1, 3 nuclear factors regulating interleukin-2 gene enhancer activity. *European Journal of Immunology* 1994; 24(11):2646-2652.

76. Jacotot E, Callebaut C, Blanco J, Rivire Y, Krust B, Hovanessian AG. HIV Envelope Glycoprotein-Induced Cell Killing by Apoptosis Is Enhanced with Increased Expression of CD26 in CD4+T Cells. *Virology* 1996; 223(2):318-330.

77. Jeremias I, Herr I, Boehler T, Debatin KM. TRAIL/Apo-2-ligand-induced apoptosis in human T cells. *European Journal of Immunology* 1998; 28(1):143-152.

78. Johnston JA, Bacon CM, Finbloom DS, Rees RC, Kaplan D, Shibuya K, Ortaldo JR, Gupta S, Chen YQ, Giri JD, Oshea JJ. TYROSINE PHOSPHORYLATION AND ACTIVATION OF STAT5, STAT3, AND JANUS KINASES BY INTERLEUKIN-2 AND INTERLEUKIN-15. *Proceedings of the National Academy of Sciences of the United States of America* 1995; 92(19):8705-8709.

79. Kameoka M, Kimura T, Zheng YH, Suzuki S, Fujinaga K, Luftig RB, Ikuta K. Protease-defective, gp120-containing human immunodeficiency virus type 1 particles induce apoptosis more efficiently than does wild-type virus or recombinant gp120 protein in healthy donor-derived peripheral blood T cells. *Journal of Clinical Microbiology* 1997; 35(1):41-47.

80. Kariko K, Kuo A, Barnathan ES. Overexpression of urokinase receptor in mammalian cells following administration of the in vitro transcribed encoding mRNA. *Gene Therapy* 1999; 6(6):1092-1100.

81. Kaslow RA, Carrington M, Apple R, Park L, Munoz A, Saah AJ, Goedert JJ, Winkler C, Obrien SJ, Rinaldo C, Detels R, Blattner W, Phair J, Erlich H, Mann DL. Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection. *Nature Medicine* 1996; 2(4):405-411.

82. Kawamura T, Gatanaga H, Borris DL, Connors M, Mitsuya H, Blauvelt A. Decreased Stimulation of CD4+ T Cell Proliferation and IL-2 Production by Highly Enriched Populations of HIV-Infected Dendritic Cells. *J Immunol* 2003; 170(8):4260-4266.

83. Kelleher AD, Carr A, Zaunders J, Cooper DA. Alterations in the immune response of human immunodeficiency virus (HIV)-infected subjects treated with an HIV-specific protease inhibitor, ritonavir. *Journal of Infectious Diseases* 1996; 173(2):321-329.

84. Kieffer TL, Finucane MM, Nettles RE, Quinn TC, Broman KW, Ray SC, Persaud D, Siliciano RF. Genotypic analysis of HIV-1 drug resistance at the limit of detection: Virus production without evolution in treated adults with undetectable HIV loads. *Journal of Infectious Diseases* 2004; 189(8):1452-1465.

85. Kinter A, McNally J, Riggin L, Jackson R, Roby G, Fauci AS. Suppression of HIV-specific T cell activity by lymph node CD25(+) regulatory T cells from HIV-infected individuals. *Proceedings of the National Academy of Sciences of the United States of America* 2007; 104(9):3390-3395.

86. Kinter AL, Hennessey M, Bell A, Kern S, Lin Y, Daucher M, Planta M, McGlaughlin M, Jackson R, Ziegler SE, Fauci AS. CD25(+) CD4(+) regulatory T cells from the peripheral blood of asymptomatic HIV-infected individuals regulate CD4(+) and CD8(+) HIV-specific T cell immune responses in vitro and are associated with favorable clinical markers of disease status. *Journal of Experimental Medicine* 2004; 200(3):331-343.

87. Krathwohl MD, Schacker TW, Anderson JL. Abnormal presence of semimature dendritic cells that induce regulatory T cells in HIV-infected subjects. *Journal of Infectious Diseases* 2006; 193(4):494-504.

88. Krowka JF, Stites DP, Jain S, Steimer KS, Georgenascimento C, Gyenes A, Barr PJ, Hollander H, Moss AR, Homsy JM, Levy JA, Abrams DI. LYMPHOCYTE PROLIFERATIVE RESPONSES TO HUMAN IMMUNODEFICIENCY VIRUS-ANTIGENS INVITRO. *Journal of Clinical Investigation* 1989; 83(4):1198-1203.

89. Kryworuchko M, Pasquier V, Thze J. Human immunodeficiency virus-1 envelope glycoproteins and anti-CD4 antibodies inhibit interleukin-2-induced Jak/STAT signalling

in human CD4 T lymphocytes. *Clinical & Experimental Immunology* 2003; 131(3):422-427.

90. Lane HC, Depper JM, Greene WC, Whalen G, Waldmann TA, Fauci AS. Qualitative-analysis of immune function in patients with the acquired immunodeficiency syndrome - evidence for a selective defect in soluble-antigen recognition. *New England Journal of Medicine* 1985; 313(2):79-84.

91. Lederman MM, Connick E, Landay A, Kuritzkes DR, Spritzler J, St Clair M, Kotzin BL, Fox L, Chiozzi H, Leonard JM, Rousseau F, Wade M, Roe JD, Martinez A, Kessler H. Immunologic responses associated with 12 weeks of combination antiretroviral therapy consisting of zidovudine, lamivudine, and ritonavir: Results of AIDS clinical trials group protocol 315. *Journal of Infectious Diseases* 1998; 178(1):70-79.

92. Letvin NL. Animal-models for AIDS. *Immunology Today* 1990; 11(9):322-326.

93. Liegler TJ, Stites DP. HIV-1 gp120 and anti-gp120 induce reversible unresponsiveness in peripheral CD4 T-lymphocytes. *Journal of Acquired Immune Deficiency Syndromes and Human Retrovirology* 1994; 7(4):340-348.

94. Liu WH, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, Gottlieb PA, Kapranov P, Gingeras TR, Fazekas de St Groth B, Clayberger C, Soper DM, Ziegler SF, Bluestone JA. CD127

expression inversely correlates with FoxP3 and suppressive function of human CD4(+) T reg cells. *Journal of Experimental Medicine* 2006; 203(7):1701-1711.

95. Loddenkemper C, Maul J, Berg E, Stein H, Zeitz M, Duchmann R. To the Editor - Eur. J. Immunol. 1/2006. *European Journal of Immunology* 2006; 36(1):245.

96. Lu W, Wu XX, Lu YZ, Guo WZ, Andrieu JM. Therapeutic dendritic-cell vaccine for simian AIDS. *Nature Medicine* 2003; 9(1):27-32.

97. Lyons AB, Parish CR. Determination of lymphocyte division by flow-cytometry. *Journal of Immunological Methods* 1994; 171(1):131-137.

98. Mantel PY, Ouaked N, Ruckert B, Karagiannidis C, Welz R, Blaser K, Schmidt-Weber CB. Molecular mechanisms underlying FOXP3 induction in human T cells. *Journal of Immunology* 2006; 176(6):3593-3602.

99. Masci AM, Galgani M, Cassano S, De Simone S, Gallo A, De Rosa V, Zappacosta S, Racioppi L. HIV-1 gp120 induces anergy in naive T lymphocytes through CD4-independent protein kinase-A-mediated signaling. *Journal of Leukocyte Biology* 2003; 74(6):1117-1124.

100. Mascola JR, Lewis MG, Stiegler G, Harris D, VanCott TC, Hayes D, Louder MK, Brown CR, Sapan CV, Frankel SS, Lu Y, Robb ML, Katinger H, Birx DL. Protection of Macaques

against Pathogenic Simian/Human Immunodeficiency Virus 89.6PD by Passive Transfer of Neutralizing Antibodies. *Journal of Virology* 1999; 73(5):4009-4018.

101. Masur H, Ognibene FP, Yarchoan R, Shelhamer JH, Baird BF, Travis W, Suffredini AF, Deyton L, Kovacs JA, Falloon J, Davey R, Polis M, Metcalf J, Baseler M, Wesley R, Gill VJ, Fauci AS, Lane HC. CD4 counts as predictors of opportunistic pneumonias in human immunodeficiency virus (HIV) infection. *Annals of Internal Medicine* 1989; 111(3):223-231.

102. Mattapallil JJ, Douek DC, Hill B, Nishimura Y, Martin M, Roederer M. Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. *Nature* 2005; 434(7037):1093-1097.

103. Mattapallil JJ, Letvin NL, Roederer M. T-cell dynamics during acute SIV infection. *AIDS* 2004; 18(1):13-23.

104. McDonald B, Martin-Serrano J. No strings attached: the ESCRT machinery in viral budding and cytokinesis. *Journal of Cell Science* 2009; 122(13):2167-2177.

105. Miedema F, Petit AJC, Terpstra FG, Schattenkerk J, Dewolf F, Al BJM, Roos M, Lange JMA, Danner SA, Goudsmit J, Schellekens PTA. Immunological abnormalities in human immunodeficiency virus (HIV)-infected asymptomatic homosexual men - HIV affects the

immune-system before CD4+ T-helper cell depletion occurs. *Journal of Clinical Investigation* 1988; 82(6):1908-1914.

106. Migueles SA, Laborico AC, Shupert WL, Sabbaghian MS, Rabin R, Hallahan CW, Van Baarle D, Kostense S, Miedema F, McLaughlin M, Ehler L, Metcalf J, Liu SY, Connors M. HIV-specific CD8(+) T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. *Nature Immunology* 2002; 3(11):1061-1068.

107. Monceaux V, Viollet L, Petit F, Fang RHT, Cumont M-C, Zaunders J, Hurtrel B, Estaquier J. CD8+ T Cell Dynamics during Primary Simian Immunodeficiency Virus Infection in Macaques: Relationship of Effector Cell Differentiation with the Extent of Viral Replication. *J Immunol* 2005; 174(11):6898-6908.

108. Montes M, Lewis DE, Sanchez C, de Castilla DL, Graviss EA, Seas C, Gotuzzo E, White AC. Foxp3+ regulatory T cells in antiretroviral-naive HIV patients. *Aids* 2006; 20(12):1669-1671.

109. Morio T, Chatila T, Geha RS. HIV glycoprotein gp120 inhibits TCR-CD3-mediated activation of fyn and lck. *International Immunology* 1997; 9(1):53-64.

110. Muhl T, Krawczak M, ten Haaft P, Hunsmann G, Sauermann U. MHC class I alleles influence set-point viral load and survival time in simian immunodeficiency virus-infected rhesus monkeys. *Journal of Immunology* 2002; 169(6):3438-3446.

111. Mwau M, Cebere I, Sutton J, Chikoti P, Winstone N, Wee EGT, Beattie T, Chen YH, Dorrell L, McShane H, Schmidt C, Brooks M, Patel S, Roberts J, Conlon C, Rowland-Jones SL, Bwayo JJ, McMichael AJ, Hanke T. A human immunodeficiency virus 1 (HIV-1) clade A vaccine in clinical trials: stimulation of HIV-specific T-cell responses by DNA and recombinant modified vaccinia virus Ankara (MVA) vaccines in humans. *Journal of General Virology* 2004; 85:911-919.

112. Nilsson J, Boasso A, Velilla PA, Zhang R, Vaccari M, Franchini G, Shearer GM, Andersson J, Chougnet C. HIV-1-driven regulatory T-cell accumulation in lymphoid tissues is associated with disease progression in HIV/AIDS. *Blood* 2006; 108(12):3808-3817.

113. Noraz N, Gozlan Jl, Corbeil J, Brunner T, Spector SA. HIV-induced apoptosis of activated primary CD4+ T lymphocytes is not mediated by Fas-Fas ligand. *Aids* 1997; 11(14):1671-1680.

114. Nowak MA, Lloyd AL, Vasquez GM, Wiltrout TA, Wahl LM, Bischofberger N, Williams J, Kinter A, Fauci AS, Hirsch VM, Lifson JD. Viral dynamics of primary viremia and antiretroviral therapy in simian immunodeficiency virus infection. *Journal of Virology* 1997; 71(10):7518-7525.

115. Oswald-Richter K, Grill SM, Shariat N, Leelawong M, Sundrud MS, Haas DW, Unutmaz D. HIV infection of naturally occurring and genetically reprogrammed human regulatory T-cells. *Plos Biology* 2004; 2(7):955-966.

116. Pantaleo G, Demarest JF, Schacker T, Vaccarezza M, Cohen OJ, Daucher M, Graziosi C, Schnittman SS, Quinn TC, Shaw GM, Perrin L, Tambussi G, Lazzarin A, Sekaly RP, Soudeyns H, Corey L, Fauci AS. The qualitative nature of the primary immune response to HIV infection is a prognosticator of disease progression independent of the initial level of plasma viremia. *Proceedings of the National Academy of Sciences of the United States of America* 1997; 94(1):254-258.

117. Pantaleo G, Graziosi C, Demarest JF, Butini L, Montroni M, Fox CH, Orenstein JM, Kotler DP, Fauci AS. HIV-infection is active and progressive in lymphoid-tissue during the clinically latent stage of disease. *Nature* 1993; 362(6418):355-358.

118. Pantaleo G, Menzo S, Vaccarezza M, Graziosi C, Cohen OJ, Demarest JF, Montefiori D, Orenstein JM, Fox C, Schrager LK, Margolick JB, Buchbinder S, Giorgi JV, Fauci AS. Studies in subjects with long-term nonprogressive human-immunodeficiency-virus infection. *New England Journal of Medicine* 1995; 332(4):209-216.

119. Park IW, Steen R, Li Y. Characterization of multiple mRNA species of simian immunodeficiency virus from macaques in a CD4+ lymphoid cell line. *Journal of Virology* 1991; 65(6):2987-2992.

120. Perelson AS, Essunger P, Cao YZ, Vesanen M, Hurley A, Saksela K, Markowitz M, Ho DD. Decay characteristics of HIV-1-infected compartments during combination therapy. *Nature* 1997; 387(6629):188-191.

121. Perkus ME, Limbach K, Paoletti E. Cloning and expression of foreign genes in vaccinia virus, using a host range selection system. *Journal of Virology* 1989; 63(9):3829-3836.

122. Persaud D, Siberry GK, Ahonkhai A, Kajdas J, Monie D, Hutton N, Watson DC, Quinn TC, Ray SC, Siliciano RF. Continued Production of Drug-Sensitive Human Immunodeficiency Virus Type 1 in Children on Combination Antiretroviral Therapy Who Have Undetectable Viral Loads. *Journal of Virology* 2004; 78(2):968-979.

123. Petrovas C, Price DA, Mattapallil J, Ambrozak DR, Geldmacher C, Cecchinato V, Vaccari M, Tryniszewska E, Gostick E, Roederer M, Douek DC, Morgan SH, Davis SJ, Franchini G, Koup RA. SIV-specific CD8(+) T cells express high levels of PD1 and cytokines but have impaired proliferative capacity in acute and chronic SIVmac251 infection. *Blood* 2007; 110(3):928-936.

124. Piatak M, Saag MS, Yang LC, Clark SJ, Kappes JC, Luk KC, Hahn BH, Shaw GM, Lifson JD. HIGH-LEVELS OF HIV-1 IN PLASMA DURING ALL STAGES OF INFECTION DETERMINED BY COMPETITIVE PCR. *Science* 1993; 259(5102):1749-1754.

125. Picker LJ, Watkins DI. HIV pathogenesis: The first cut is the deepest. *Nature Immunology* 2005; 6(5):430-432.

126. Pierson T, McArthur J, Siliciano RF. Reservoirs for HIV-1: Mechanisms for Viral Persistence in the Presence of Antiviral Immune Responses and Antiretroviral Therapy. *Annual Review of Immunology* 2000; 18(1):665-708.

127. Pinto LMD, Garcia S, Lecoeur H, Rapp C, Gougeon ML. Increased sensitivity of T lymphocytes to tumor necrosis factor receptor 1 (TNFR1)- and TNFR2-mediated apoptosis in HIV infection: relation to expression of Bcl-2 and active caspase-8 and caspase-3. *Blood* 2002; 99(5):1666-1675.

128. Pope M, Gezelter S, Gallo N, Hoffman L, Steinman RM. Low-level of HIV-1 infection in cutaneous dendritic cells promote extensive viral replication upon binding to memory CD4(+) T cells. *Journal of Experimental Medicine* 1995; 182(6):2045-2056.

129. Rinaldo J, Charles R, Liebmann JM, Huang X, Fan Z, Al, Shboul Q, McMahon DK, Day RD, Riddler SA, Mellors JW. Prolonged Suppression of Human Immunodeficiency Virus Type 1 (HIV-1) Viremia in Persons with Advanced Disease Results in Enhancement of CD4 T Cell Reactivity to Microbial Antigens but Not to HIV-1 Antigens. *The Journal of Infectious Diseases* 1999; 179(2):329-336.

130. Roggero R, Robert-Hebmann V, Harrington S, Roland J, Vergne L, Jaleco S, Devaux C, Biard-Piechaczyk M. Binding of human immunodeficiency virus type 1 gp120 to CXCR4 induces mitochondrial transmembrane depolarization and cytochrome c-mediated apoptosis independently of Fas signaling. *Journal of Virology* 2001; 75(16):7637-7650.

131. Rossio JL, Esser MT, Suryanarayana K, Schneider DK, Bess JW, Vasquez GM, Wiltrout TA, Chertova E, Grimes MK, Sattentau Q, Arthur LO, Henderson LE, Lifson JD. Inactivation of human immunodeficiency virus type 1 infectivity with preservation of conformational and functional integrity of virion surface proteins. *Journal of Virology* 1998; 72(10):7992-8001.

132. Schwartz O, Alizon M, Heard J-M, Danos O. Impairment of T Cell Receptor-Dependent Stimulation in CD4+ Lymphocytes after Contact with Membrane-Bound HIV-1 Envelope Glycoprotein. *Virology* 1994; 198(1):360-365.

133. Schwartz RH. T cell anergy. *Annual Review of Immunology* 2003; 21(1):305-334.

134. Selliah N, Finkel TH. Cutting Edge: JAK3 Activation and Rescue of T Cells from HIV gp120-Induced Unresponsiveness. *J Immunol* 1998; 160(12):5697-5701.

135. Selliah N, Finkel TH. Cutting edge: JAK3 activation and rescue of T cells from HIV gp120-induced unresponsiveness. *Journal of Immunology* 1998; 160(12):5697-5701.

136. Sequar G, Britt WJ, Lakeman FD, Lockridge KM, Tarara RP, Canfield DR, Zhou SS, Gardner MB, Barry PA. Experimental coinfection of rhesus Macaques with rhesus cytomegalovirus and Simian immunodeficiency virus: Pathogenesis. *Journal of Virology* 2002; 76(15):7661-7671.

137. Sho M, Kishimoto K, Harada H, Livak M, Sanchez-Fueyo A, Yamada A, Zheng XX, Strom TB, Basadonna GP, Sayegh MH, Rothstein DM. Requirements for induction and maintenance of peripheral tolerance in stringent allograft models. *Proceedings of the National Academy of Sciences of the United States of America* 2005; 102(37):13230-13235.

138. Shoukry NH, Lavoie PM, Thibodeau J, Dsouza S, Sekaly RP. MHC class II-dependent peptide antigen versus superantigen presentation to T cells. *Human Immunology* 1997; 54(2):194-201.

139. Sieg SF, Bazdar DA, Harding CV, Lederman MM. Differential expression of interleukin-2 and gamma interferon in human immunodeficiency virus disease. *Journal of Virology* 2001; 75(20):9983-9985.

140. Sieg SF, Harding CV, Lederman MM. HIV-1 infection impairs cell cycle progression of CD4(+) T cells without affecting early activation responses. *Journal of Clinical Investigation* 2001; 108(5):757-764.

141. Sieg SF, Mitchem JB, Bazdar DA, Lederman MM. Close link between CD4(+) and CD8(+) T cell proliferation defects in patients with human immunodeficiency virus disease and relationship to extended periods of CD4(+) lymphopenia. *Journal of Infectious Diseases* 2002; 185(10):1401-1408.

142. Staprans SI, Barry AP, Silvestri G, Safrit JT, Kozyr N, Sumpter B, Nguyen H, McClure H, Montefiori D, Cohen JI, Feinberg MB. Enhanced SIV replication and accelerated progression to AIDS in macaques primed to mount a CD4 T cell response to the SIV envelope protein. *Proceedings of the National Academy of Sciences of the United States of America* 2004; 101(35):13026-13031.

143. Staprans SI, Dailey PJ, Rosenthal A, Horton C, Grant RM, Lerche N, Feinberg MB. Simian Immunodeficiency Virus Disease Course Is Predicted by the Extent of Virus Replication during Primary Infection. *Journal of Virology* 1999; 73(6):4829-4839.

144. Stevenson M. Dissecting HIV-1 through RNA interference. *Nat Rev Immunol* 2003; 3(11):851-858.

145. Tamma SML, Chirmule N, McCloskey TW, Oyaizu N, Kalyanaraman VS, Pahwa S. Signals transduced through the CD4 molecule interfere with TCR/CD3-mediated ras activation leading to T cell anergy/apoptosis. *Clinical Immunology and Immunopathology* 1997; 85(2):195-201.

146. Tateyama M, Oyaizu N, McCloskey TW, Than S, Pahwa S. CD4 T lymphocytes are primed to express Fas ligand by CD4 cross-linking and to contribute to CD8 T-cell apoptosis via Fas/FasL death signaling pathway. *Blood* 2000; 96(1):195-202.

147. Trautmann L, Janbazian L, Chomont N, Said EA, Gimmig S, Bessette B, Boulassel MR, Delwart E, Sepulveda H, Balderas RS, Routy JP, Haddad EK, Sekaly RP. Upregulation of PD-1 expression on HIV-specific CD8(+) T cells leads to reversible immune dysfunction. *Nature Medicine* 2006; 12(10):1198-1202.

148. Tryniszewska E, Nacsa J, Lewis MG, Silvera P, Montefiori D, Venzon D, Hel Z, Parks RW, Moniuszko M, Tartaglia J, Smith KA, Franchini G. Vaccination of Macaques with Long-Standing SIVmac251 Infection Lowers the Viral Set Point After Cessation of Antiretroviral Therapy. *J Immunol* 2002; 169(9):5347-5357.

149. Tsunemi S, Iwasaki T, Imado T, Higasa S, Kakishita E, Shirasaka T, Sano H. Relationship of CD4+CD25+ regulatory T cells to immune status in HIV-infected patients. *Aids* 2005; 19(9):879-886.

150. Valentin A, von Gegerfelt A, Rosati M, Miteloudis G, Alicea C, Bergamaschi C, Jalah R, Patel V, Khan AS, Draghia-Akli R, Pavlakis GN, Felber BK. Repeated DNA therapeutic vaccination of chronically SIV-infected macaques provides additional virological benefit. *Vaccine* 2010; 28(8):1962-1974.

151. Veazey RS, Klasse PJ, Ketas TJ, Reeves JD, Piatak M, Kunstman K, Kuhmann SE, Marx PA, Lifson JD, Dufour J, Mefford M, Pandrea I, Wolinsky SM, Doms RW, DeMartino JA, Siciliano SJ, Lyons K, Springer MS, Moore JP. Use of a small molecule CCR5 inhibitor in macaques to treat simian immunodeficiency virus infection or prevent simian-human immunodeficiency virus infection. *Journal of Experimental Medicine* 2003; 198(10):1551-1562.

152. Velu V, Titanji K, Zhu BG, Husain S, Pladevega A, Lai LL, Vanderford TH, Chennareddi L, Silvestri G, Freeman GJ, Ahmed R, Amara RR. Enhancing SIV-specific immunity in vivo by PD-1 blockade. *Nature* 2009; 458(7235):206-U205.

153. Viollet L, Monceaux V, Petit F, Fang RHT, Cumont M-C, Hurtrel B, Estaquier J. Death of CD4+ T Cells from Lymph Nodes during Primary SIVmac251 Infection Predicts the Rate of AIDS Progression. *J Immunol* 2006; 177(10):6685-6694.

154. Vlahakis SR, Algeciras-Schimnich A, Bou G, Heppelmann CJ, Villasis-Keever A, Collman RG, Paya CV. Chemokine-receptor activation by env determines the mechanism of death in HIV-infected and uninfected T lymphocytes. *Journal of Clinical Investigation* 2001; 107(2):207-215.

155. Vlahakis SR, Villasis-Keever A, Gomez T, Vanegas M, Vlahakis N, Paya CV. G Protein-Coupled Chemokine Receptors Induce Both Survival and Apoptotic Signaling Pathways. *J Immunol* 2002; 169(10):5546-5554.

156. von Boehmer H. Mechanisms of suppression by suppressor T cells. *Nature Immunology* 2005; 6(4):338-344.

157. von Gegerfelt AS, Rosati M, Alicea C, Valentin A, Roth P, Bear J, Franchini G, Albert PS, Bischofberger N, Boyer JD, Weiner DB, Markham P, Israel ZR, Eldridge JH, Pavlakis GN, Felber BK. Long-Lasting Decrease in Viremia in Macaques Chronically Infected with Simian Immunodeficiency Virus SIVmac251 after Therapeutic DNA Immunization. *Journal of Virology* 2007; 81(4):1972-1979.

158. Wahren B, Morfeldtmansson L, Biberfeld G, Moberg L, Sonnerborg A, Ljungman P, Werner A, Kurth R, Gallo R, Bolognesi D. CHARACTERISTICS OF THE SPECIFIC CELL-MEDIATED IMMUNE-RESPONSE IN HUMAN IMMUNODEFICIENCY VIRUS-INFECTION. *Journal of Virology* 1987; 61(6):2017-2023.

159. Watson A, Ranchalis J, Travis B, McClure J, Sutton W, Johnson P, Hu S, Haigwood N. Plasma viremia in macaques infected with simian immunodeficiency virus: plasma viral load early in infection predicts survival. *Journal of Virology* 1997; 71(1):284-290.

160. Weiner HL. Oral tolerance: immune mechanisms and the generation of Th3-type TGF-beta-secreting regulatory cells. *Microbes and Infection* 2001; 3(11):947-954.

161. Weiss L, Donkova-Petrini V, Caccavelli L, Balbo M, Carbonneil C, Levy Y. Human immunodeficiency virus-driven expansion of CD4+CD25+ regulatory T cells, which suppress HIV-specific CD4 T-cell responses in HIV-infected patients. *Blood* 2004; 104(10):3249-3256.

162. Weiss R. How does HIV cause AIDS? *Science* 1993; 260(5112):1273-1279.

163. Weissman D, Ni H, Scales D, Dude A, Capodici J, McGibney K, Abdool A, Isaacs SN, Cannon G, Kariko K. HIV Gag mRNA Transfection of Dendritic Cells (DC) Delivers Encoded Antigen to MHC Class I and II Molecules, Causes DC Maturation, and Induces a Potent Human In Vitro Primary Immune Response. *J Immunol* 2000; 165(8):4710-4717.

164. Wells AD, Gudmundsdottir H, Turka LA. Following the fate of individual T cells throughout activation and clonal expansion - Signals from T cell receptor and CD28 differentially regulate the induction and duration of a proliferative response. *Journal of Clinical Investigation* 1997; 100(12):3173-3183.

165. Westendorp MO, Frank R, Ochsenbauer C, Stricker K, Dhein J, Walczak H, Debatin KM, Krammer PH. Sensitization of t-cells to CD95-mediated apoptosis by HIV-1 tat and gp120. *Nature* 1995; 375(6531):497-500.

166. Wildin RS, Ramsdell F, Peake J, Faravelli F, Casanova JL, Buist N, Levy-Lahad E, Mazzella M, Goulet O, Perroni L, Bricarelli FD, Byrne G, McEuen M, Proll S, Appleby M, Brunkow

ME. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nature Genetics* 2001; 27(1):18-20.

167. Williams MA, Trout R, Spector SA. HIV-1 gp120 modulates the immunological function and expression of accessory and co-stimulatory molecules of monocyte-derived dendritic cells. *Journal of Hematotherapy & Stem Cell Research* 2002; 11(5):829-847.

168. Wu L, KewalRamani VN. Dendritic-cell interactions with HIV: infection and viral dissemination. *Nature Reviews Immunology* 2006; 6(11):859-868.

169. Wyatt R, Sodroski J. The HIV-1 Envelope Glycoproteins: Fusogens, Antigens, and Immunogens. *Science* 1998; 280(5371):1884-1888.

170. Yue Y, Kaur A, Eberhardt MK, Kassis N, Zhou SS, Tarantal AF, Barry PA. Immunogenicity and Protective Efficacy of DNA Vaccines Expressing Rhesus Cytomegalovirus Glycoprotein B, Phosphoprotein 65-2, and Viral Interleukin-10 in Rhesus Macaques. *Journal of Virology* 2007; 81(3):1095-1109.

171. Zhang ZQ, Fu TM, Casimiro DR, Davies ME, Liang XP, Schleif WA, Handt L, Tussey L, Chen MC, Tang AM, Wilson KA, Trigona WL, Freed DC, Tan CY, Horton M, Emini EA, Shiver JW. Mamu-A*01 allele-mediated attenuation of disease progression in simian-human immunodeficiency virus infection. *Journal of Virology* 2002; 76(24):12845-12854.

172. Zou WP. Regulatory T cells, tumour immunity and immunotherapy. *Nature Reviews Immunology* 2006; 6(4):295-307.

173. zur Megede J, Sanders-Beer B, Silvera P, Golightly D, Bowlsbey A, Hebblewaite D, Sites D, Nieves-Duran L, Srivastava R, Otten GR, Rabussay D, Zhang L, Ulmer JB, Barnett SW, Donnelly JJ. A Therapeutic SIV DNA Vaccine Elicits T-Cell Immune Responses, But No Sustained Control of Viremia in SIVmac239-Infected Rhesus Macaques. *AIDs Research and Human Retroviruses* 2008; 24(8):1103-1116.