Early localized SIV-specific CD8 T cells response to pathogenic SIV is correlated with successful SIV vaccine

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This thesis is dedicated to my grandmother, Oak Tae Kim, and parents, Jong Soo Hong and Hyun Woo Ryoo, who made sacrifices for me and supported my education in the United States.

ABSTRACT

Live-attenuated lentivirus vaccines are the most effective in inducing protection against subsequent challenge with pathogenic lentiviruses. For example, infection with live-attenuated simian immunodeficiency viruses (SIVs) including nonpathogenic simian-human immunodeficiency virus (SHIV) 89.6, and SIVΔnef in macaques provides protection against subsequent challenge with highly pathogenic strains of SIV. The mechanism by which live-attenuated SIVs induce protection is not well understood. My central hypothesis is that an early SIV-specific CD8 T cell response in lymph nodes and at the site of infection is responsible for the protection induced from live-attenuated SIV vaccines. The rationale for this hypothesis stems from our previous studies showing that SIV disseminates throughout the body by one week post-infection but SIV-specific CD8 T cell responses are too late and not detected in situ until the second and third weeks post-infection. For the present study, rhesus macaques were immunized intravenously with live non-pathogenic SHIV89.6, or SIVΔnef and then challenged intravaginally with pathogenic SIVmac239, or SIVmac251. Using the experimental approach of in situ tetramer staining combined with immunohistochemistry, confocal microscopy and quantitative image analyses, I determined: 1) localization and quantification of virus-specific CD8 T cells; 2) phenotypic changes in lytic granule contents of virus-specific $CD8^+$ T cells; and 3) virus-specific CD8 T cells interacting with $CD83^+$cells in tissues from these animals. My main results show that an early but not necessarily robust SIV-

specific CD8 T cell response localized to lymph nodes and genital tissues correlated with protection in these animals. In addition, the majority of SIV-specific CD8 T cells that I observed in vaccinated animals showed little to no perforin expression, compared to cells from non-vaccinated and pathogenic SIV-infected animals. Lastly, I also observed an increase in the interaction of vaccine-induced SIV-specific T cells with $CD83^{+}$ dendritic cells after pathogenic SIV challenge in immunized animals. These results indicate that vaccine-induced protection correlates with early localized SIV-specific CD8 T cells that show little to no perforin expression at the portal of viral entry and lymph nodes. Additionally my studies indicate that vaccine-induced protection correlates with increased interactions of SIV-specific CD8 T cells with mature dendritic cells. These studies provide a better understanding of immune correlates involved in the protection afforded by live-attenuated SIV vaccines, and provide insights into what is needed to create a successful HIV vaccine.

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List of Abbreviations

1. HIV: human immunodeficiency virus
2. AIDS: acquired immune deficiency syndrome
3. CTL: cytotoxicity T lymphocyte
4. PCP: pneumocystis carinii pneumonia
5. LAV: lymphadenopathy-associated virus
6. HTLV: human T-lymphotropic virus
7. CCR : CC chemokine receptor
8. CXCR : CXC chemokine receptor
9. SHIV : simian-human immunodeficiency virus
10. MHC : major histocompatibility complex
11. TAP : transporter associated with antigen processing
12. ER: endoplasmic reticulum
13. SIV: simian immunodeficiency virus
14. ERAP: aminopeptidase
15. TRAIL : Tumor necrosis factor-related apoptosis-inducing ligand
16. Ig: immunoglobulin
17. APC: antigen-presenting cell
18. CNAR: non-cytotoxic antiviral response
19. SDF-1 : stromal cell-derived factor-1
20. DC: dendritic cell
21. DC-SIGN : DC-specific intercellular adhesion molecule-3 grabbing non-integrin
22. PALS : periarteriolar lymphoid sheaths
23. Th : T helper
24. IFN-□: interferon gamma
25. TNF-α : tumor necrosis factor alpha
26. IL: interleukin
27. CAF: CD8 T cell activating factor
28. TCR: T cell receptor
29. PBS-H: phosphate buffered saline containing heparin
30. NGS: normal goat serum

Chapter 1. Introduction

The History and Global spread of HIV

Around 30 years ago, acquired immunodeficiency syndrome (AIDS) first appeared in the medical literature reporting five young homosexual men with *Pneumocystis pneumonia* (PCP) who displayed a profound depletion of CD4-positive T-lymphocytes (1). A couple years later, in May 1983, Dr. Luc Montagnier found a suspect virus named lymphadenopathy-associated virus (LAV) (2) and, in December 1984, Dr. Robert Gallo published that human T-lymphotropic virus (HTLV) was the cause of AIDS (3). The same year, Dr. Jay Levy and colleagues published a report of this virus isolate as AIDS-associated retrovirus (4). In the ensuing years, they had gotten into arguments over what to officially call the newly discovered virus. Finally, all three of these designations were identified as Human Immunodeficiency Virus (HIV) that causes AIDS by the International Committee on the Taxonomy of Viruses in 1986.

Although, since the peak in 1995, recent epidemiological estimates have shown a steep decline in AIDS mortality, HIV has caused one of the most devastating epidemics since 1981(5). Despite accumulated knowledge of the disease, the HIV pandemic continues unabated. World Health Organization (WHO) estimated that 33.2 million individuals are living with HIV, 2.5 million are newly infected, primarily through sexual transmission, and 2.1 million died from AIDS in 2007(5). Of these 33.2 million people living with HIV, approximately 65% live in Sub-Saharan Africa (5). In addition, of these 2.5 million new infections, approximately 1.9 million occurred in Sub-Saharan Africa (5). In the countries of South and South-East Asia, 5.0 million are HIV positive (5).

Eastern Europe and other areas of Asia are next area of great concern because of the absence of effective control programs (5).

Origins and Structure of HIV/ SIV

HIV genome is composed of three major genes (gag, pol and env), two regulatory genes (tat and rev) and four accessory genes (*nef, vpr, vpu,* and *vif)* (Figure 1A). The *gag* gene encodes precursor polyproteins, resulting in the formation of the viral particles: the matrix protein, the capsid protein, and the nucleocapsid protein (6). The *pol* gene encodes the viral enzymes including reverse transcriptase, integrase, and protease. Together gag and pol proteins form the mature, infectious viral particles (Figure 1B). The *env* gene encodes precursor proteins for the viral envelope proteins such as gp120 and gp41 spike proteins, which interact with CD4 receptors and specific co-receptors in target cells (7).

The *tat* gene encodes small protein that is essential for efficient transcription of viral genes and for viral replication in infected host cells (8). The *rev* gene encodes the phosphoprotein expressed in the early stage of HIV-1 replication (9, 10). This protein functions to increase stability and translation of HIV RNA (11, 12).

Four accessory genes produce accessory proteins that affect the course and severity of viral infection, resulting in promotion of viral life cycle and disease progression (13, 14). The *nef* gene encodes the nef protein that is essential for viral replication and development of disease in HIV-1 infected patients (15). The vpu gene encodes the vpu protein that functions not only to downregulate CD4 in host cells, but also to efficiently release viral particles from infected cells (16). The vpr gene encodes a small protein, which functions to promote HIV-1 pathogenesis (17), but its specific

function has not been clarified. The *vif* gene encodes the vif protein that is essential for efficient viral replication and neutralizing host cell antiviral protective proteins such as ABOBEC3G (18).

The envelope of mature HIV-1viral particles is composed of a lipid bilayer (Figure 1B). Underlying this lipid envelope, matrix surrounds the inner structure which contains ribonucleoprotein (nucleocapsid + diploid RNA genome) as well as viral enzymes (integrase, reverse transcriptase, and protease proteins) all surrounded by capsid (Figure 1B).

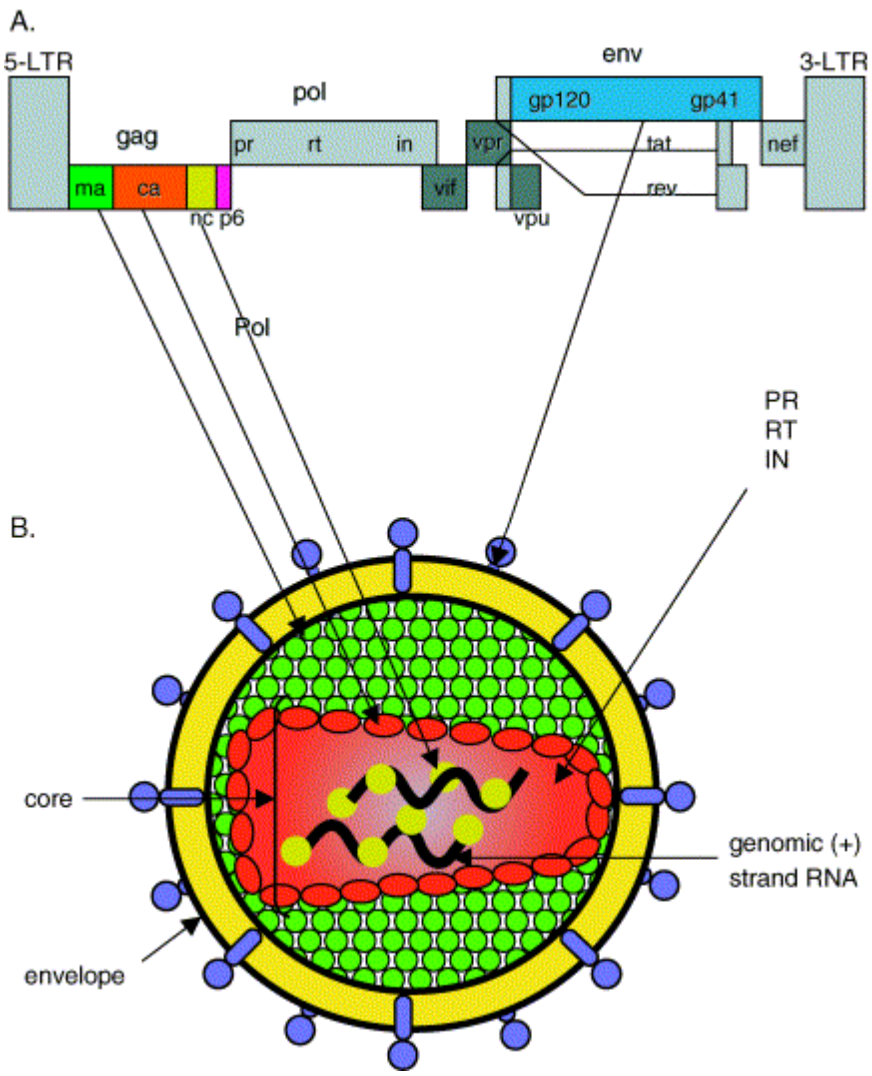

Figure 1. HIV genome and Structure of the HIV. (A) HIV-1 proviral genome. (B) Structure of the HIV-1 mature viral particle. capsid (ca), the matrix (ma), nucleocapsid (nc),reverse transcriptase (rt), integrase (in), protease (pr), transactivator of transcription (tat), regulator of expression of the virion (rev), viral infectivity factor (vif), viral protein U (vpu), viral protein R (vpr), negative effector (nef) and and long terminal repeats (LTR), which play a role in the initiation of transcription. Figure adapted from (6).

Like HIV, simian immunodeficiency virus (SIV) belongs to the genus of lentivirus and the retrovirus family. Lentivirus is considered a “slow virus” because the virus takes a long time to produce symptoms of disease in a number of animals (19). There are two main types of HIV: HIV-1 and HIV-2. It is believed that the strains of HIV-1 originated from SIV found in chimpanzees and can be classified into major groups M (responsible for more than 90% of HIV infections worldwide), N (extremely rare), and O (found only in West-Central Africa) (20-22). The strain HIV-2 is much rarer as well as less pathogenic in humans than HIV-1 (22). HIV-2 originated from SIV found in sooty mangabeys and is restricted to West Africa (22).

SIV is closely related to HIV in sequence and shows similar pathogenicity in the rhesus macaque animal model (23, 24). SIV infection is asymptomatic in the natural host despite high level of virus. However, if SIV from chimpanzees and sooty mangabeys infects Asian rhesus macaques, they show lymphoma and development of disease similar to AIDS patients (25). The SIV isolated from rhesus macaques, Macaca mulatta, is called SIVmac. SIVmac251 is a pathogenic wild-type viral isolate from a rhesus monkey with malignant lymphoma (26). SIVmac239 is a pathogenic molecular clone derived from SIVmac251 (27). Both SIVmac251 and SIVmac239 are widely used in the field of HIV research and were used in my studies. In summary, rhesus macaques infected with SIV serve as a good animal model for HIV and are now widely used for understanding HIV pathogenesis.

HIV Replication Cycle

The first step in HIV infection is binding of viral envelope glycoproteins to CD4 on the surface of susceptible cells such as $CD4^+$ T cells, macrophages and dendritic cells (28). Following the virions-CD4 binding, a new epitope exposed by gp120 conformational changes interacts with HIV coreceptors, either CCR5 or CXCR4 (29-31). Virion-CD4 interaction is essential for HIV infectivity, and virion-coreceptor interaction is required for membrane fusion and entry (32, 33). After the fusion and entry has taken place, the viral RNA is uncoated in the cytoplasm. This single-stranded HIV RNA is transcribed into double-stranded HIV DNA by use of viral reverse transcriptase. The viral DNA then is inserted into the host cell genome by the viral integrase. This integrated DNA complex is called a provirus (34). Once integrated, the provirus will produce few to no copies of HIV for several years and thus latently infected cells may evade the host adaptive immune system (14, 35). When the provirus is activated, viral RNA or protein can be synthesized by using host cell machinery (14). These viral particles are gathered and assembled beneath the host cell membrane (36). The viral envelope proteins such as gp120 and gp41 are inserted into host cell plasma membrane (36). The assembled virus particles are then wrapped by host cell plasma membrane during the budding (36). Finally, a number of newly assembled viral particles are released from virus-infected host cells. These essential steps in the life cycle of HIV are presented in Figure 2.

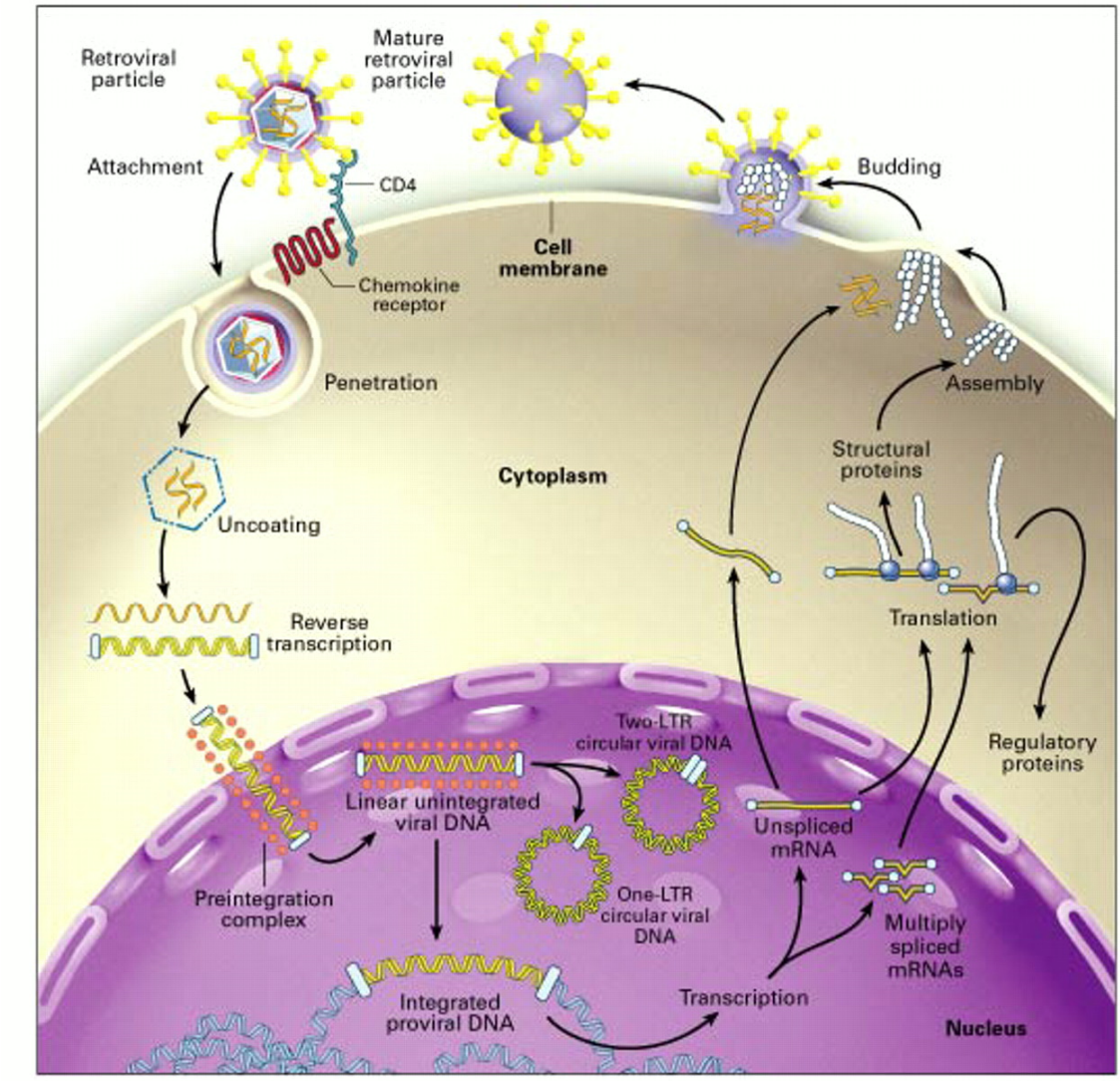

Figure 2. The Essential Steps in the Life Cycle of HIV-1. Figure adapted from (37).

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Current Antiretroviral Drugs for HIV Infection

Recent progress in understanding the viral replication cycle has resulted in the development of antiretroviral therapies for HIV infection. First, inhibiting HIV entry and fusion with the target cell membrane can be done by use of soluble HIV receptors such as drugs that bind to the chemokine receptor CCR5 and by drugs that bind to the HIV viral envelope gp41 (38, 39). Second, anti-retroviral agents that are currently approved inhibit reverse transcriptase, protease and integrase in the viral replication cycle (40). Most inhibitors of reverse transcriptase are nucleoside analogs that lack the 3' hydroxyl moiety needed for DNA chain elongation. They act as competitor with natural deoxynuclotide substrates, thus interfering with viral DNA synthesis (41). In contrast, non-nucleoside reverse transcriptase inhibitors induce conformational change by attaching the enzyme itself, thus inhibiting the catalytic activity of the enzyme (42). Therefore, these inhibitors block viral replication in a different way. Protease inhibitors block HIV Gag proteolytic processing which is necessary for viral infectivity (43). Integrase inhibitors prevent provirus from integrating into the host DNA (44).

To date, highly active antiretroviral therapy (HAART) combinations of antiretroviral regimens have been widely used (19). (19)This therapeutic regimen has significantly decreased HIV/AIDS-related morbidity and mortality (45, 46). Studies have shown that targeting at least three different components of the HIV replication cycle results in plasma viral load suppression, significant increase in CD4 cell count, and decrease in the incidence of HIV-associated infection and diseases (46-49). However, unfortunately, current antiretroviral treatments have readily led to side effects such as multiple neurologic disorders and antiretroviral resistance to these drugs (41, 50).

Failure of HIV vaccines

Since the discovery of HIV in 1983, several types of HIV vaccines have been evaluated. Vaccine developers have tried to design effective vaccines to elicit broadly neutralizing antibodies and/or robust cellular immune responses because studies on the natural history of SIV have provided evidence that these immune factors are closely associated with the control of virus replication in nonhuman primate model (51-53). However, unfortunately, to date all HIV vaccines have failed in clinical trials (54, 55).

Purified recombinant protein of gp120 made by VaxGen Inc. of Brisbane was tried as a vaccine candidate in the United States and Thailand and its failure was announced in 2003. This gp120-based vaccine was expected to generate neutralizing antibodies that correlates with prevention of HIV infection. However, this viral surface-protein vaccine did not induce neutralizing antibodies in the majority of volunteers and thus failed to protect trial participants in two phase III trials (56-59). Another promising approach to developing an HIV vaccine is to investigate vaccine candidates that elicited T cell responses to better control viral replication after HIV infection. The importance of T cell-based vaccine has been suggested by studies in SIV model (55). However, recent failure of an adenovirus type 5(Ad5)-based vaccine designed for strong T cell response was announced in human phase 2b efficacy trials (STEP) (60). This Ad5-based vaccine developed by Merck had elicited cell-mediated immunity against HIV-1 gag, pol and nef protein in phase I trials and thus was considered to be strong vaccine candidate (61). In the STEP trial, the vaccine neither prevented HIV-1 infection nor reduced early plasma virus levels in vaccines compared with the placebo recipients (62). In addition, in the STEP trial, higher virus levels in HIV-infected vaccines were observed compared to

those in infected placebo groups. It is possible that the failure might be due to the pre-existing immunity to adenovirus vector, thus reducing the sufficient breadth of vaccine-induced HIV-specific CD8 T cells (55). However, the reasons for this unexpected observation are still unknown. Interestingly, a recent AIDS vaccine clinical trial performed by the U.S. Military HIV Research Program in collaboration with the Thai Ministry of Public Health was announced on the 24th of September, 2009 (63). They showed the first evidence that a vaccine could offer some protection from HIV infection (63). However, this vaccine had no impact on virus levels in people who reported the highest risk behavior. In addition, they used strains of HIV common in Thailand, thus it is still unclear whether such a vaccine would be effective against other strains.

Live attenuated SIV vaccines in non-human primate models

Infection of rhesus monkeys with SIV has been generally considered the best model for HIV/AIDS in humans. Especially important are rhesus macaques infected by the genital mucosal route which is used as an animal model for the homosexual or heterosexual transmission of HIV because of the similarities in reproductive biology between humans and macaques (64, 65). In this SIV model, to date, the most successful vaccines have been live-attenuated lentiviruses (55, 66). Immunization with these attenuated viruses has provided protection in rhesus macaques challenged with pathogenic SIV. For example, previous studies have shown that attenuation of SIV by deletion of nef sequences from SIVmac239 has proven to be non-pathogenic in rhesus macaques (67, 68). Animals immunized with SIV□nef showed 100% protection from challenge with pathogenic SIV (67). Other live attenuated lentiviruses that provide

protection against pathogenic challenge with SIV are chimeric lentiviruses, namely, simian-human immunodeficiency viruses (SHIVs) which contain human env gene incorporated into SIVmac239 genome (69). In the case of SHIV89.6, SHIV-immunized animals showed 60% protection after vaginal challenge with pathogenic SIV (70, 71). Although these live attenuated lentiviruses are not used in humans due to a number of safety concerns (72), understanding the immune correlates of protection against pathogenic SIV strains in the immunized animals may be useful for developing new vaccine strategies. However, the mechanisms by which attenuated SIV strains provide protection remains poorly understood.

Chapter 2. Immune responses to viruses in mucosal tissues

General Overview of T cells and Dendritic Cells in Viral Infection

Many human and animal viruses initially cross one of a number of available host portals and replicate at sites near the portal of entry including the mucosal surfaces of reproductive tracts (73, 74). Local components of innate immunity non-specifically detect and destroy viruses, but once the viruses evade the numerous early defense mechanisms, development of a virus-specific immune response is triggered by the virus antigens via adaptive immune mechanisms (74). To best understand the immune response to live attenuated SIV vaccination, it is beneficial to have a comprehensive overview of the biology of immune cells involved in HIV/SIV infection. However, due to space constraints, I here concentrate mainly on the biology of dendritic cells (DCs) and T cells and their role in the immune system after viral infection. This is important background for the understanding of virus-specific CD8 T cell response involved in the protection afforded by live attenuated lentivirus vaccines.

Dendritic Cells

Dendritic cells (DCs) were first identified in 1973 (75) as potent professional antigen presenting cells (APCs) that, in the site of infection, encounter viruses and can take up them. There are two main DC subsets are derived from $CD34^+$ hematopoietic stem cells: myeloid DCs, characterized by CD1a, $CD11c^+$, and $CD33^+$ expression (76), and plasmacytoid DCs (pDCs), characterized by $CD123^+$ and $CD304^+$ (76, 77). Myeloid

DCs are located in most tissues including the entry site of pathogens (78), while plasmacytoid DCs are predominantly found in the peripheral blood and lymphoid tissues in very low numbers (79). However, the origin of different DC populations is still not completely understood. During viral infection, immature DCs take up viral antigen by phagocytosis, receptor-mediated endocytosis and macropinocytosis in the peripheral tissues. After taking up antigens, immature DCs differentiate into mature DCs and migrate to the draining lymph node or mucosal associated lymphoid tissues (80). Mature DCs up-regulate the expression of CCR7 on their cell surfaces and enter T cell-rich areas in lymph tissues where they present processed peptide to both naive $CD4^+$ and $CD8^+$ T cells to generate effector cells by direct cell-cell contact (76). Thus, DCs as antigen presenting cell are central to the development of adaptive immunity to invading pathogens. However, DCs could also be exploited by some viruses such as HIV to facilitate the spread of virus. During HIV infection, DCs can contribute to the spread of HIV infection by binding directly to virions through interaction with CCR5 and DC-SIGN on DC surfaces and transporting infectious virions from sites of infection to secondary lymphoid organs (81, 82).

T cell Recognition of antigen bound to MHC proteins

Both cytotoxic T cells and helper T cells are initially activated in response to viral infection by recognizing peptide antigens in association with major histocompatibility complex (MHC) class I and II molecules, respectively. In both cases, two signals are required for the activation of T cells; T cell antigen receptor complex (TCR/CD3) interacts with the peptide-MHC complexes expressed on the surface of an antigen-

presenting cell or virus-infected cells (First signal) (83). Interaction between co-stimulatory molecules on the surface of antigen presenting cells and T cells is the second signal. Once T cells become fully activated, MHC Class I-restricted $CD8^+$ T cells are generally involved in the cellular immunity such as elimination of virus-infected cells, while MHC class II-restricted $CD4^+$ T cells are involved in the induction and maintenance of the adaptive immune response such as the production of antibodies and the activation of CD8 T cells (84-86). There are two distinct pathways in which viral peptides are processed for display to T cells. First, in the MHC class I antigen processing pathway, intracellular microbes including virus can be degraded by proteasome. After this proteasomal breakdown, the peptides diffuse to the transporter associated with antigen processing (TAP) transporter at the endoplasmic reticulum (ER) membrane, where they are loaded onto MHC class I molecules. TAP-associated glycoprotein (Tapasin) presented in the lumen of ER acts to not only link the MHC class I-chaperon complex to TAP, but also facilitates binding of TAP-transported peptides to the MHC class I molecule (87). After peptide loading, an aminopeptidase (ERAP) trims peptides down 8 to 10 amino acid in length. Finally, the assembled MHC class I molecules dissociate from TAP and are transported via Golgi apparatus to the cell surface (88), where they can be presented to cytotoxic T-lymphocytes.

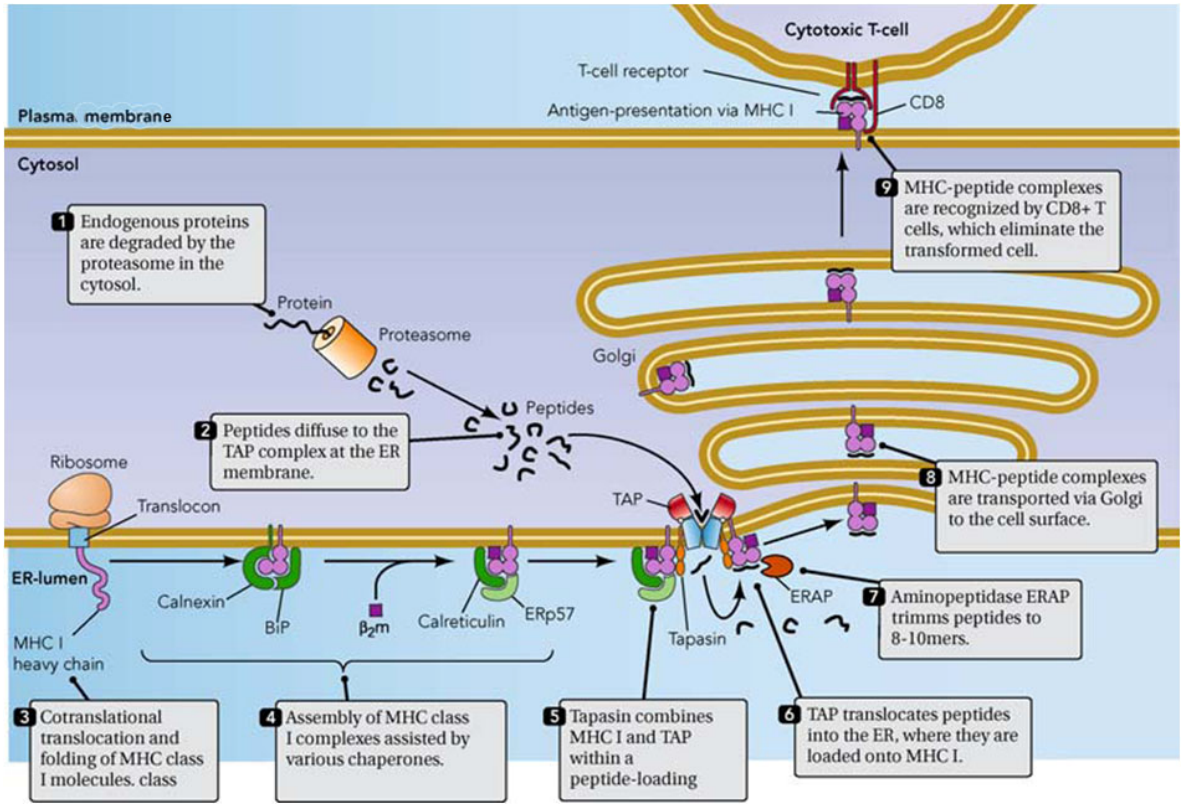


Figure 3. MHC class I antigen processing pathway. Figure adapted from (89).

Second, in the MHC class II antigen processing pathway, pathogens or exogenous proteins can be internalized by antigen presenting cells via endocytosis such as pinocytosis and phagocytosis. After uptake, antigens are degraded into oligo-peptides of 13 to18 amino acids within endocytic compartments, where they then bind to class II MHC molecules. This assembly of peptide with class II MHC molecules is exocytosed to the cell surface, where they can be recognized by $CD4^+$ T cells (90, 91).

$CD4^+$ T Cells

$CD4^+$ T cells play a key role in the development and maintenance of cellular and humoral immune responses (92). In the lymph nodes as well as mucosal sites, production of interleukin-12 (IL-12) by DCs or other antigen presenting cells, or the production of IL-4 by an unknown source, or the production of TGF-β and IL-6 by T-cells and macrophages induce naive CD4 T cells to become T helper type 1 (Th1), T helper type 2 (Th2), and T helper type 17 (Th17) cells, respectively (92-94). Th1 cells produce cytokines including IL-2, TNF-α and interferon- γ (INF-γ), resulting in the activation and proliferation of $CD8^+$ T cells (92, 93). Th2 cells secrete cytokines such as IL-4, IL-5, IL-6, IL-10 and IL-13 which promote B cell-dependent humoral immunity (92, 93). Th17 cells produce IL-17 and IL-22 that are believed to regulate inflammatory responses due to the extracellular bacterial infection as well as autoimmune disease (95-97). Although it is commonly accepted helper T cell function, there is also evidence that some populations of $CD4^+$ T cells can directly kill the virus-infected cells, independent of $CD8^+$ T cells and B cells (98). However, whether these cytolytic $CD4^+$ T cells can be novel target for effective therapy against viral infection remains to be resolved. In addition to the enhancement of the immune response, some $CD4^+$ T cells called regulatory T cells suppress T cell effector responses (99).

$CD8^+$ T Cells

After viral infection, the immune system develops a virus-specific response that is mediated by cytotoxic $CD8^+$ T cells which leads to the clearance of viruses. The primary expansion and memory response of $CD8^+$ T cells requires $CD4^+$ T cell help (100, 101).

However, in some viruses including choriomeningitis virus, herpes simplex virus-1, and sendai virus, cytotoxic $CD8^+$ T cells response is independent of $CD4^+$ T cell help (102-104). $CD8^+$ T cells suppress viral replication by two main mechanisms inducing apoptosis: I) Granule dependent cytotoxic mechanism, II) death-receptor dependent cytotoxic mechanism (105). Of these, the first mechanism involves lytic molecules such as perforin and granzymes accessing virus-infected target cells through an immunological synapse (105, 106). By this mechanism, cytotoxic $CD8^+$ T cells directly kill virus-infected cells by releasing perforin which forms pores on the membrane of target cells. This granule-dependent cytotoxicity mechanism is further described below. In the second mechanism, Fas (CD95), tumor necrosis factor or TRAIL interact with their ligands expressed on cytotoxic $CD8^+$ T cells, leading to the intracellular signalization of the apoptotic process in infected cells (105, 107). In addition, cytotoxic $CD8^+$ T cells can produce antiviral cytokine INF-γ. This cytokine not only induces the increase in the expression of class I MHC molecules on infected cells (108, 109), but also inhibit viral replication (110, 111) and thus enhance the efficiency of CTL-mediated cytotoxicity of these cells. This cytokine also stimulate the isotype shift to a specific subclass of IgG in viral infection (112).

$CD8^+$ T cells also suppress retrovirus replication in non-MHC restricted and non-cytotoxic manners (113, 114). The $CD8^+$ cell non-cytotoxic antiviral response (CNAR) has been shown to suppress HIV-1(115), HIV-2(116) and SIV replication (117). CNAR is mediated by soluble antiviral factors (118), such as the $CD8^+$ cell antiviral factor (CAF) that can inhibit HIV transcription within the infected cells (119, 120). Some studies reported other natural anti-HIV factors including β chemokines (121) and stromal

cell-derived factor-1 (SDF-1) (122). These factors bind to the co-receptors that virus use and thus block the virus entry into cells.

Role of Cytolytic Enzymes

Lytic enzymes include perforin and several granule-associated proteases, such as the granzymes. In vitro, perforin is found in cytotoxic T cells granules and released by exocytosis (123). This enzyme forms pores around 15 to 16 nm in the membranes of target cells after $CD8^+$ T cell-target cell recognition (124, 125). Studies in humans and mouse models involving defects in granule killing have also provided critical clues to the important role of perforin. Perforin-deficient mice displayed spontaneous lymphoma (126) and defects in the human perforin gene mediate familial hematophagocytic lymphoshitiocytosis and involve diminution of immune responses to infections by intracellular pathogens (127). Thus, perforin secretion from the lytic granules has an essential role in inducing target cell death.

Other components of the lytic granules have been identified as serine proteases: granzymes A, B, H, K and M. Following the secretion of cytotoxic granules, these granzymes enter the target cells through membrane pores formed by perforin (128, 129) and trigger cell-death by either a caspase-dependent or a caspase-independent pathway (105, 130). Granzyme B induces cell death by caspase cleavage (131), whereas other granzymes induce caspase-independent cell death (132, 133). These granzymes can be internalized and detected together with perforin in the cytoplasm of the target cells (105). However, whether only perforin-mediated pores are necessary for the entrance of granzymes is still unclear. This original hypothesis has been challenged by a recent

alternative hypothesis, suggesting granzymes can be endocytosed into target cells by perforin-independent pathway (134). Despite inconsistent findings, the original hypothesis that perforin is critical for delivery of granzymes still remains a feasible model.

General Overview of the Structure of mucosa and associated immune system

The mucosal surfaces can be divided into two types based on function and anatomical features. Type I mucosal surfaces include the intestine, lung, and upper female reproductive tracts are covered by simple epithelium. The main role of type I mucosa is to provide physiological functions such as absorption and respiration. Type II mucosal surfaces represent those of oral, esophageal and lower female reproductive tracts which are covered by stratified squamous epithelia. These surfaces function as physiological barriers in the body (135).

The mucosal immune system can be also divided into two parts, the inductive site and the effector site. Inductive sites in the mucosa are mucosal associated lymphoid tissues where the mucosal immune response can be initiated (Figure 4). The inductive sites of mucosal-associated lymphoid tissues in the gut include the Peyer's patches in the ileum and the appendix (136). Inductive sites in the respiratory tract include the palatine tonsil, bronchus-associated lymphoid tissue, and other lymphoepithelial structures of Waldeyer's pharyngeal ring in human nasopharynx-associated lymphoid tissue (137). Inductive sites are dome shaped, are covered by specialized epithelial cells, and contain B cell follicles and other immune cells (Figure 4). Mucosal antigens are preferentially taken up at the inductive sites by the follicle-associated epithelium which contains specialized

M (membrane) cells. M cells are thin and bell-shaped cells that non-specifically transport live and dead luminal particles (138) and microbial antigens by pattern recognition receptor-mediated uptake (139). Professional antigen-presenting cells, including DCs, B cells, macrophages, as well as antigen-retaining follicular DCs within B cell follicle are localized in inductive sites (137, 140). Luminal peptides are presented by professional mucosal antigen-presenting cells to subsets of naïve intraepithelial and subepithelial T cells that enter mucosal inductive site via high endothelial venules (137, 141). In B cell follicles of inductive sites, B cells can differentiate and undergo antibody class switch from expressing IgM or IgD to expressing other isotypes, predominantly IgA within the mucosa.

Mucosal effector sites are areas such as the lamina propria where activated immune cells migrate to and wait to encounter specific antigens such as viruses (Figure 4). After being primed in mucosal inductive sites or local lymph nodes, B and T cells migrate to mucosal effector sites such as lamina propria of the respiratory tract, intestinal tract, and other mucosa tissues via lymphatic vessel and peripheral blood (73, 142). Lamina propria and the epithelial compartment principally form effector sites which include B lymphocytes, J chain-expressing IgA and IgM plasma cells, IgG plasma cells with a variable –chain level, $CD8^+$ T cells and $CD4^+$ T cells. In addition, intraepithelial lymphocytes, which are mainly $\alpha+/\beta+$ $CD8^+$ T cells, are located near the lumen in lamina propria (142). In these sites, secretory IgA, secretory IgM and serum-derived IgG antibodies are generated and enter the mucosal lumen. Epithelium on type I mucosa contain polymeric Ig receptor, and transport IgA to the mucosal lumen. In contrast, type II epithelium does not transport IgA due to lack of polymeric Ig receptor (135).

However, these immune responses are not completely separated between inductive and effector sites, because microbial peptide can initially induce immune response in lamina propria of effector sites. For example, viruses can be sampled by lamina propria DCs, which present antigens to naïve T cells in draining lymph nodes (135, 143) (Figure 4).

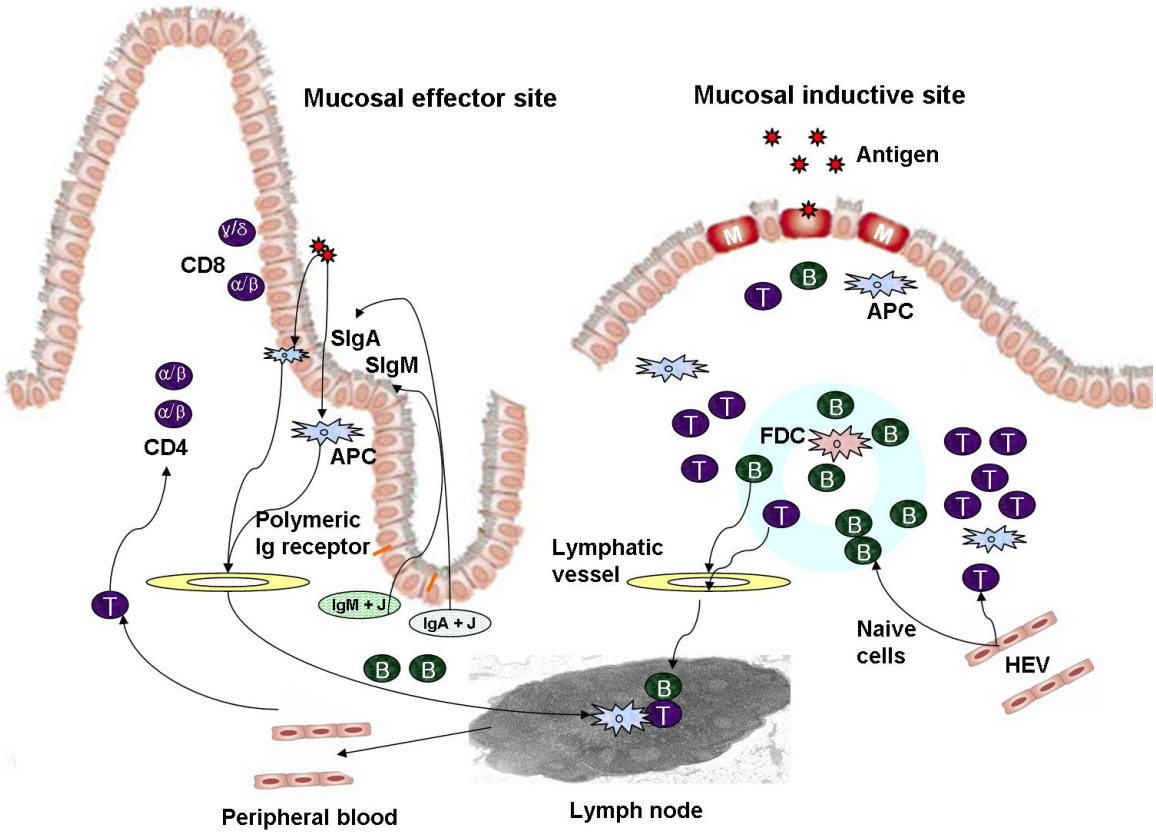


Figure 4. Schematic overview of Inductive and Effector Sites in Mucosa. Luminal antigens are transported through M cells to the professional antigen-presenting cells (APCs), including dendritic cells located under the epithelium. In addition, intra- or subepithelial dendritic cells in mucosal-associated lymphoid tissues and lamina propria can capture antigens and migrate via lymphatic vessels to draining lymph nodes where they become mature dendritic cells, which stimulate naïve T cells. Naïve B and T cells

enter inductive site via high endothelial venules (HEVs). They become effector B and T cells in the inductive sites and draining lymph nodes and then migrate to mucosal effector sites via thoracic duct and peripheral blood. Finally, the effector sites such as mucosal lamina propria have a number of immune cells, including plasma cells secreting J chain-expressing IgA and IgM (IgA+J and IgM+J), $CD4^+$ T cells, and $CD8^+$ T cells. Secretory IgA and IgM are generated by binding polymeric IgR and then release into the lumen.

Vaginal transmission of HIV/SIV

Many human and animal viruses initially cross one of a number of available host portals and replicate at sites near the portal of entry. Once inside the host, the mucosal surfaces of the gastrointestinal, respiratory, and reproductive tracts provide significant barriers to local or systemic infection. In these sites, local components of innate immunity non-specifically detect and destroy viruses, but once the viruses evade the numerous early defense mechanisms, development of a virus-specific immune response is triggered by the virus antigens via adaptive immune mechanisms. In the case of HIV, these mucosal surfaces are the most common natural route of infection and may be target for HIV vaccine development. In North America and Europe where the majority of patients are infected through homosexual contact, the rectal mucosa is a major portal for HIV entry (144). However, worldwide, most HIV infection occurs through heterosexual transmission and vaginal intercourse is the predominant mode of HIV transmission (145). Of mucosal sites, the reproductive genital tract may act as a barrier to HIV infection and its disruption dramatically increase the access of HIV into the submucosa, where susceptible target cells are more concentrated. This concept is supported by studies in

rhesus macaques, which show that thin epithelial layer of genital tract is more susceptible site to SIV infection after the treatment of progesterone (146, 147).

The epithelium and lamina propria of reproductive genital tract in human or rhesus macaques show immune cells including Langerhans cells, macrophages, $CD4^+$ and $CD8^+$ T cells (76, 148). Although the exact mechanism of HIV transmission through DCs remains to be established, recent evidence indicates that conjugates of Langerhan's cells and $CD4^+$ T cells are found in the lamina propria, suggesting the role of DCs in transfer of HIV to T cells in genital mucosa (149). Langerhans cells are a subset of DCs found in Type II mucosal surfaces. In the vaginal epithelial, Langerhans cells can be infected with HIV similar to $CD4^+$ T cells (150, 151). However, these recent studies also show that Langerhans cells are not main target cells for HIV trans-infection in mucosal site. Most HIV-infected cells are $CD4^+$ T cells that reside in lymphoid tissues (145).

Many studies using SIV infected animals provide insights about cellular immune mechanisms associated with control of viral replication in the genital tract. In the case of $CD8^+$ T cells, this cell population is mainly found in deep layers of the vaginal epithelium and lamina propria (148). During acute and chronic SIV infections, SIV-specific $CD8^+$ T cells are found predominantly in the vaginal tissues (152, 153), which is consistent with human study showing the presence of these cells in the cervical tissues of HIV-1-infected women (154). However, $CD8^+$ T-cell responses are not detectable until 14 days post-challenge, when infection has already spread systemically (153). Therefore, I suspect that an early mucosal memory T-cell response induced by an effective vaccine contains viral replication at the portal of entry and prevents systemic infection or, if the virus spreads systemically, improves control and elimination of HIV infected cells.

Chapter 3: Early localized SIV-specific CD8 T cells response to pathogenic SIV is correlated with successful SIV vaccine

Introduction and Rationale

With over 33 million people infected with human immunodeficiency virus (HIV) worldwide and over 2 million new infections occurring each year (5), there is a critical need to develop an effective HIV vaccine. However, there is currently no effective vaccine that provides immune control against HIV infection (55). To date, the most successful SIV vaccines have been live-attenuated lentiviruses (55, 66). For example, rhesus macaques immunized with live-attenuated non-pathogenic simian human immunodeficiency virus (SHIV) are largely protected from intravaginal challenge with highly pathogenic simian immunodeficiency virus (SIV) (70, 71). Another example is that immunization with live-attenuated SIVΔnef not only diminishes virus replication in most rhesus macaques, but also substantially provide protection against a subsequent challenge with pathogenic SIV such as the molecular clone SIVmac239 and viral isolate SIVmac251(67, 155, 156). Although such non-human primate models for AIDS vaccine have contributed to understanding immune responses against pathogenic SIV and aid in the development of HIV vaccines, the mechanisms by which attenuated SIV strains provide protection are poorly understood.

Although passive administration of broadly neutralizing monoclonal antibodies successfully prevented infection (51), many vaccine strategies aimed at inducing HIV or SIV-specific antibodies have largely failed to induce broad neutralization activity because

of the vast diversity of the viral envelope (157, 158). Moreover, recent studies have provided evidence that CD8 T cell responses are responsible for much of the reduction in virus load after vaccination (55, 159). Wilson et al. (160) showed that DNA vaccine-induced cellular immune responses, in the absence of neutralizing antibodies, can control replication of a highly pathogenic SIVmac239 to a significant degree, suggesting that virus-specific CD8 T cell responses are more important mediators of this protection. Vaccine studies are therefore currently focused on inducing potent cellular immune response, which is dependent on virus-specific T cells.

There is a lot of evidence that virus-specific CD8 T cells clearly play a central role in suppressing HIV and SIV replication. Many studies have shown the appearance of SIV and HIV-specific CD8 T cell responses appear concurrently with the reduction in peak viremia during the acute stages of infection (161-163). Transient depletion of CD8 T cells during primary SIV infection in macaques showed a dramatical increase in viral loads (164, 165). Patients with high levels of functional HIV-specific CD8 T cells often showed slower progression of disease than those with low levels (166, 167). In addition, ex vivo expansion of HIV-specific CD8 T cell clones followed by re-administration of these cells to HIV-1 patients led to a temporary decrease in HIV-1 viral load (168). However, despite the clear importance of CD8 T cells in controlling HIV and SIV infections, CD8 T cells are unable to fully contain and clear virus-infected cells during HIV and pathogenic SIV infections. In our previous study, we demonstrated that, in SIV-infected macaques, a robust virus-specific CD8 T cell response was not seen until around 14 days post infection, whereas productive systemic infection was already observed in lymph tissues at 6 days post-infection (153, 169). The temporal gap between productive

systemic infection and emergence of virus-specific CD8 T cell response suggests that this cellular response may occur too late in lymph tissues as well as genital tract to control the initial spread of viremia. These findings suggest that successful SIV and HIV vaccines need the induction of a robust, early virus-specific CD8 T cell response in secondary lymph nodes and genital mucosal tissues.

In order to directly visualize antigen-specific T cells in situ, the staining method in tissue sections with MHC class I tetramers has been developed (170, 171). Combining in situ tetramer staining with immunohistochemistry allows researchers to determine the localization of antigen-specific CD8 T cells in specific tissue compartments, to determine the relationship of antigen-specific T cells to other cells, and to correlate the phenotype of antigen-specific T cells to specific tissue locations (Figure 5). In previous studies, we used in situ tetramer staining to characterize virus-specific T cells in tissues from primates (153, 172), and humans (173).

Here, I hypothesize that protection provided by live attenuated SIV immunization requires the induction of an early virus-specific CD8 T cell response to pathogenic SIV in secondary lymphoid organs and the portal of viral entry. To test this hypothesis, I used the experimental approach of in situ tetramer staining combined with immunohistochemistry in order to detect and determine the phenotype of SIV-specific CD8 T cells in vaccinated and non-vaccinated animals. In this study, using in situ tetramer staining, I examined the time and magnitude of SIV-specific T cell responses from rhesus macaques that were intravenously vaccinated with live attenuated SIVs and intra-vaginally challenged with highly pathogenic SIVs.

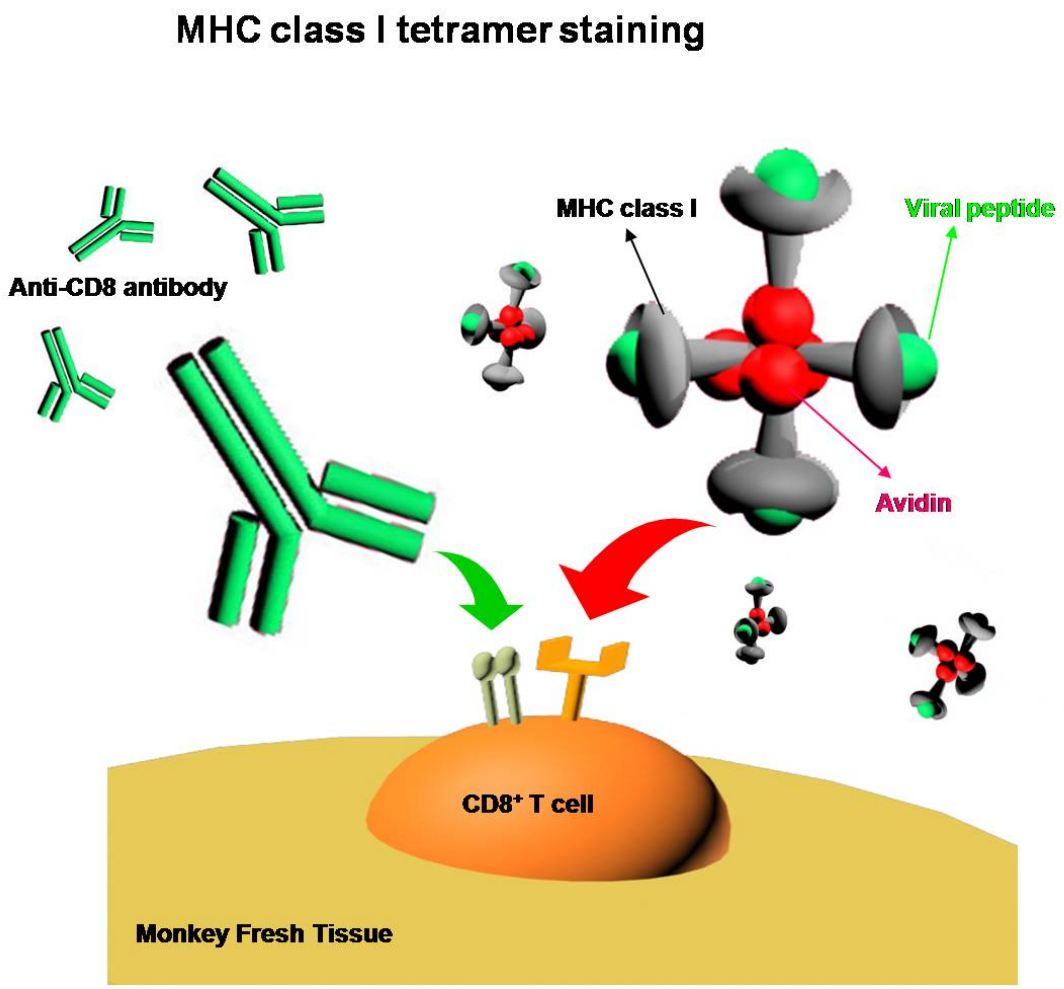


Figure 5: The detection of virus-specific CD8$^+$ cells in fresh tissue sections using MHC class I tetramers and anti-CD8 antibodies. MHC class I tetramers consist of 4 biotinylated MHC-class I molecules that are attached to a fluorescently labeled avidin molecule. MHC-molecules contain a particular viral epitope such as SIV gag, tat, and/or an irrelevant peptide.

Thus, tetramers can bind to T cell receptors that recognize specific viral epitopes. Sections are counterstained with CD8 or other antibodies.

Materials and Methods

Animals, live attenuated SIV immunization, and pathogenic SIVchallenge

For SHIV89.6 live vaccine study (Figure 6), female rhesus macaques (*Macaca mulatta*) were housed at the California National Primate Research Center in accordance with the American Association for Accreditation of Laboratory Animal Care standards. Twelve Mamu-A*01 rhesus macaques were intravenously inoculated with SHIV89.6 live vaccine, and 6 to 12 months later, intra-vaginally challenged with SIVmac239 and sacrificed post-SHIV89.6 inoculation and various time points post-challenge as described (174-176). Freshly dissected tissues were shipped on ice in RPMI containing 100 μg/ml heparin over night from the National Primate Research Center in California to the Skinner lab in Minnesota. As controls, four rhesus macaques were not vaccinated and intra-vaginally inoculated with SIVmac239 and sacrificed from 3 to 14 days post-infection. For the SIVΔnef live vaccine study (Figure 7), adult female rhesus macaques (*Macaca mulatta*) were maintained at the New England Primate Research Center in accordance with the regulations of the American Association of Accreditation of Laboratory Animal Care standards. Six Mamu-A*01 rhesus macaques first immunized intravenously to SIVΔnef and subsequently challenged intra-vaginally with SIVmac251 and sacrificed post- SIVΔnef inoculation and various time points post-challenge. Freshly dissected tissues were shipped on ice in RPMI containing 100 μg/ml heparin over night from the New England Primate Research Center to the Skinner lab in Minnesota. All experiments were approved by local institutional animal use and care as well as bio-safety review boards.

In situ tetramer staining

In situ tetramer and immunohistochemistry were performed on the tissues essentially as described previously (171, 177). In brief, fresh tissues were shipped on ice in RPMI tissue culture media containing 100mg/ml heparin to our lab overnight. Immediately upon arrival, the tissues were cut into 200 micron thick sections using a vibratome and put in a tissue chamber set in a 24-well tissue culture plates containing 1ml of cold phosphate buffered saline containing 100mg/ml heparin (PBS-H). Tetramers were generated by adding FITC-labeled ExtraAvidin (Sigma Aldrich) to biotinylated-MHC Class I monomers loaded with immunodominant SIV peptides over the course of 8 hours to a final molar ratio of 4.5:1. Tetramers were incubated with fresh tissue sections at a concentration of 0.5ug/ml with 2% normal goat serum (NGS). Based on the haplotype of the monkeys, we used tetramers including Mamu A*01 SIV gag CM9 (CTPYDINQM) and Mamu A*01 SIV tat SL8 (STPESANL). At the same time, purified mouse-anti-human CD8 (DAKO), at a concentration of 1:200, was added to a subset of the sections. All sections were incubated at 4°C overnight. Sections were then washed in chilled PBS-H, fixed with 4% paraformaldehyde for 2 hours at room temperature, and incubated with rabbit-anti-FITC antibodies and purified rat-anti human CD3 (Serotec) for the counterstaining in PBS-H with 2% NGS. Antibodies specific for these epitopes were then added along with rabbit-anti-FITC antibody in 2% NGS, and incubated at 4°C for 1 to 3 days. Sections were washed three times with PBS-H for at least 20 min and then incubated with Cy3-conjugated goat anti-rabbit antibodies and Alexa 488-conjugated

goat anti-mouse antibodies, both diluted 1:5000 and 1:2000, respectively in PBS-H with 2% NGS, for 1 to 3 days. On the final day, the sections were washed three times for at least 20 min in PBS-H, post-fixed with 4% paraformaldehyde to secure the tetramer and antibodies in place for subsequent in situ hybridization, and then mounted on slides with glycerol gelatin containing 4 mg/ml n-propyl galate, fluorophore preservative. Mamu A*01 flp (FLPSDYFPSV) tetramers and isotype antibody (mouse IgG) or no antibody were used as negative controls. Stained sections were analyzed using a Bio-Rad 1000, Bio-Rad1024, and Olympus Fluoview1000 confocal microscopes. Digital images were collected and analyzed using Confocal Assistant version 4.02 and Adobe Photoshop 7.01.

Quantification of tetramer-binding cells

Confocal images of tissue sections stained with MHC-tetramers and CD8 antibodies were collected using a 20X objective. At least three and up to nine (when available) fields were collected for each tissue. Digital images were analyzed using Olympus Fluoviewer (FV10-ASW 1.7 viewer), confocal assistant (version 4.02) and image J software (version 1.37) and tetramer-binding cells were manually counted in each field to determine the concentration of tetramer-binding cells (cells/mm^2).

Results

In order to gain understanding of antiviral effector T cells response associated with live attenuated lentivirus immunization and to identify correlates of protective immunity provided by live attenuated lentivirus immunization, we examined rhesus macaques that were vaccinated with attenuated lentiviruses and challenged with highly pathogenic SIVs and here I report our findings concerning correlates of protection from pathogenic simian immunodeficiency virus (SIV) vaginal challenge (174, 175). I focus on my contribution to these studies which included the determination of the magnitude and localization of SIV-specific CD8 T cells in situ in tissues.

In this study, I localized and quantified SIV-specific T cells in situ in lymph node, spleen, vagina, and cervix tissues from Mamu A*01 rhesus macaques that were vaccinated with attenuated lentiviruses SHIV89.6 and SIVΔnef and challenged with highly pathogenic SIVmac239 and SIVmac251, respectively, and sacrificed at various time points (Figures 6 and 7). As a control, I examined tissues from animals that were not vaccinated and infected with SIVmac239 and sacrificed at 3, 7, and 14 days post-infection (Figure 6).

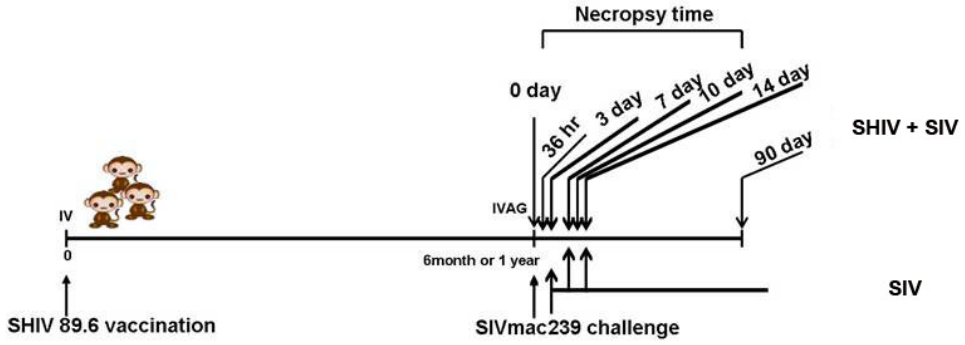


Figure 6: Timeline for SHIV89.6 vaccination and SIVmac239 challenge. Female rhesus macaques were vaccinated intravenously with attenuated SHIV89.6 and subsequently challenged intravaginally at 6 months or one year post-SHIV89.6 inoculation with pathogenic SIVmac239. Animals were sacrificed at 0. 1.5, 3, 7, 10, and 14 days points post-challenge. As a control, a set of macaques were not vaccinated and infected with SIVmac239.

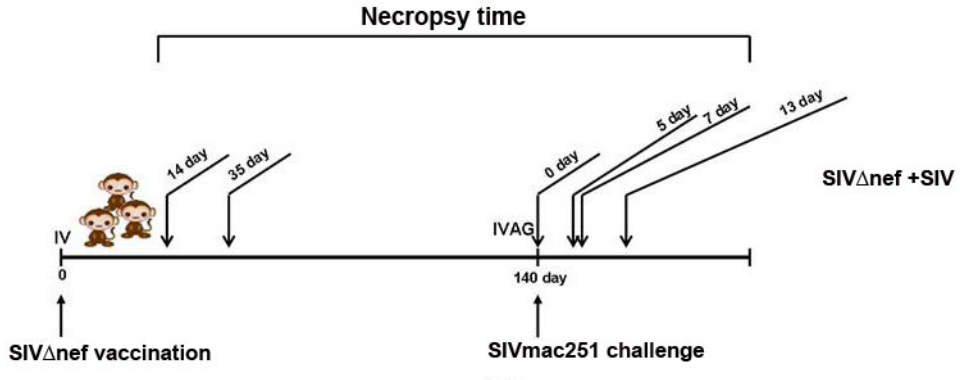


Figure 7: Timeline for SIVΔ*nef* vaccination and SIVmac251 challenge. Female rhesus macaques were vaccinated intravenously with attenuated SIVΔnef and subsequently challenged intravaginally with pathogenic SIVmac251. Animals were sacrificed at 14, 35, and 140 days post- SIVΔnef inoculation and at 5, 7, and 13 days post-challenge.

Mamu A*01/gag and Mamu A*01/tat tetramer-binding cells were evaluated in this study because we have previously demonstrated that we can successfully visualize these cells in situ, and because 70% of the CD8 T cell response is directed to gag and tat in MamuA*01 animals (172). Representative images of tetramer-binding cells in each tissue and each time point for macaques that were vaccinated and challenged are shown in Figure 8. The concentration of Mamu A*01/gag and Mamu A*01/tat tetramer-binding cells in tissues are presented in Figure 9.

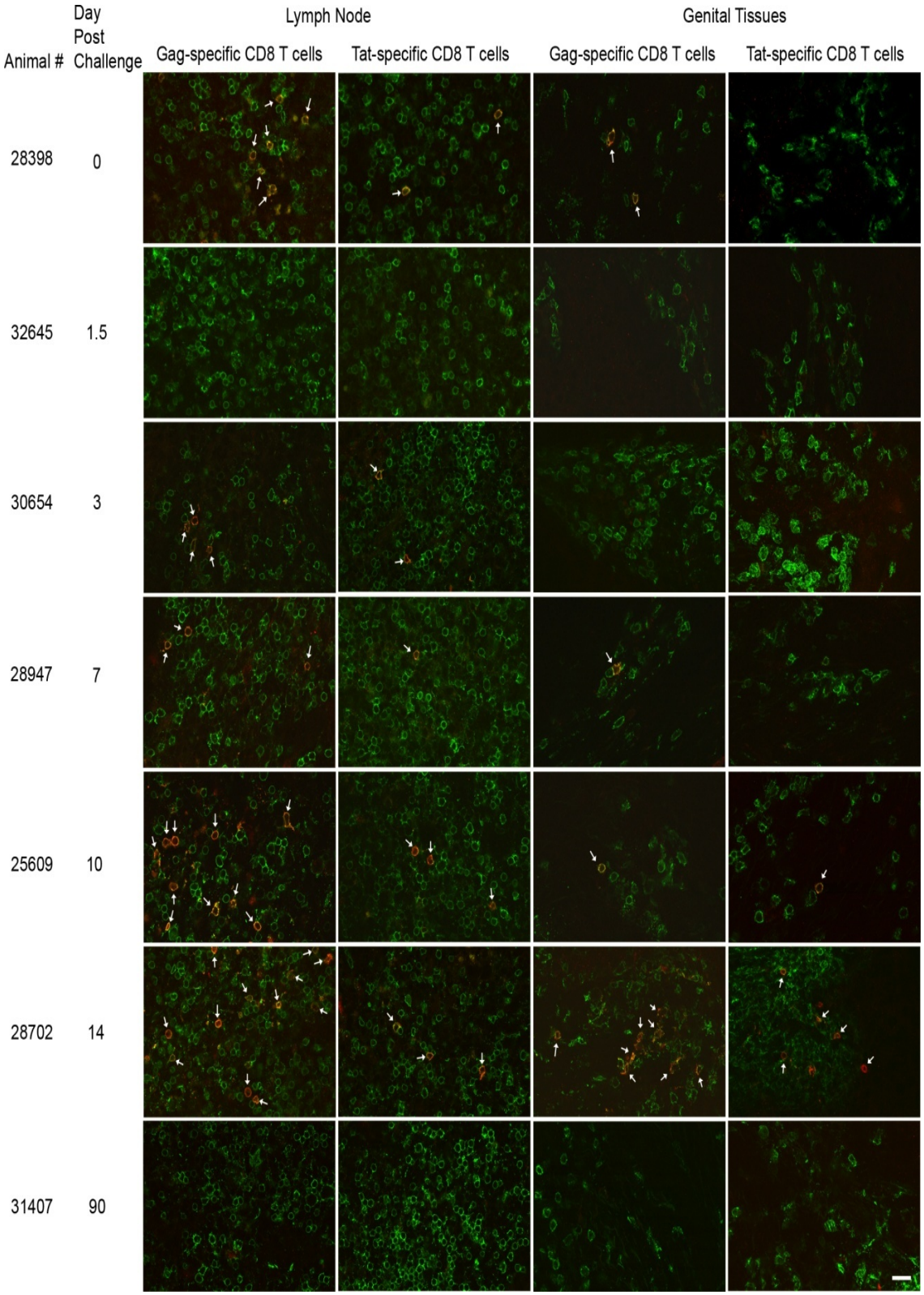

Day Post Challenge
Lymph Node
Genital Tissues
Animal #
Gag-specific CD8 T cells
Tat-specific CD8 T cells
Gag-specific CD8 T cells
Tat-specific CD8 T cells
28398
0
32645
1.5
30654
3
28947
7
25609
10
28702
14
31407
90

Figure 8. SIV-specific CD8 T cells are present in lymph nodes and Genital tissues of SHIV89.6 vaccinated rhesus macaques at early time points post-SIV challenge. Representative images of Mamu-A*01 Gag181-189 CM9 and Mamu-A*01Tat28-35 SL8 tetramer-binding cells (red) and CD8 antibody binding cells (green) from lymph nodes and the vagina of SHIV 89.6-vaccinated-SIVmac239-challenged rhesus macaques, collected at days 1, 1.5, 3, 7, 10, 14, and 90, as indicated. Tetramer-binding cells are indicated by white arrows. All images are confocal Z-scans collected using a 60X objective. Scale bar = 20 microns.

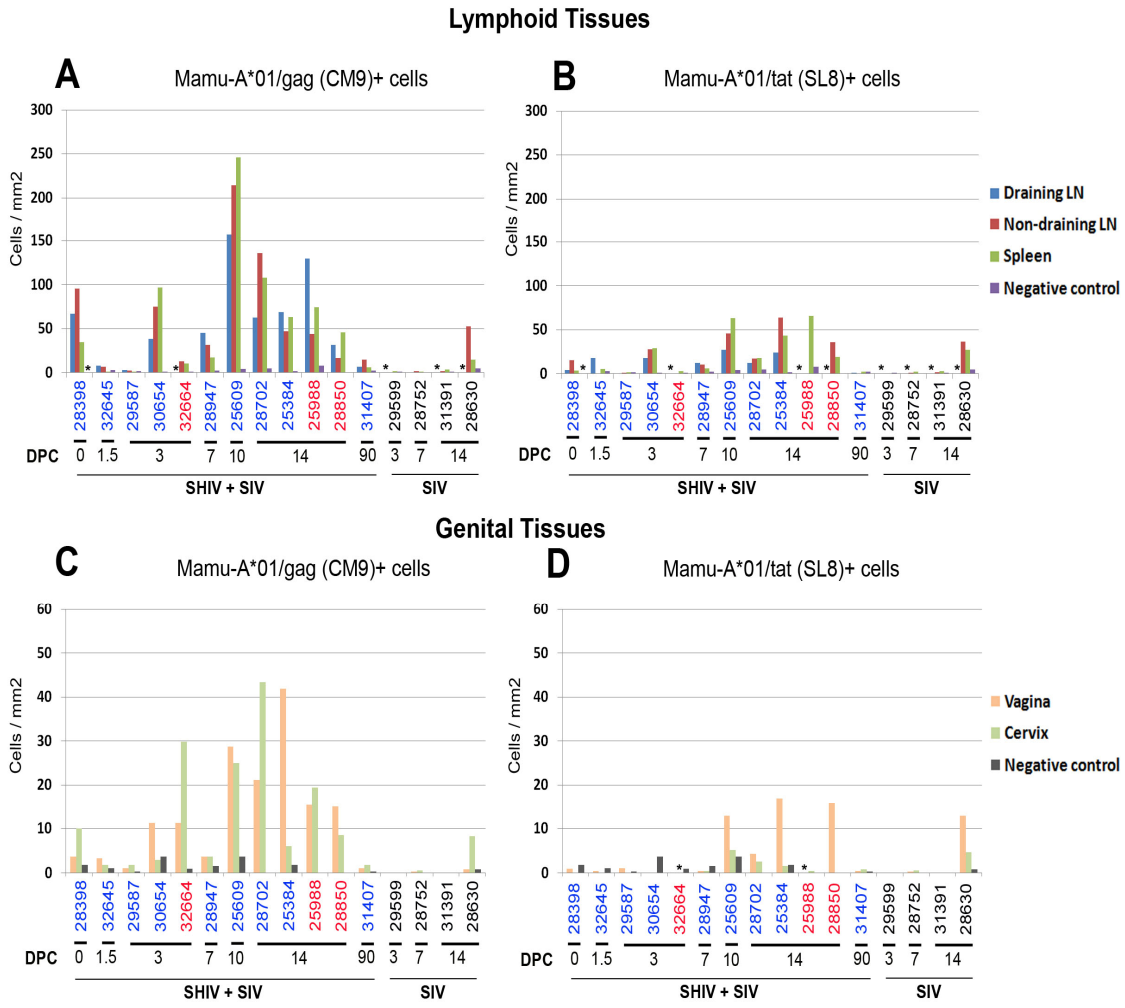


Figure 9. Concentrations of SIV-specific CD8$^+$ T cells in tissues of vaccinated and non-vaccinated SIVmac239-infected Mamu-A*01 macaques. Concentrations of Mamu-A*01 Gag181-189 CM9$^+$ cells in lymphoid and genital tissues are shown in panels A) and C), respectively. Concentrations of Mamu-A*01Tat28-35 SL8$^+$ cells in lymphoid and genital tissues are shown in panels B) and D), respectively. Concentrations of positive cells identified in negative control staining with Mamu-A*01 FLP is shown in each graph. Asterisks (*) indicate that stained tissues sections were not available. Animal numbers are indicated at the bottom of each graph and are colored red for

animals that were challenged with SIVmac239 6-8 months after vaccination with SHIV89.6 and colored blue for animals challenged approximately 12 months post-vaccination.

In unvaccinated control animals infected with highly pathogenic SIVmac239, no tetramer-binding cells were seen in any tissues at 3 and 7 days post-infection and tetramer-binding cells were detected in 1 of 3 animals at 14 days post-infection. In contrast, in the SHIV 89.6 vaccinated macaques tetramer-binding cells were readily detectable at the time of challenge with SIVmac239, in 1 of 3 animals at 3 days post-challenge, and at 7, 10, and 14 days-post challenge (Figure 9). Very few tetramer-binding cells were detected at 90 days post-challenge. Curiously, only a few tetramer-binding cells were evident in 1 animal at 1.5 days post-challenge, and in 2 of 3 animals at 3 days post-challenge (Figure 9). This may indicate that there is a loss of detection of SIV-specific CD8 T cells in situ at early times post-challenge, or simply be due to animal variability. Mamu A*01/gag tetramer-binding cells peaked in lymphoid tissues at 10 days post-challenge with 246 cells/mm^2 (Figure 9A). The magnitude of the Mamu A*01/tat response detected in situ was substantially smaller in the lymphoid tissues with maximum values reaching 63 cells/mm^2 at 10 and 14 days post-challenge (Figure 9B). In the genital tissues, Mamu A*01/gag tetramer-binding cells peaked at 14 days post-infection with over 40 cells/mm^2 (Figure 9C). Similar to the lymphoid tissues, the Mamu A*01/tat cells were less concentrated than the Mamu A*01/gag binding cells, and peaked at 14 days post-challenge with over 15 cells/mm^2 (Figure 9D). Three of four non-vaccinated SIVmac239 infected animal showed little or no response of Mamu A*01/gag cells or Mamu A*01/tat-binding cells in all of tissues at the early time points (Figure 9). In

contrast to non-vaccinated animals, SIV-specific CD8 T cells in SHIV 89.6 vaccinated MamuA*01 animals were detectible during the first week post-challenge with pathogenic SIVmac239 and thus, correlated with protection.

In order to determine whether CD8 T cells are required for the protection provided by SHIV 89.6 immunization, we temporarily depleted a group of SHIV 89.6 immunized animals of CD8 T cells using anti-CD8 antibodies at the time of challenge with SIVmac239. We found that $CD8^+$ T cells were effectively depleted. Representative images showing tetramer and CD8 staining are presented in Figure 13. Our collaborator, Dr. Chris Miller, and his group, found that the vaccinated animals that were depleted of CD8 T cells were unable to control viral replication after pathogenic SIV challenge (174). These findings demonstrate that protection induced by the SHIV 89.6 vaccine is mediated by $CD8^+$ cells.

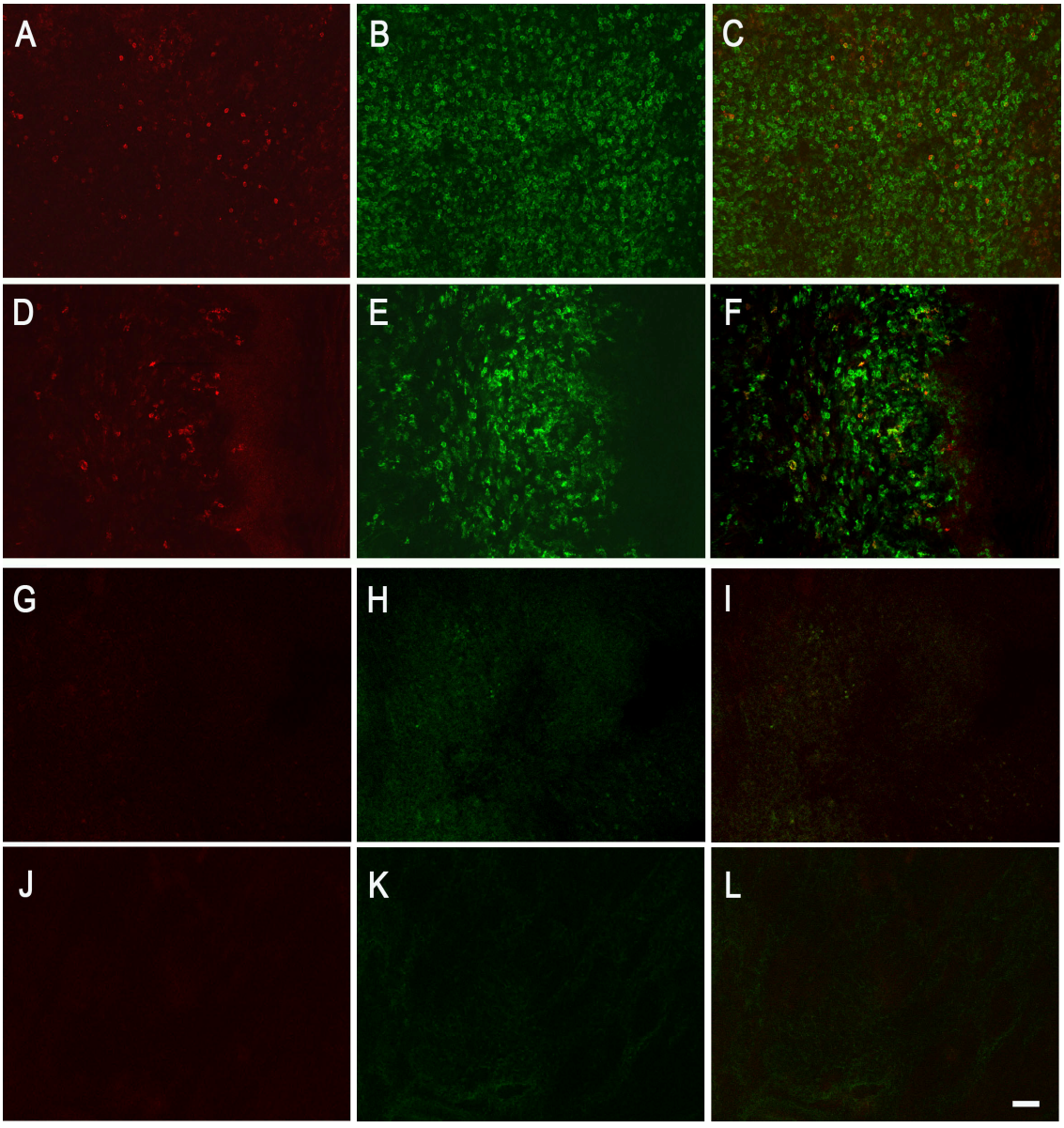


Figure 10. MHC class I tetramers (left panels) and CD8 (middle panels) and merged images (right panels) in tissue sections from SHIV-immunized CD8$^+$-depleted rhesus macaques after challenge. Panels A to F are representative images showing positive staining of tetramer and CD8 in lymph node (A to C) and cervix (D to F) at 14 day post-challenge. Panels G to L are representative images showing spleen (G to I) and cervix (J to L) of SHIV89.6-vaccinated animal depleted of CD8 T cells at 14 day post-challenge and shows no detectible tetramer+ or CD8 T cells. All images are

confocal Z-scans collected at 34 and 31um into the tissues, respectively, using 20X objective. Scale bar = 50 microns.

To further test my hypothesis that protection provided by live attenuated SIV immunization requires the induction of an early virus-specific CD8 T cell response to pathogenic SIV in secondary lymphoid organs and the portal of viral entry, I also determined the location and quantity of virus-specific CD8 T cells in rhesus macaques that were vaccinated with SIVΔnef and challenged with the highly pathogenic viral isolate SIVmac251.

In order to get more detailed information on the location of SIV-specific CD8 T cells and other cells, I triple-stained fresh tissues with tetramers and CD8 and CD3 antibodies during the course of this vaccine study. This antibody counterstaining provided visualization of distinct T cell zones in lymphoid tissues (Figure 10) and T cell populations in genital tissues (Figure 11). My results together with our previous findings (153, 178) showed that in the lymph nodes tetramer-binding cells were most highly concentrated in the T cell rich areas (Figure 10) and in the submucosa of genital tissues (Figure 11). There appeared to be no difference in the location of tetramer-binding cells in each tissue between vaccinated and non-vaccinated groups. Moreover, negative control staining of sections with tetramers loaded with an irrelevant peptide did not show staining pattern similar to tetramers containing gag or tat peptide in any tissue, indicating that tetramer staining with tetramers loaded with viral peptides was specific (data not shown).

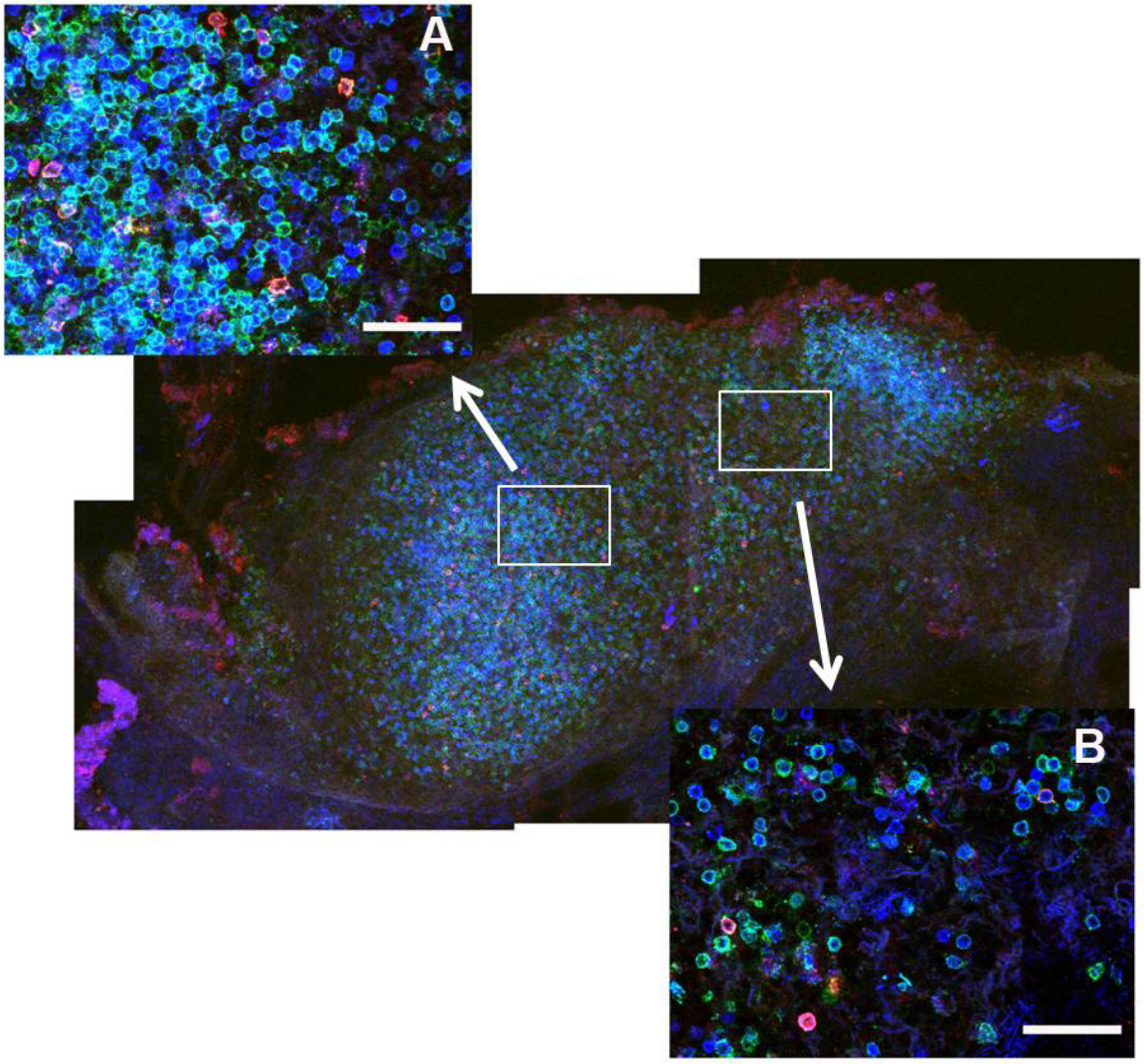


Figure 11. Montage of whole lymph node sections showing CD8 staining (green), CD3 staining (blue) and MHC class I tetramer$^+$ cells (red). All panels are representative images of axillary lymph node from animal #119.96 at 35 day post-infection of SIVΔnef, with panels (A) and (B) showing a higher magnification of the area indicated by the white box (arrow) in the montage. Enlarged images (arrows) illustrate that CD8$^+$ CD3$^+$ tetramer-stained cells are present in both the T cell rich zone (A) and outside of the T cell zone (B). Montage shows projected confocal z-scan images collected

from 16 to 26 in 1um steps using a 20x objective and a 60x objective (A and B). Bar = 50 microns

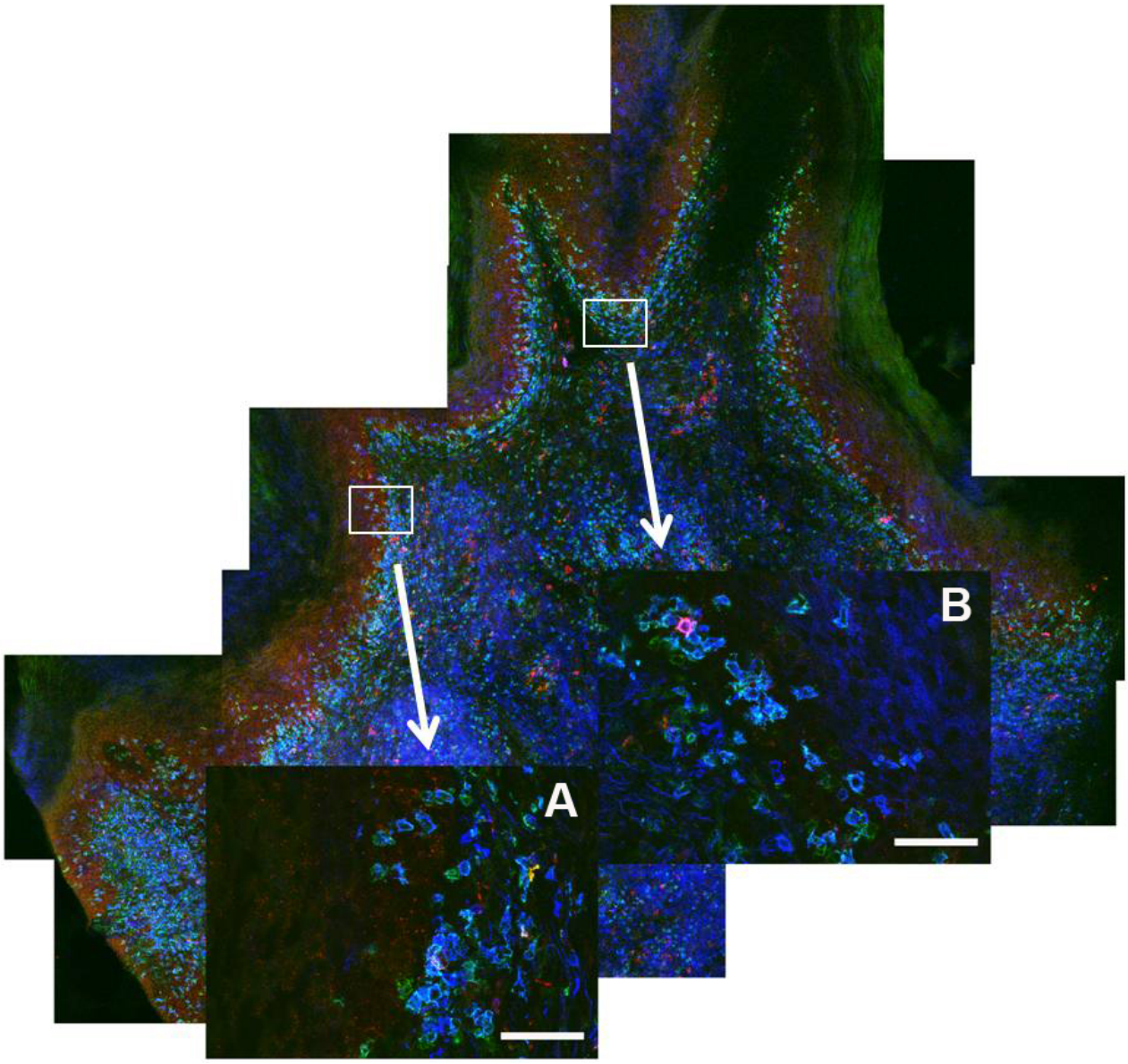


Figure 12. Montage of whole vagina section showing CD8 staining (green), CD3 staining (blue) and MHC class I tetramer^{+} cells (red). All panels are representative images of axillary lymph node from animal #126.00 at 14 day post-infection of SIVΔnef, with panels (A) and (B) showing a higher magnification of the area indicated by the white box (arrow) in the montage. Enlarged images (arrows) illustrate

that $CD8^+$ $CD3^+$ tetramer-stained cells are mainly present in the submucosa. Montage shows projected confocal z-scan images collected from 8 to 24 at 1um steps using a 20x objective and a 60x objective (A and B). Bar = 50 microns

Similar to the results found from SHIV 89.6 vaccination and SIVmac239 challenge, tetramer-binding cells were detectable at the early time points (5, 7, 13 days-post challenge with SIVmac251) in all animals immunized with SIVΔnef (Figure 12). In the genital tissues, Mamu A*01/gag and tat tetramer-binding cells were also less concentrated than those in lymphoid tissues (Figure12). We also found that challenge with SIVmac251resulted in no to very little increase in the number of Mamu-A*01gag and tat -specific T cells in immunized animals, compared to immunized animals that were not challenged with SIVmac251(Figure 12). Thus, these results demonstrate that early localized SIV-specific CD8 T cells correlate with protection in vaccinated animals challenged with highly pathogenic SIV.

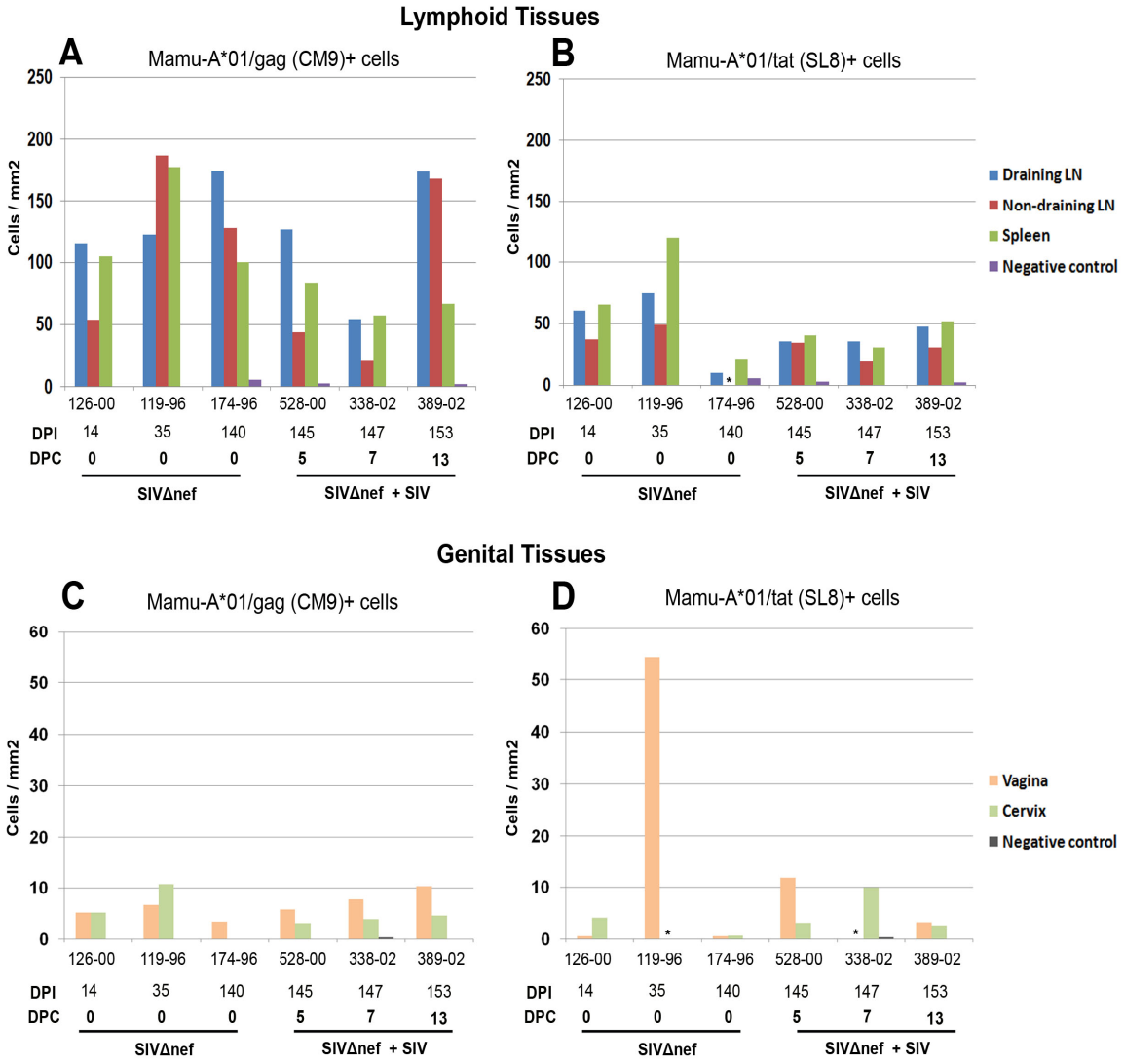


Figure 13. Concentrations of SIV-specific CD8$^+$ T cells in tissues of vaccinated and SIVmac251-challenged Mamu-A*01 macaques. Concentrations of Mamu-A*01 Gag181-189 CM9$^+$ cells in lymphoid and genital tissues are shown in panels A) and C), respectively. Concentrations of Mamu-A*01Tat28-35 SL8$^+$ cells in lymphoid and genital tissues are shown in panels B) and D), respectively. Concentrations of stained cells identified in negative control staining with Mamu-A*01 FLP is shown in each graph. Asterisks (*) indicate tissues were not available. Animal numbers are indicated at the

bottom of each graph.(DPI: day post infection with SIVΔnef; DPC: day post challenge with SIVmac251)

Discussion

During the acute phase of primary SIV infection in vaginally inoculated rhesus macaques, the adaptive virus-specific CD8 T cell response is not seen in situ until 14-21 days post-infection, whereas productive systemic SIV infection is observed in tissues by 6-7 days post-infection (153, 179). The temporal gap between productive systemic infection and emergence of virus-specific CD8 T cell response indicates that CD8 T cells response occurs too late to control the initial spread of SIV. This suggests that a successful CD8 T cell-mediated vaccine will need to induce virus-specific CD8 T cells during the first week of infection in order to prevent or reduce the spread of virus. Based on this concept, our published studies (174, 175) done in collaboration with Dr. Christopher Miller's group at UC Davis indicate that protection provided by SHIV 89.6 immunization is due to the presence of virus-specific CD8 T cells in the genital tissues and in secondary lymphoid tissues. Further in the $CD8^+$ T cell-depleted animals, plasma or tissue levels of SIV replication after challenge were significantly higher than those of CD8 T cell intact SHIV-immunized animals (180). Taken together with our recent findings, these support my hypothesis that successful live attenuated SIV immunization requires the induction of early virus-specific CD8 T cell in response to pathogenic SIV in secondary lymph nodes and genital mucosal tissues. Here, my contribution to our recent studies was to determine the magnitude and localization of SIV-specific CD8 T cells in situ in tissues.

The major goal of effective T cell-based vaccines is to acquire anti-viral T cell immunity including proliferative expansion and activation of virus-specific T cells after the challenge. In our studies, interestingly, although early localized SIV-specific $CD8^+$ T cells in vaccinated and protected animals were observed after the challenge, there was no widespread expansion of T cell response in any tissue examined (Figure 9). These findings demonstrate that although vaccine-induced protection requires CD8 T cells, it does not require SIV-specific CD8 T cell expansion.

In situ hybridization performed by our collaborator, Dr. Ashley Haase, and his group at the University of Minnesota revealed that most vaccinated animals (eleven of twelve) showed no replicating SIVmac239 in all tissues (unpublished data). In contrast, all of the non-vaccinated control Mamu A*01 animals sacrificed at 7 and 14 days post-infection showed replicating SIVmac239 in lymphoid and genital tissues (unpublished data). Thus, in contrast to non-vaccinated animals, in SHIV 89.6 vaccinated MamuA*01 animals that were challenged with SIVmac239, replicating virus may be largely controlled in the presence of early localized SIV-specific T cells in lymphoid and genital tissues. These results suggest the possibility that there is little systemic dissemination of SIV in vaccinated animals after challenge with pathogenic SIV, and that the vaccine induced virus-specific CD8 T cells are sufficient to clear most virus-producing cells at the portal of virus entry and draining lymph nodes. This possibility is supported by findings of our collaborator's, Dr. Miller's group, that vaccinated animals had significantly higher number of polyfunctional virus-specific CD8 T cells at the portal of viral entry and in secondary lymphoid tissues, compared to the non-vaccinated SIV control animals. (174).

Like the results from our studies of SHIV 89.6 vaccinated and SIVmac239 challenged animals, in situ examination of SIV-specific T cells from SIVΔnef immunized animals challenged with pathogenic SIVmac251 provide further support of the importance of SIV-specific CD8 T cells in protective immunization induced by a live-attenuated SIV based vaccine. SIVΔnef-immunized animals showed SIV-specific CD8 T cells localized in lymphoid and genital tissues before and following vaginal challenge with highly pathogenic SIVmac251. Our collaborator's, Dr. Haase and his group, examined replicating virus using in situ hybridization and found that there was no detectable replicating SIVmac251 in all SIVΔnef -vaccinated animals (unpublished data). Moreover, all vaccinated animals showed that there was also no evidence of a substantial expansion of SIV-specific CD8 T cells after the vaginal challenge (Figure 12), again suggesting that a vaccine-induced protection does not require robust CD8 T cell expansion to pathogenic SIV in order for SIV-specific CD8 T cells to control the spread of virus in animals immunized with live attenuated lentiviruses. These findings will lead us to important questions how localized virus-specific T cells control replicating challenge virus and which memory T cells at early time points are associated with protection from vaginal SIV challenge.

Chapter 4: Vaccine-Induced Protection is Associated with SIV-Specific T Cells that Show Little to No Perforin Expression in situ

Introduction and Rationale

$CD8^+$ T cells play a critical role in the clearance of viruses in humans and animals by their ability to kill the virus-infected cells (181, 182). After the initial antigen stimulation in secondary lymph node, naïve $CD8^+$ T cells divide and differentiate into effector and memory cells (183, 184). Peripheral effector $CD8^+$ T cells exert their immune function through the direct cytolysis of infected cells and by secreting cytokines in response to their targets (185, 186). With regard to recall responses, memory T cells in secondary lymphoid organs rapidly divide and become central memory and effector memory subsets are identified according to the distinct capacities (187, 188). After antigenic stimulation, effector memory subsets are associated with immediate effector functions such as increased cytokine production and cytolytic activity, compared with central memory subsets.

Perforin is a membrane perturbing protein found in cytolytic granules in effector $CD8^+$ T cells and its expression is required for perforin mediated killing in virus-infected cells during viral infections including HIV (189-191). Pores formed in membranes of target cells by perforin allow the delivery of other granule-associated proteases, including granzymes, into virus-infected cells, resulting in the direct lysis of target cells by

apoptosis (192, 193). The classification of virus-specific $CD8^+$ T cells based on perforin expression can be evaluated for effector function, and to distinguish effector cells from memory cells. For instance, perforin is produced in very large amounts by effector T cells in primary infections, whereas low levels of perforin expression are characteristic of memory T cell subsets present in healthy as well as virus-infected humans (194-196). However, in protective memory responses against viruses, changes in lytic granule contents of virus-specific $CD8^+$ T cells have remained poorly understood.

In this study, we analyzed expression levels and subcellular localization of perforin in SIV-specific CD8 T cells in situ to identify perforin profiles that correlate with protective CD8 T cell responses induced by SHIV 89.6 vaccination. During acute SIV infection, SIV-specific $CD8^+$ T cells become detectible in situ during the second to third week post-infection (197), whereas, in SHIV 89.6 vaccinated animals, SIV-specific CD8 T^+ cells are detectible prior to SIV challenge and during the first week post-challenge (174, 175). It is not known to what degree SIV-specific CD8 T cells express perforin, an important mediator in killing SIV infected cells. Also, several recent studies have shown that subsets of effector and memory T cells can be identified based on the expression of cytolytic proteins, such as granzyme B and perforin (198, 199). In order to provide insights into SIV-specific CD8 T cell function and to distinguish memory and effector subsets of CD8 T cells, in this study, I determined the levels of perforin expression within SIV-specific CD8 T cells in vaccinated and non-vaccinated animals infected with SIVmac239.

Materials and Methods

Animals

Adult female rhesus macaques (Macaca mulatta) were maintained at the California National Primate Research Center and the Wisconsin National Primate Research Center in accordance with the regulations of the American Association of Accreditation of Laboratory Animal Care standards. The experiments were approved by local institutional animal use and care as well as biosafety review boards. I used six Mamu-A*01 rhesus macaques that were either intravaginally or intrarectally infected with SIVmac239 and sacrificed during the acute stages of infection as previously described (179, 197). I also investigated six Mamu-A*01 rhesus macaques that were immunized intravenously with attenuated SHIV89.6 and subsequently challenged intravaginally with SIVmac239 and sacrificed post-SHIV89.6 inoculation and various time points post-challenge. Freshly dissected lymph nodes and vagina tissues were shipped on ice in RPMI containing 100 μg/ml heparin over night from either the National Primate Research Center in either California or in Wisconsin to the Skinner lab in Minnesota.

In situ tetramer staining and immunohistochemistry

In situ tetramer staining was performed essentially as previously described (153, 171) to detect SIV-specific T cells in tissues from rhesus macaques. MHC class I tetramers were generated from monomers by adding six aliquots of FITC-labeled ExtraAvidin (Sigma) to biotinylated Mamu-A*01 molecules loaded with SIV gag

(CTPYDINQM) peptides (Immunomics) over the course of 8 h to a final molar ratio of 4.5:1. Fresh lymph node and vaginal tissues were cut into approximately 0.5cm pieces and embedded in 4% low melting agarose. Tissue blocks were placed in a vibratome bath containing 0 to 4°C PBS with 100 ug/ml heparin (PBS-H), and 200 um thick sections were generated. To examine the expression levels and intracellular localization of perforin in SIV-specific T cells, first we stained macaque tissues with Mamu A*01 gag tetramers overnight at 4°C. Subsequently, the sections were washed and fixed with 4% paraformaldehyde for 2 hours at room temperature. Sections were again washed and then permeabilized with PBS containing 0.1% triton-X and 2% normal goat serum. After the permeabilization, we incubated sections with perforin antibody diluted 1:100 (Novocastra) and rabbit-anti-FITC antibodies diluted 1:10,000 (BioDesign) in permeabilizing buffer overnight at 4°C. We washed three times with PBS-H and then incubated with Cy3-conjugated goat anti-rabbit antibodies diluted 1:5000 (Jackson ImmunoResearch) and Alexa 488-conjugated goat anti-mouse antibodies diluted 1:2000 (Molecular Probes), in PBS-H with 2% NGS, for 1 to 3 days. Sections were mounted using warmed glycerol gelatin (Sigma) containing 4 mg/ml n-propyl gallate (Fluka, Switzerland).

Subsets of sections were stained with Hoescht to label cell nuclei and these sections used to collect representative images. For this staining, the mounted sections were put on the heat plate at 60 ~ 70 °C and the coverslips were removed. The sections were washed 2 times for 5 min in water at room temperature. The slides were incubated in Hoechst 33342 diluted to 0.0025% in water (Invitrogen) for 10 min at room

temperature. The sections were washed 3 times for 5 min and mounted to slides with glycerol gelatin (Sigma) containing 4 mg/ml n-propyl gallate (Fluka, Switzerland).

Image analysis

Digital images showing intracellular expression of perforin (green) within gag-specific T cells (red) were collected with an Olympus FluoView1000 confocal microscope using 20X and 60X objectives and analyzed using Fluoview software program. From the surface of section, Z-series images were collected with 1 micron steps as far into the tissue as perforin staining permitted. Using Fluoview software, tetramer positive cells were examined as being perforin positive or perforin negative. Tetramer$^+$ perforin$^+$ cells were divided into three groups: perforin high cells showed much higher perforin staining than most perforin$^+$ cells in the field, perforin low cells showed only 1 or 3 perforin granules within cell or less perforin than most cells in the field with staining just above what is seen in negative control stained sections and perforin medium cells were neither high or low. Tetramer$^+$ cells in which perforin staining was polarized to one face of the cell, or in which perforin staining was only located in association with the cell membrane were also noted and quantified. Statistical analyses were performed using unpaired two-tailed T-test.

Results

Perforin expression levels within SIV-specific T cells in vaccinated and non-vaccinated SIV-infected animals

In the present study, in situ tetramer staining combined with immunohistochemistry showed that perforin$^+$ Mamu A*01 gag-specific T cells were present in the lymph nodes and vagina of all of the vaccinated and non-vaccinated animals examined (Figure 14 and Figure 15A). Representative images are shown in Figure 13. I found that the majority of SIV-specific T cells induced by immunization and challenge showed no perforin staining (Figure 15B and D). In contrast, the majority of SIV-specific T cells in non-vaccinated SIV-infected animals were perforin$^+$ (Figure 15B and D). Quantification of tetramer$^+$perforin$^+$ and tetramer$^+$perforin$^-$ cells showed that the percentage of SIV-specific T cells that were perforin$^+$ in tissues of vaccinated/challenged animals was significantly lower than that observed during primary SIV infection (37.94% ± 14% vs 72.16 ± 7 % in lymph node; $p<0.0005$ and 24.86% ± 11% vs 70.16 ± 25 %; $p<0.005$ in vagina, respectively) (Figure 15B and D). I also scored tetramer-binding perforin$^+$ cells as being perforin high, medium and low based on levels of intracellular perforin accumulation and found that all of the non-vaccinated SIV control animals showed a subset of cells that were tetramer$^+$ perforin high in lymph node and vagina, while only four of six in lymph nodes and only one of six in that vagina of vaccinated/challenged animals did (Figure 14C and E). In addition, vaccinated/challenged animals showed significantly fewer tetramer$^+$ perforin high and perforin medium cells, and significantly more tetramer$^+$ perforin low cells than non-vaccinated animals (Figure 15C and E). Thus, protection in the vaccinated animals was

associated with SIV-specific CD8 T cells that were predominantly perforin negative and perforin low.

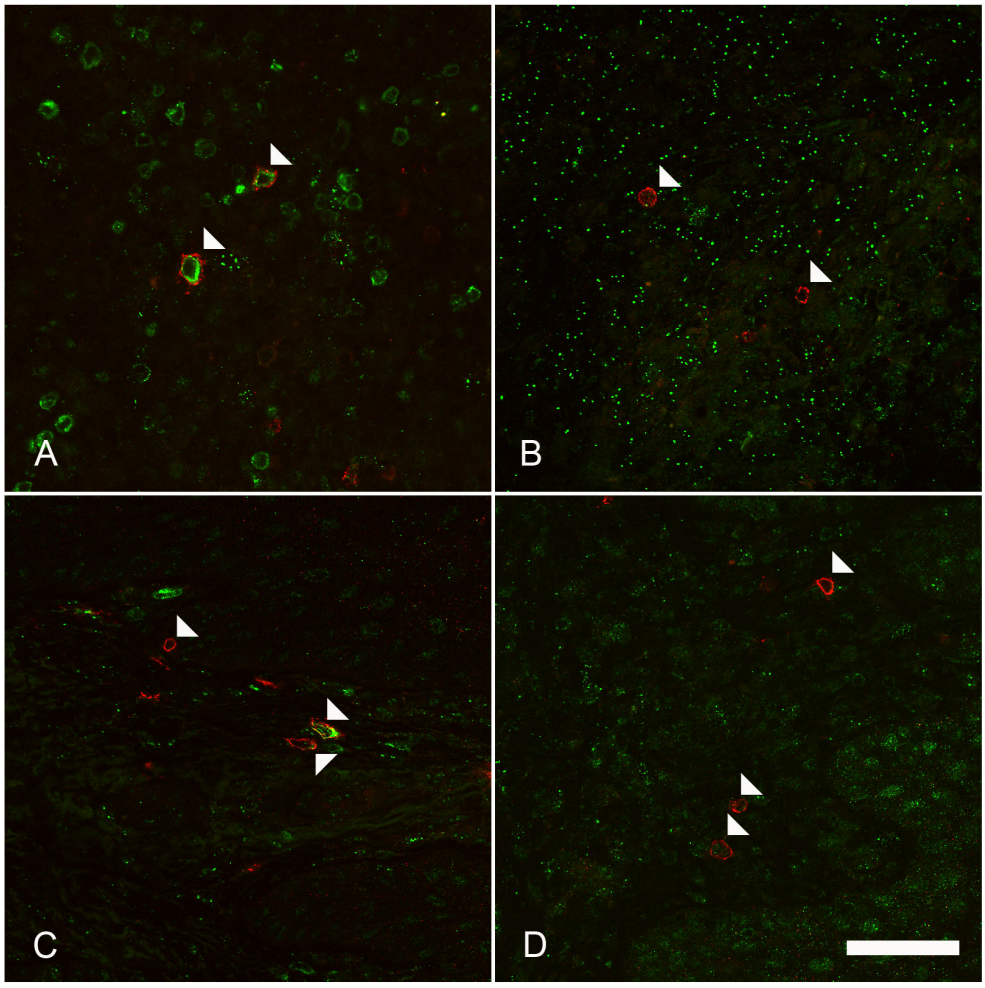


Figure 14. Perforin expression lower within SIV-specific T cells and fewer tetramer$^+$perforin$^+$ cells in tissues of SHIV-immunized compared to non-immunized animals infected with pathogenic SIV. Representative staining patterns are shown for perforin (green) and Mamu-A*01 Gag CM9-specific T cells (red) in the lymph node (A and B) and vagina (C and D). The representative images are from SIVmac239-infected rhesus macaque #24037, at 14 days post-infection (A), #80072 at 21 days post-infection (C) and SHIV-immunized rhesus macaque #28702 at 14 day post- SIVmac239 challenge

(B and D), respectively. Arrows indicate tetramer positive cells. All images are confocal Z-scans collected using a 60X objective. Scale Bar = 50 microns.

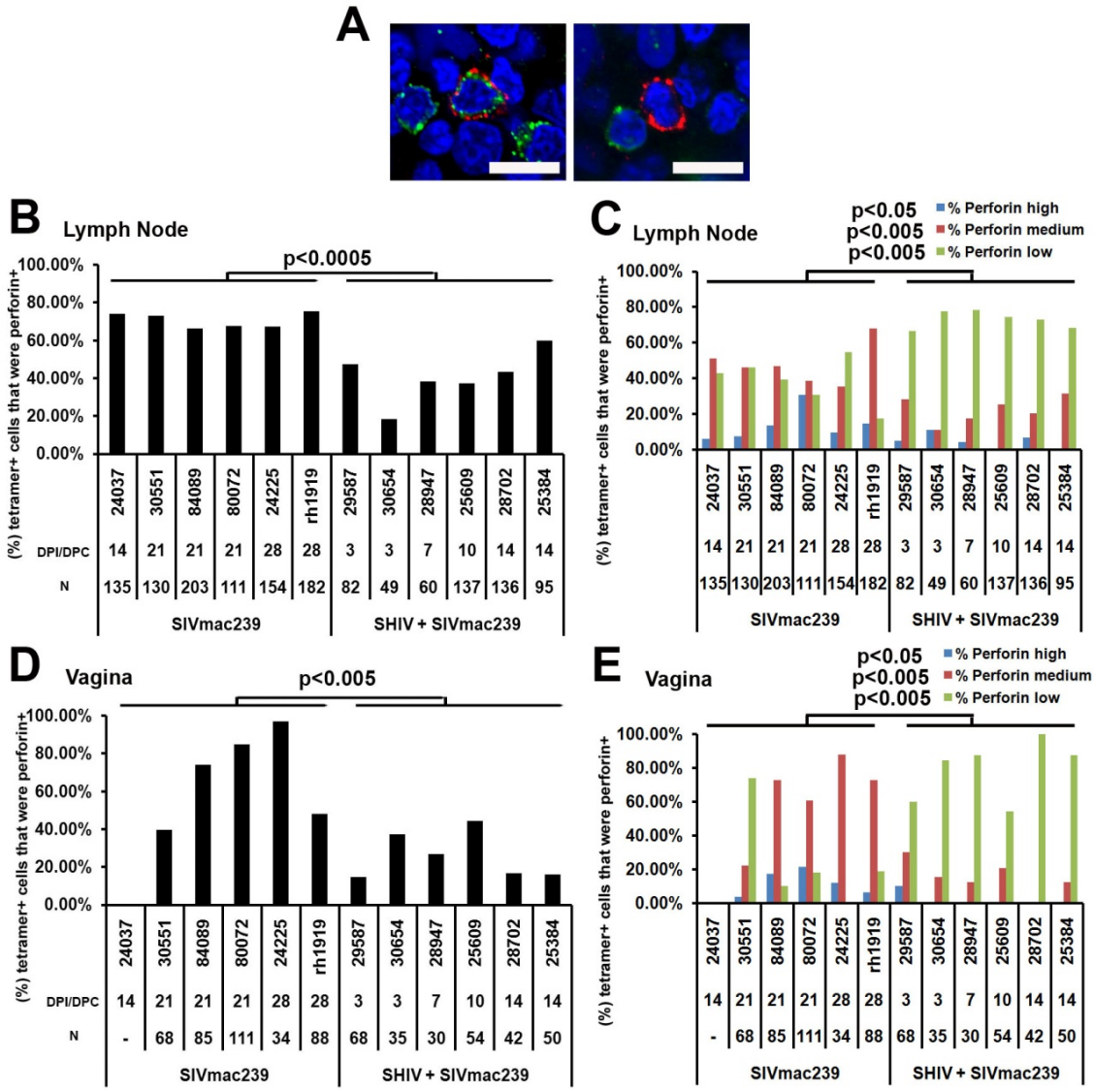


Figure 15. Fewer SIV-specific T cells showed perforin expression and perforin expression was lower in lymph node and vagina tissues from SHIV89.6-immunized animals compared to non-immunized SIV-infected animals. A) shows representative staining patterns for perforin (green), Mamu-A*01 Gag CM9-specific T cells (red) and cell nucleus (blue) in the tissues. Left panel shows a perforin positive tetramer stained cell, while right panel shows a perforin negative tetramer stained cell. Images are confocal Z-scans collected using a 60X objective and 3X zoom. Scale Bar = 20 microns. B and D) Shows the percentage of Mamu-A*01 Gag $CM9^{+}$ cells that were $perforin^{+}$ cells in lymph node and vagina tissues from each animal. Significant differences were seen in the percentage of $perforin^{+}$ $tetramer^{+}$ cells between vaccinated and non-vaccinated groups. C and E) shows the percentage of $tetramer^{+}$ $perforin^{high}$, $tetramer^{+}$ $perforin^{medium}$, and $tetramer^{+}$ $perforin^{low}$ cells found in lymph nodes and vagina. Significant differences were seen in the percentage of lymph node or vaginal SIV-specific T cells expressing high, medium, or low level of perforin between vaccinated and non-vaccinated groups. DPI/DPC: Day Post Infection / Day Post Challenge, N: Number of Mamu-A*01 Gag CM9-specific T cells examined.

Polarization of perforin within virus-specific CD8 T cells

To better understand difference of molecular anatomy relevant to cytolytic granules in SIV-specific T cells between two groups, I next examined the pattern of perforin expression observed within SIV-specific T cells. It is known that when a cytotoxic CD8 T cell is in the process of killing an infected cell, the perforin within the cell becomes polarized towards the infected cell (200, 201). I found that in a subset of cells perforin staining was polarized and localized to one region in cytoplasm and cell membrane of Mamu A*01 gag-specific T cells (Figure 16A). I also found that all of the non-vaccinated SIV-infected animals showed a subset of tetramer-binding cells in which perforin was polarized, while five of six in lymph node and only one of six in vagina of vaccinated/challenged animals showed a subset of tetramer-binding cells with perforin localized (Figure 16B and D). Quantitative analysis showed that the percentage of SIV-specific T cells that were perforin polarized in tissues of vaccinated/challenged animals was significantly lower than that observed during primary SIV infection (Out of total $tetramer^+$ cells, 4.10 ± 3% vs 16.70% ± 6% in lymph node; $p<0.001$ and 0.49 ± 3% vs 12.73% ± 2%; $p<0.01$ in vagina, respectively) (Figure 16B and D). In addition, there was significant difference in the percentage of SIV-specific T cells showing polarized perforin in $perforin^+$ $tetramer^+$ cells between vaccinated/challenged and non-vaccinated SIV-control animals (9.61 ± 7% vs 23.86% ± 9% in lymph node; $p<0.05$ and 3.33 ± 13% vs 17.13% ± 5%; $p<0.05$ in vagina, respectively) (Figure. 16C and E). These results indicate that more SIV-specific CD8 T cells were actively killing SIV-infected cells in unvaccinated animals compared to vaccinated animals challenged with pathogenic SIV, and are consistent findings from Ashley Haase's group showing that there were more

virus-producing cells present in the unvaccinated animals compared to vaccinated/challenged animals (unpublished data).

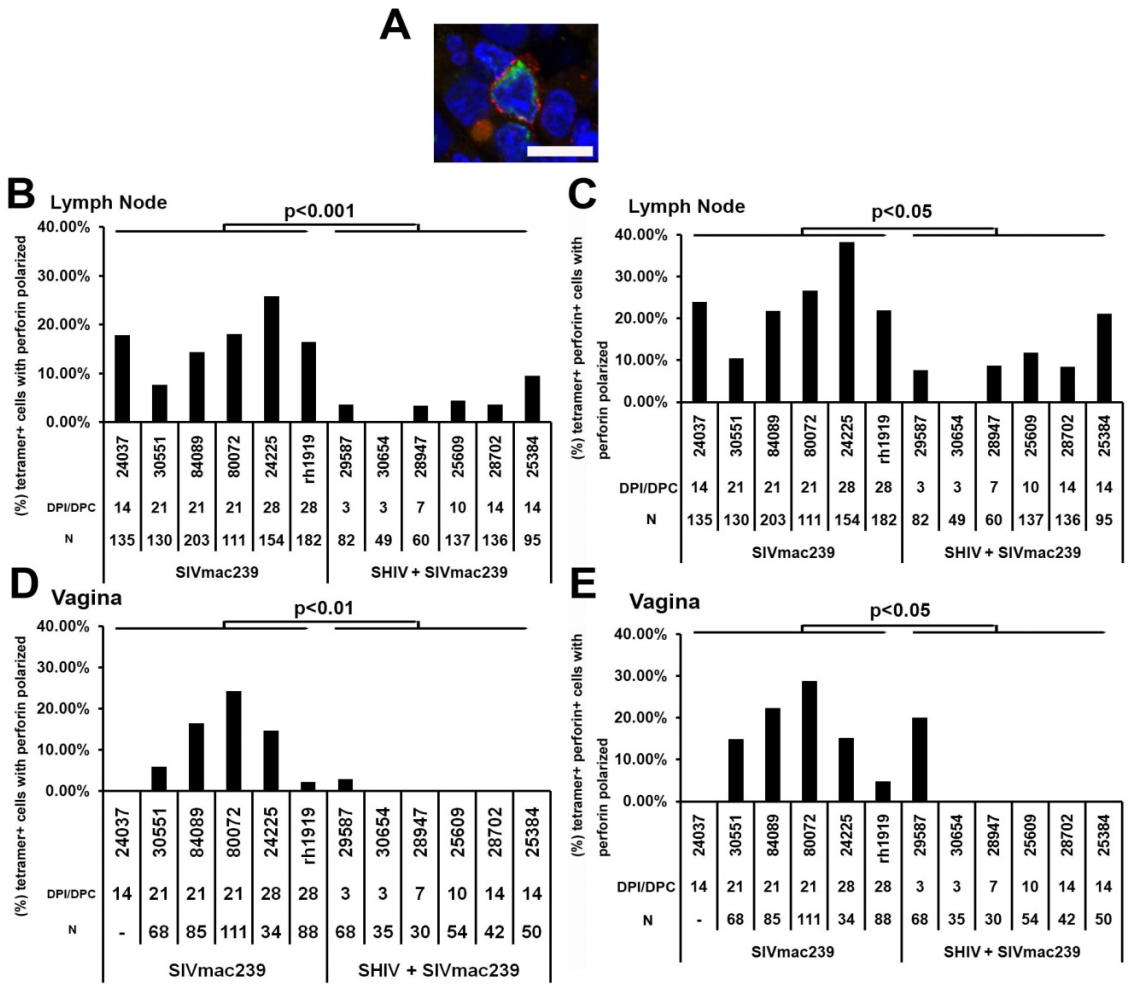


Figure 16. Fewer vaccine-induced SIV-specific CD8 T cells showed polarized perforin in lymph nodes and vagina. A) Shows representative staining patterns of tetramer^{+} perforin^{+} cells in which the perforin was polarized to one side of the cell. Images are confocal Z-scans collected using a 60X objective and 3X zoom. Scale Bar =

20 microns B) Shows the percentage of Mamu-A*01 Gag CM9^{+} cells in which perforin was found to be polarized among total tetramer^{+} cells in tissues of each animal. C) shows the percentage of tetramer^{+} cells in which perforin was polarized in each animal. Lymph node and vaginal SIV-specific T cells in non-vaccinated animals showed significantly higher percentages of SIV-specific T cells with perforin polarized than vaccinated animals. DPI/DPC: Day Post Infection / Day Post Challenge, N: Number of Mamu-A*01 Gag CM9-specific T cells examined.

In addition, I also found a subset of virus-specific CD8 T cells showing perforin that was localized within one or a few granules only on the T cell membrane (Figure 16A). Of the SIV-specific T cells that were perforin^{+} in the vaccine/challenged animals, this staining pattern was dominant (Figure 17C and E). In contrast, in non-vaccinated SIV infected animals, this pattern of perforin staining was relatively rare (Figure 17C and E). Of total tetramer^{+} cells, quantitative analysis showed that the percentage of SIV-specific T cells showing this pattern in vaccinated/challenged animals was significantly greater than that observed during primary SIV infection (17.08% ± 7% vs 4.82 ± 6% in lymph node; $p<0.01$ and 10.01% ± 3% vs 1.66 ± 2%; $p<0.001$ in vagina, respectively) (Figure 17B and D). In addition, there was a significant difference in the percentage of SIV-specific T cells showing this pattern out of perforin^{+} tetramer^{+} cells between vaccinated/challenged and SIV-control groups (45.18% ± 10% vs 7.08 ± 9% in lymph node; $p<0.0001$ and 0.49% ± 13% vs 3.18 ± 5%; $p<0.001$ in vagina, respectively) (Figure 16C and E).

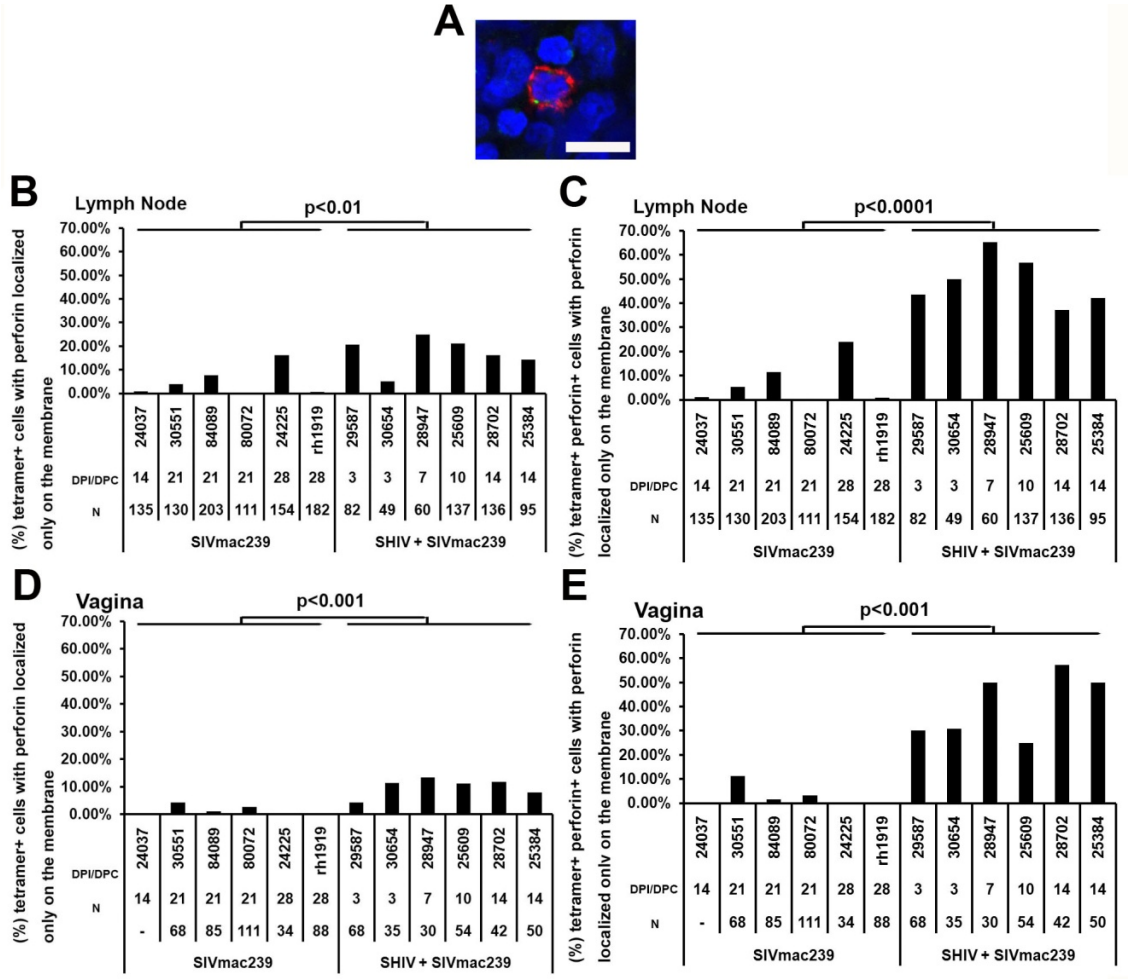


Figure 17. Vaccine-induced perforin$^+$ SIV-specific CD8 T cells frequently showed perforin localized only in cell membranes in lymph nodes and vagina. A) Shows representative staining patterns of tetramer+ perforin+ cells in which the perforin was localized only in the membrane of the cell. Image is a confocal Z-scan collected using a 60X objective and 3X zoom. Scale Bar = 20 microns B) shows the percentage of Mamu-A*01 Gag CM9+ cells expressing perforin localized only in the cell membrane among total tetramer+ cells in tissues of each animal. C) shows the percentage of Mamu-A*01 Gag CM9$^+$ cells showing this membrane pattern among total tetramer+ perforin+ cells in tissues of each animal. Vaccine-induced perforin+ SIV-specific T cells showed significantly higher percentage of this membrane pattern, compared to counterparts in

non-vaccinated animals. DPI/DPC: Day Post Infection / Day Post Challenge, N: Number of Mamu-A*01 Gag CM9-specific T cells examined.

As a negative control, I examined tissue sections stained with tetramers and no mouse primary antibodies. I found that in negative control lymph node and vagina sections, all Mamu-A*01 Gag CM9^{+} cells showed no green staining (data not shown), indicating that perforin staining within SIV-specific T cells was specific.

Discussion

Virus-specific CD8 T cells expressing effector molecules play an important role in the control of virus infections (193). Although the role of these effector T cells and their lytic enzymes during the primary infection are well understood, the phenotypic change of virus-specific $CD8^+$ T cells in protective memory responses against viruses remains poorly understood. Recent studies have suggested that there are phenotypic differences between effector T cells and memory T cells in mice and human. For example, during chronic or secondary virus infections, memory virus-specific T cells lost or showed significantly decreased levels of cytotolytic effector molecules compared to primary effector T cells (188, 202). In individuals infected with EBV and HCMV, a large number of central memory and effector memory $CD8^+$ T cell subsets were perforin negative or expressed low levels of perforin (198, 203). Moreover, in a primate model that closely resembles memory T cell development of humans (204), animals elicited and maintained populations of central and effector memory T cell subsets one year after immunization with SHIV 89.6 and after challenge with pathogenic SIV(174, 205), whereas non-vaccinated animals infected with pathogenic SIV mainly showed increased perforin expression in spleen and blood mononuclear cells during acute infection and CD8 T cells predominantly containing this lytic enzyme in the spleen (206). Taken together, these findings suggest that animals that are immunized and challenged with pathogenic SIV have more memory and fewer effector virus-specific CD8 T cells, compared to animals experiencing primary SIV infections.

In this study, I examined whether there were differences in perforin expression in virus-specific CD8 T cells in vaccinated and challenged animals, compared to non-vaccinated animals infected with pathogenic SIV in situ. I found that, in lymph node and vagina tissues from vaccinated animals challenged with SIVmac239, the majority of SIV-specific CD8 T cells showed little to no perforin expression and showed statistically significantly less perforin expression per cell and lower percentages of perforin positive cells relative to SIV-specific CD8 T cells from non-vaccinated SIVmac239 infected animals. This is consistent with the idea that central memory T cells as well as some effector memory T cell subsets typically express at no or low level of perforin (195, 198), and indicate that these subsets of memory T cells were dominant in the vaccinated/challenged animals.

Although our study did not demonstrate in situ which memory and effector subsets of SIV-specific T cells were distinguished with respect to perforin expression, it is now clear that the generation of virus-specific T cells expressing little or no perforin was more observed in vaccinated animals compared to non-vaccinated animals. Several lines of evidence have supported our observation. For example, expression of perforin was not detected in the subset of central memory cells (198) and, in both mice and nonhuman primates, this subset provided protection from systemic viral challenge (188, 207, 208). Other evidence includes that perforin^{+} virus-specific T cells were able to kill targets even though they expressed a low level of perforin (198). In addition, SHIV-immunized animals showed polyfunctional T cell responses including the degranulation capacity in virus-specific CD8 T cells in genital tissues at early time points, 3, 7, and 14 days after the SIV challenge (174). Indeed, in situ hybridization for SIV RNA performed

by our collaborators on the same cohort of animals that I examined, showed that the majority of vaccinated animals showed no detectable pathogenic SIV-producing cells, while all of the non-vaccinated animals showed detectable in these cells in situ (unpublished data). These results taken together suggest that vaccine-induced SIV-specific T cells have characteristics more similar to memory T cell subsets expressing little to no perforin and that SIV-specific CD8 T cells with little or no perforin correlate with vaccine-induced protection. However, future studies are still necessary to elucidate which T cell memory subsets are closely involved in the protection afforded by live-attenuated virus in this nonhuman primate model. I also quantified levels of perforin within tetramer-binding cells and found that vaccinated/challenged animals showed a significantly lower percentage of tetramer^{+} perforin high or medium cells compared to non-vaccinated animals. Of interest, our findings of decreased perforin expression in virus-specific T cells in the non-human primate system were consistent with recent observations in HIV-1 infected humans. In humans, HIV-1 specific CD8 T cells showed a progressive shift to lower perforin expression after vaccination, but not during acute HIV-infection (209).

Previously, McGavern et al. showed that perforin is polarized inside virus-specific T cells when virus-specific CD8 T cells are in contact with and killing a target cell (200). In this study, I found that, in a subset of cells, perforin was polarized and localized to one region in cytoplasm and cell membrane of Mamu A*01 gag-specific T cells, indicating interactions between SIV-specific T cells and target cells. I also found non-vaccinated animals showed significant greater percentage of subset of tetramer-binding cells in which perforin was polarized than vaccinated/challenged animals. As mentioned above,

SIV-producing cells were detectable in all of non-vaccinated animals, not in majority of vaccinated animals (unpublished data). Thus, our observations show that the increased perforin polarization within the virus-specific CD8 T cells in unvaccinated animals compared to vaccinated animals infected with pathogenic SIV is correlated with the presence of virus producing cells in situ, and indicate that more SIV-specific CD8 T cells were actively killing infected cells in unvaccinated compared to vaccinated animals.

Our examination of perforin abundance and localization within SIV-specific CD8 T cells led us to the interesting discovery that virus-specific CD8 T cells in which perforin is localized only on the T cell membrane were more observed in the vaccine/challenged animals compared to those in non-vaccinated animals. This unique pattern of perforin expression has never been reported. It is possible that perforin expression on the membrane of SIV-specific CD8 T cells might allow rapid release of granules that can effectively kill target cells in successfully vaccinated animals. These results demonstrate that a subset of SIV-specific CD8 cells, most likely a subset of memory cells, express low levels of perforin located only in the cell membrane. They further indicate that SIV-specific T cells in successfully vaccinated animals are phenotypically different from their counterparts in non-vaccinated SIV-infected animals.

In summary, we have shown that immune protection elicited by attenuated SHIV against mucosal challenge with a pathogenic SIV is mediated by CD8 T cells and is associated with SIV-specific $CD8^+$ T-cell responses localized in mucosal and lymphoid tissues at the time of challenge (174). In addition, in animals that were vaccinated with SHIV and challenged with SIVmac239, most virus-specific CD8 T cells showed little to no perforin expression in situ and that in $perforin^+$ virus-specific CD8 T cells, the

perforin was typically associated with the cell membrane. Taken together, our findings suggest that vaccine-induced protection is mediated by memory CD8 T cells expressing little to no perforin.

Chapter 5: Vaccine-induced early protection against pathogenic SIV: interactions between vaccine-Induced SIV-Specific T Cells and CD83^{+} cells

Introduction and Rationale

In HIV (210-213) and SIV (214) infections, professional antigen presenting cells (APCs) including DCs and activated B cells are involved in the priming of virus-specific T cell-mediated immunity. For example, following mucosal exposure, DCs located beneath the epithelium may efficiently uptake viral antigens at the first. These cells then mature in response to the endogenous stimuli, resulting in up-regulation of DC adhesion and co-stimulatory molecules including CD83 (215). During the maturation, DCs are highly specialized at presenting viral antigens to naïve CD4 and CD8 T cells and memory T cells reside in the lymphoid tissues (216).

Finally, mature DCs, not immature DCs, can enhance proliferation and multiple types of HIV-specific T cell functions including the secretion of cytokines (217). In addition, central and effector memory T cells can be activated during a secondary infection, particularly in tissues where pathogen-specific T cells interact with antigen bearing DCs (218). Together, these findings show that, after vaccination, antigen presentation by mature DCs is essential for naïve and memory T cell activation, leading to the partial control of viral spread in HIV and SIV infections. In our study, we used the live-attenuated SIVΔnef strain that is deleted of the nef gene as a SIV vaccine. Immunization with the attenuated virus SIVΔnef provides protection against subsequent challenge with pathogenic SIV such as the molecular clone SIVmac239 and the biological isolate

SIVmac251 (67, 156, 219). This model for AIDS vaccine would be valuable to track viral antigen recognition between APCs- T cells in lymphoid tissues of a successful vaccine.

CD83 is a conserved immunoglobulin superfamily member present on mature DCs as well as activated lymphocytes (220-224). Immunohistologic analysis revealed that CD83 expression is highly restricted to the lymphoid tissues (225). The CD83 lymphocytes showing weak staining were located in the follicular mantle and , whereas CD83 DCs showing strong staining were distributed in T cell zone of lymphoid tissues (225). CD83 is also an adhesion molecule that binds a subset of activated $CD8^+$ T cells (226), resulting in their long-term survival and expansion (227). In addition, impaired function of $CD4^+$ T cells was observed in CD83 knock-out mice (228, 229), whereas enhanced function of these cells was found in transgenic mice that overexpress CD83 (228). These studies indicate that $CD83^+$ DCs are important for T cell activation and survival.

Physical interactions of $CD83^+$ DCs and $CD83^+$ activated lymphocytes with T cells in situ may be involved in the vaccine-induced protection. Here, to understand better the mode of vaccine induced protection from pathogenic SIV by SIV-specific CD8 T cells, I determined the location and quantity of physical interactions between $CD83^+$ cells and Gag CM9 or Tat SL8 tetramer-stained cells in the spleen of rhesus macaques immunized with SIVΔnef before and after challenge with SIVmac251. I hypothesize that, **in vaccinated animals upon pathogenic SIV challenge, there are increased interactions between SIV-specific T cells and $CD83^+$ cells.**

Materials and Methods

Animals

Adult female rhesus macaques (Macaca mulatta) were maintained at the New England Primate Research Center in accordance with the regulations of the American Association of Accreditation of Laboratory Animal Care standards. The experiments were approved by local institutional animal use and care as well as biosafety review boards. Spleen tissues were collected from four Mamu-A*01 rhesus macaques first immunized intravenously to SIVΔnef and subsequently challenged intravaginally with SIVmac251 and sacrificed post-SIVΔnef inoculation and various time points post-challenge. Freshly tissues were shipped on ice in RPMI containing 100 μg/ml heparin over night from either the New England Primate Research Center to the Skinner lab in Minnesota.

In situ tetramer and immunohistochemical staining

Fresh spleen tissues were used throughout these studies. In situ tetramer staining combined with immunohistochemistry was performed essentially as previously described (153, 171) to detect SIV-specific T cells, $CD83^+$ cells and $CD3^+$ cells in tissues from rhesus macaques. We purchased biotinylated Mamu-A*01 molecules loaded with SIV gag (CTPYDINQM) peptides (Immunomics), and loaded with SIV tat (STPESANL) (National Institute of Allergy and Infectious Diseases tetramer facility). Tetramers were generated by adding six aliquots of FITC-labeled ExtraAvidin (Sigma) to biotinylated Mamu-A*01/β2m/peptide monomers over the course of 8 h to a final molar ratio of 4.5:1.

Fresh spleen tissues were sectioned using a vibratome to a thickness of 200 um. Sections were incubated at 4°C overnight with tetramers (0.5 ug/ml) and mouse anti-human CD83 (Santa Cruz), and rat anti-CD3 (AbD serotec) antibodies diluted 1:200 in PBS-H with 2% normal goat serum (NGS). Mamu A*01 tetramers loaded with an irrelevant peptide and isotype control antibodies (mouse IgG) were used as negative controls. Sections were then washed with chilled PBS-H and fixed with 4% paraformaldehyde for 2 h at room temperature. Sections were again washed with PBS-H, incubated with rabbit anti-FITC antibodies (BioDesign) diluted 1:10,000 in PBS-H with 2% NGS, and incubated at 4°C on a rocking platform overnight. Sections were washed three times with PBS-H for at least 20 min and then incubated with Cy3-conjugated goat anti-rabbit antibodies diluted 1:5000 (Jackson ImmunoResearch), Cy5-conjugated goat anti-rat antibodies diluted 1:2000 (Jackson ImmunoResearch), and Alexa 488-conjugated goat anti-mouse antibodies diluted 1:2000 (Molecular Probes), in PBS-H with 2% NGS, for 1 to 3 days. Finally, sections were mounted using warmed glycerol gelatin (Sigma) containing 4 mg/ml n-propyl gallate (Fluka, Switzerland). Stained sections were analyzed using a Olympus FluoView1000 confocal microscope. Digital images were collected and analyzed using FluoView software 1.7 and Adobe Photoshop 7.01.

Quantitative Image Analysis

For each tissue section stained with tetramers, CD83 and CD3 antibodies, images were collected with an Olympus FluoView1000 confocal microscope using a 20X and 60X objectives using the same confocal parameters for each tissue section. Separate fields were selected for each tissue containing all colors, and if possible more fields (from

eight to fifteen) were collected. Using FluoView software (Olympus), $CD83^+$ cells were quantified in the $CD3^+$ or $CD3^-$ area as being DCs morphology and lymphocyte morphology cells. DCs morphology $CD83^+$ cells were defined as showing undulating process, bright, and CD3 negative staining in the membrane that was just above background levels, while lymphocyte morphology $CD83^+$ cells were defined as showing spherical and undisturbed outline of cell membrane above background levels, which have the size as same as $CD3^+$ cells. In the same images, the interactions between $CD83^+$ cells (green) and tetramer stained cells (red) was also noted. In the z-scan images, physical contacts were quantified by measuring the merged color (yellow) between green and red as previously described by in situ methods (230, 231). As a negative control for CD83 staining, tissue sections were stained with tetramers and isotype control mouse IgG antibodies.

Results

Identification of CD83$^+$ cells that show DC morphology or lymphocyte morphology in the spleen

As anticipated, we found two distinct morphology types of CD83$^+$ cells in spleen sections stained with anti-CD83 monoclonal antibodies (shown green) and anti-CD3 monoclonal antibodies (shown blue). Our in situ tetramer staining combined with immunohistochemistry revealed that one subset has strong staining and irregular interdigitating cells (DC morphology CD83$^+$ cells), whereas the other has week staining and round shape like B and T lymphocyte (lymphocyte morphology CD83$^+$ cells) (Figure 18). Most CD83$^+$ cells were not CD3 positive and many interactions between CD83$^+$ cells and CD3$^+$ cells were observed (Figure 18 C and F).

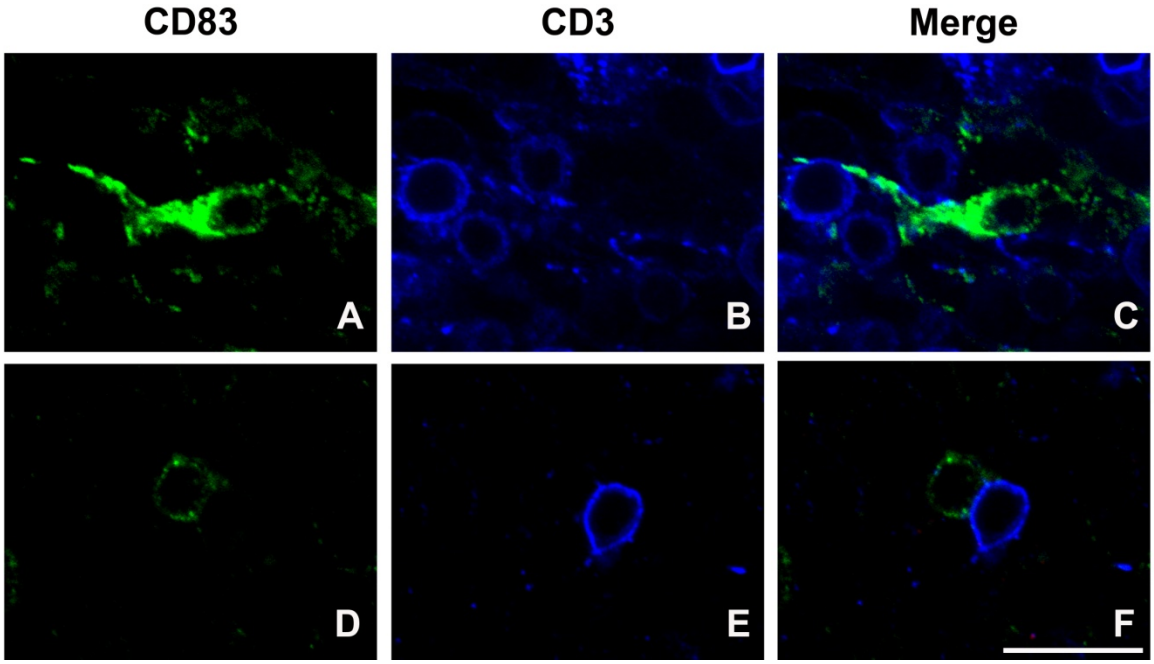


Figure 18. Identification of CD83$^+$ cells with DC morphology and lymphocyte morphology.

In situ tetramer staining combined with immunohistochemistry displayed the two types of cells based on the morphology of the membrane. In each set of panels, the left images (A and D) are CD83 antibody stain (green), the middle image (B and E) are CD3 antibody stain (blue), and right images (C and F) are merged images of the left and middle images. Upper panels (A and C) show DC morphology CD83$^+$ cells that are CD3 negative and bottom panels (D and F) show lymphocyte morphology CD83$^+$ cells that are CD3 negative. Images are all confocal Z-scans collected using a 60X objective with a 3X zoom. Bar, 50 µm.

Increased number of CD83$^+$ cells in situ after the challenge with pathogenic SIV

To determine whether the challenge of pathogenic SIV causes the change in the number of CD83$^+$ cells, we examined the concentration of CD83$^+$ cells in situ during the time frame after the challenge. First, I defined the morphology of CD83$^+$ cells as being DCs or lymphocytes as presented in Figure 18. In spleen from three vaccinated and challenged animals, CD83$^+$ cells with DC morphology or lymphocyte morphology were 1.6- to 5.14-fold more concentrated compared to before the challenge (Figure 19). Thus, in SIVΔnef immunized animals, there was an increase in the concentration of CD83$^+$ cells in spleen after the challenge with pathogenic SIV.

To further address the anatomical localization of CD83$^+$ cells within the spleen, we quantified cells as being within the T cell zone or within B cell follicles, based on the CD3 staining in four animals. We found that CD83$^+$ cells with DC morphology tended to be clustered together and were mainly present in CD3$^+$ T cell area including red pulp and periarteriolar lymphoid sheaths (PALS) of spleen, while majority of lymphocyte morphology CD83$^+$ cells were frequently found in lymphoid follicles areas that showed little to no staining with CD3 antibodies and many CD83$^+$ B cells (Figure 20). In addition, there was no alteration of CD83$^+$ cells staining pattern after the challenge (Figure 20).

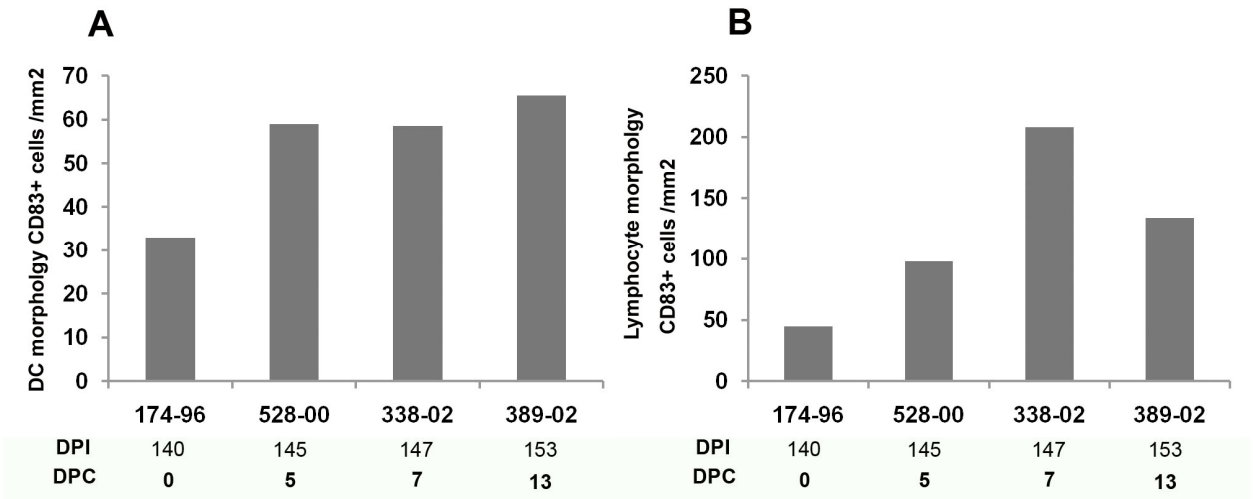
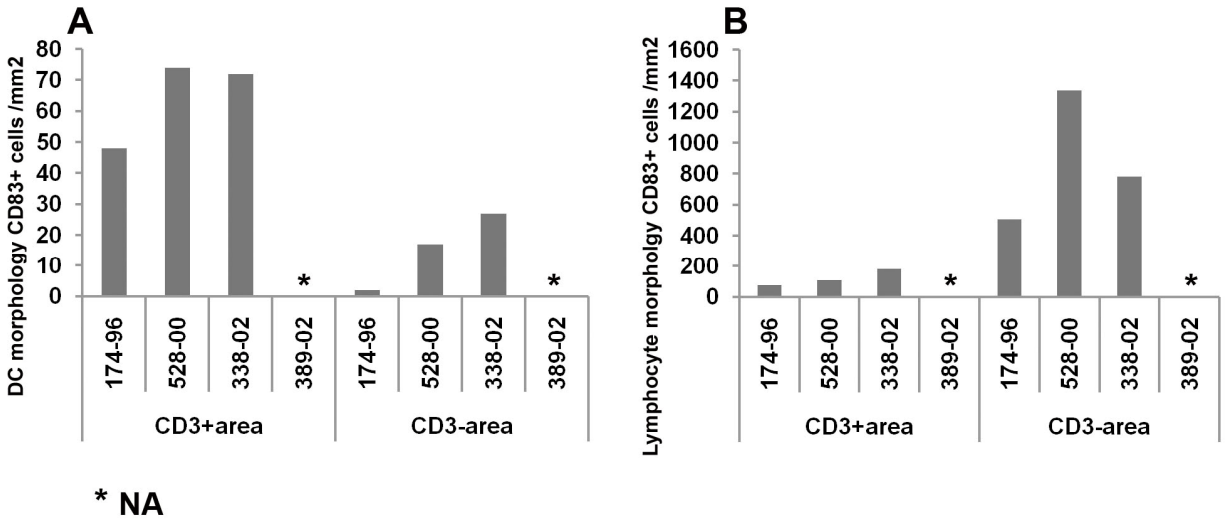


Figure 19. Concentration of CD83$^+$ cells in the spleen. This graph shows the concentration (cells/mm^2) of CD83$^+$ cells with A) DC morphology and B) lymphocyte morphology found in the spleen from each animal analyzed. DPI: day post infection with SIVΔnef; DPC: day post challenge with SIVmac251.



* NA

Figure 20. Concentration of CD83$^+$ cells in T cell zones and B cell follicles of spleen sections. This graph shows the concentration (cells/mm^2) of CD83$^+$ cells with A) DC morphology and B) lymphocyte morphology found in CD3 rich T cells zones and in CD3

low/negative regions of B cell follicles of spleen sections. *NA indicates CD3 staining was “Not Available.

DC morphology CD83$^+$cells undergo the increase in the interactions with SIV-specific T cells after the challenge with pathogenic SIV

To determine whether there was a change in the number of interactions between CD83$^+$ cells and SIV-specific T cells in vaccinated animals after challenge with pathogenic SIV, I next examined physical interactions in the spleen tissues before and after the challenge. In spleen tissue sections stained with Gag CM9 or Tat SL8 tetramers and anti-CD83 antibodies, I counted the number of cell-cell contacts made between tetramer stained SIV-specific red cells and CD83+ green cells. The interactions with SIV-specific T cells were quantified for both CD83$^+$ cells with DC morphology and lymphocyte morphology in spleens of all animals (Figure 21). Interactions between CD83$^+$ cell with DC morphology and SIV-specific T cells were typically found localized in T cell zones (areas enriched in CD3$^+$ cells), whereas interactions between CD83$^+$ cells with lymphocyte morphology and SIV-specific T cells were mainly detected in the B cell follicles (CD3 little or negative areas) in the spleen (Figure 22). We also found that, in the spleen of animals that were vaccinated with SIV and challenged with pathogenic SIV, the percentage of tetramer$^+$ cells that were interacting with CD83$^+$ cells with DC morphology was 2.2 fold to 5 fold more than that in the non-challenged animal (Figure 23). However, the percentage of tetramer$^+$ cells that were interacting with CD83$^+$ cells with lymphocyte morphology was increased in only one vaccinated/challenged animal relative to the non-challenged animal (Figure 24). In addition, the vaccinated and

challenged animals showed a higher percentage of tetramer-stained cells interacting with $CD83^+$ cells with DC morphology (13 to 30%), than tetramer-stained cells with $CD83^+$cells with lymphocyte morphology (0 to 11%). Both Tat28-35 SL8 (Tat SL8) and Gag CM9 tetramers stained cells contact a subpopulation of $CD83^+$ cells in situ, indicating that the interaction with $CD83^+$ cells was not specific for one particular type of antigen-specific T cell (Figures 23 and 24). Thus, in SIVΔnef immunized animals, there was an increase in the number of interactions of $CD83^+$ cells, particular DCs, with SIV-specific T cells in spleen after the challenge.

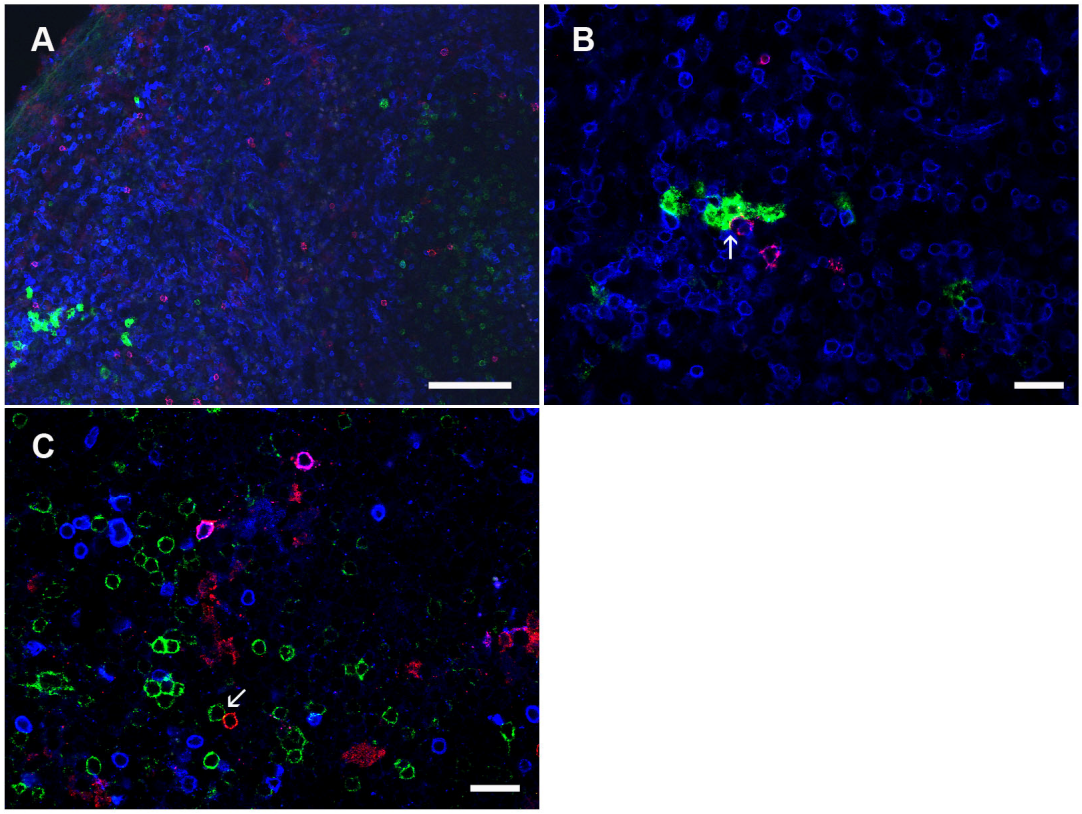


Figure 21. In situ detection of interactions between CD83$^+$ cells and SIV-specific T cells in the spleen. Representative staining patterns are shown for CD83$^+$ cells (green), Mamu-A*01 Gag CM9-specific T cells (red) and CD3$^+$ cells (blue) in the spleen (A-C). The representative images are from SIVΔnef -infected rhesus macaque #174.96 at 140 days post-vaccination. Arrows indicate interaction between CD83$^+$ cells with DC morphology and tetramer-stained cells (B) and between CD83$^+$ cells with lymphocyte morphology and tetramer-stained cells (C). All images are confocal Z-scans collected using a 20X (A) or 60X (B and C) objective. In A, the scale bar corresponds to 100 micron; in B-C, to 20 micron.

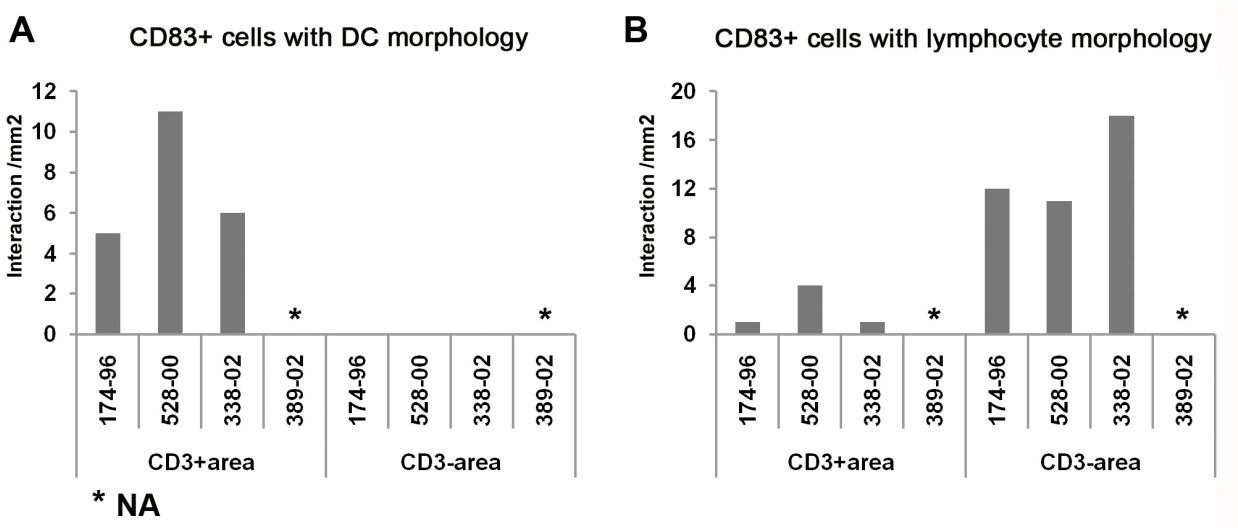


Figure 22. Quantification of interaction between CD83$^+$ cells and SIV-specific T cells in T cell zones and B cell follicles of spleen sections. (A) Interaction between CD83$^+$ cells with DC morphology and Mamu-A*01 Gag CM9 or Tat SL8-specific T cells (B) Interaction between CD83$^+$ cells with lymphocyte morphology and Mamu-A*01 Gag CM9 or Tat SL8-specific T cells. *NA indicates "Not Available".

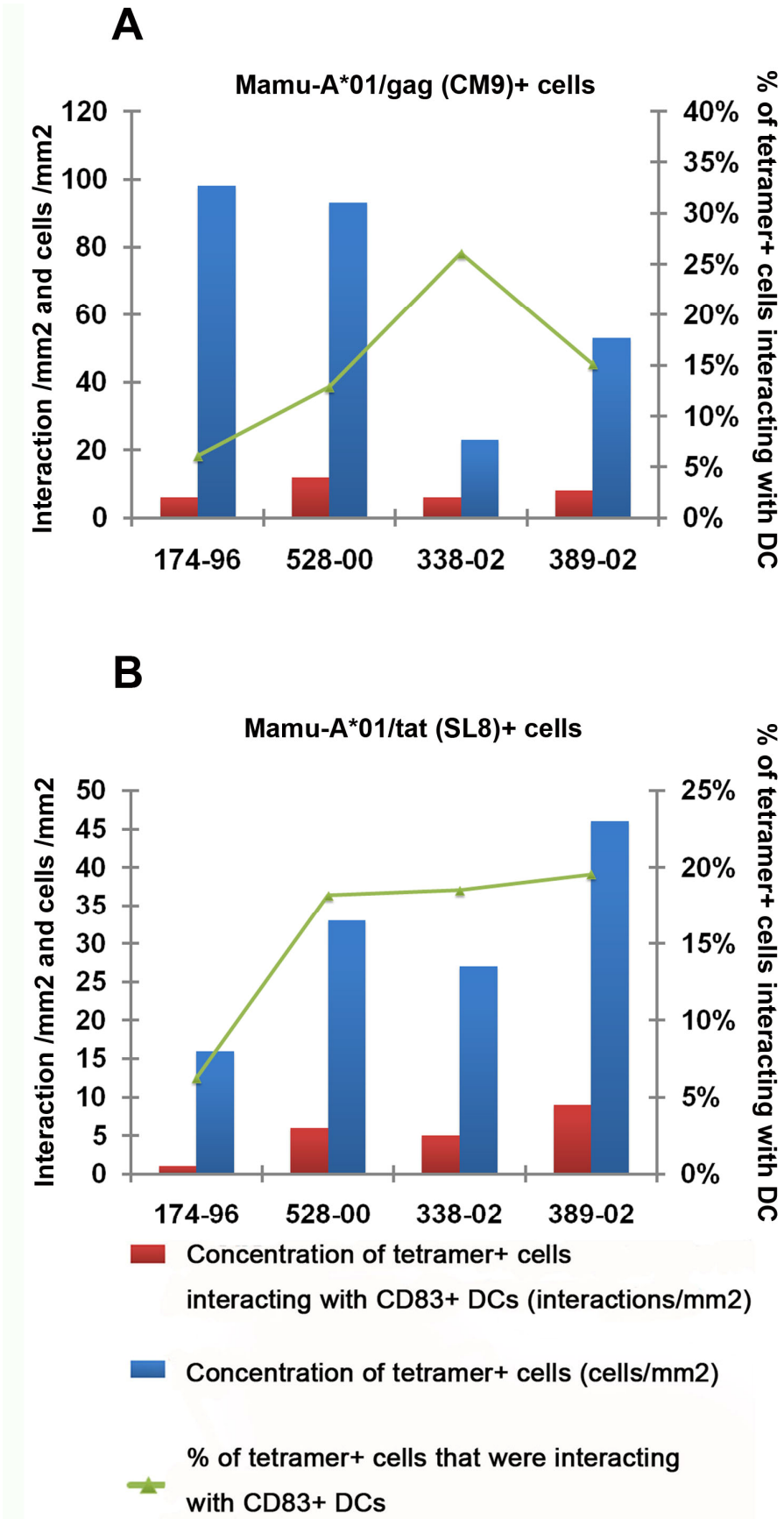

A
Mamu-A*01/gag (CM9)+ cells
Interaction /mm2 and cells /mm2
% of tetramer+ cells interacting with DC
120
100
80
60
40
20
0
40%
35%
30%
25%
20%
15%
10%
5%
0%
174-96
528-00
338-02
389-02
B
Mamu-A*01/tat (SL8)+ cells
Interaction /mm2 and cells /mm2
% of tetramer+ cells interacting with DC
50
45
40
35
30
25
20
15
10
5
0
25%
20%
15%
10%
5%
0%
174-96
528-00
338-02
389-02
Concentration of tetramer+ cells interacting with CD83+ DCs (interactions/mm2)
Concentration of tetramer+ cells (cells/mm2)
% of tetramer+ cells that were interacting with CD83+ DCs

Figure 23. Quantification of in situ interactions between CD83$^+$ cells with DC morphology and SIV-specific T cells. (A) Interactions between CD83$^+$ cells with DC morphology and Mamu-A*01 Gag CM9-specific T cells (B) Interaction between CD83$^+$ cells with DC morphology and Mamu-A*01 Tat SL8-specific T cells.The graphs show the concentration of tetramer$^+$ cells (blue bars), the concentration of tetramer$^+$ cells interacting with CD83$^+$ DCs (red bars) and the percentage of tetramer$^+$ cells interacting with CD83$^+$ cells with DC morphology.

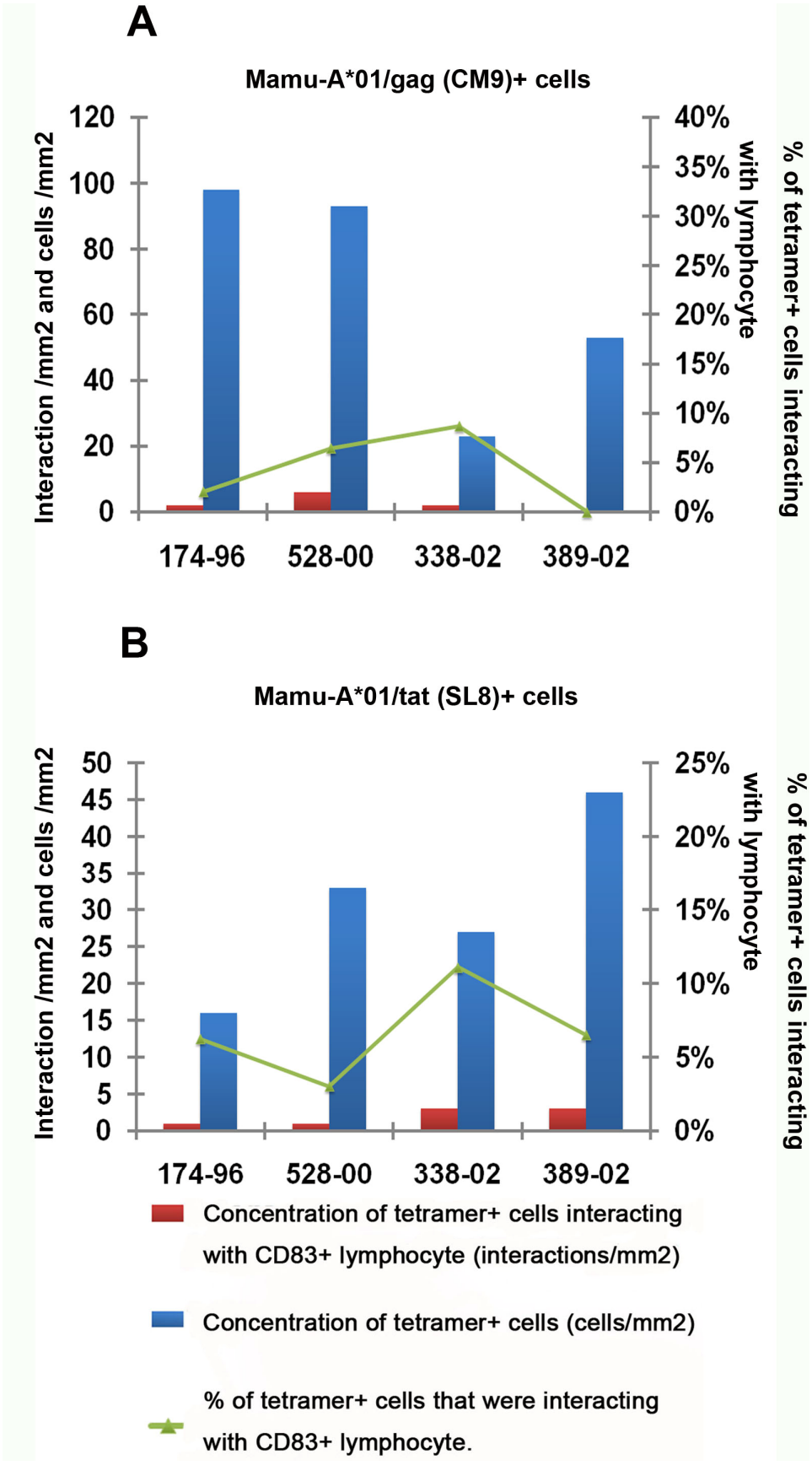

A
Mamu-A*01/gag (CM9)+ cells
Interaction /mm2 and cells /mm2
% of tetramer+ cells interacting with lymphocyte
120
100
80
60
40
20
0
40%
35%
30%
25%
20%
15%
10%
5%
0%
174-96
528-00
338-02
389-02
B
Mamu-A*01/tat (SL8)+ cells
Interaction /mm2 and cells /mm2
% of tetramer+ cells interacting with lymphocyte
50
45
40
35
30
25
20
15
10
5
0
25%
20%
15%
10%
5%
0%
174-96
528-00
338-02
389-02
Concentration of tetramer+ cells interacting with CD83+ lymphocyte (interactions/mm2)
Concentration of tetramer+ cells (cells/mm2)
% of tetramer+ cells that were interacting with CD83+ lymphocyte.

Figure 24. Quantification of in situ interaction between CD83$^+$ cells with lymphocyte morphology and SIV-specific T cells. (A) Interaction between CD83$^+$ cells with lymphocyte morphology and Mamu-A*01 Gag CM9-specific T cells (B) Interaction between CD83$^+$ cells with lymphocyte morphology and Mamu-A*01 Tat SL8-specific T cells. The graphs show the concentration of tetramer$^+$ cells and the percentage of tetramer$^+$ cells interacting with lymphocyte morphology CD83$^+$ cells.

Discussion

During acute HIV/SIV infection (232, 233), there is an increase in the number of DCs in lymph node because of migration of epidermal Langerhans cells from the skin to the lymph node. In acute SIV infection, expression of $CD83^+$ interdigitating DCs was significantly increased in the lymph node as compared with normal animals or animals with AIDS (232). Presumably, the same situation exists in the spleen. In addition, pathogenic SIV challenge showed an increase in DC-$LAMP^+$ and $CD83^+$ DCs in the mucosal lymphoid tissue and lymph nodes which are active sites for viral replication at early time points in rhesus macaques immunized with SIVΔnef (234)**.** Thus, the $CD83^+$ with DC morphology that I observed are presumably mature DCs that may have originated from Langerhans cells in the female genital tract where pathogenic SIV first contacted DCs in the vaginally-challenged animals in our study. This suggestion is supported by Zhou and his colleagues' finding ((220, 225)). Using the immunohistologic analysis, they showed that two phenotypically distinct types of $CD83^+$ cells have been identified from the blood and lymphoid tissues ((220, 225)). One showed strong CD83 staining and expressed the DC marker CD1 and was found in the T cell zone of lymphoid tissues, whereas another showed weak CD83 staining and expressed CD11c in the follicular mantle. In this study, I refer to the former cells showing unique cellular morphology (235) as $CD83^+$ cells with DCs morphology and the latter as $CD83^+$ cells with lymphocyte morphology. I also found that, in all animals, $CD83^+$ cells with DC morphology were mainly localized in CD3 rich area including red pulp and periarteriolar

lymphoid sheaths (PALS) of spleen, while $CD83^+$ cells with lymphocyte morphology were concentrated in CD3 low or negative area including lymphoid follicles. These previous studies may explain our findings that, using our approach, CD83 expressed cells can be distinguished not only by the phenotypic features but also by the anatomical localization respectively.

Several studies have implicated the function of $CD83^+$ cells as being antigen presenting cells. For example, virus-mediated (herpes simplex virus type 1 and human cytomegalovirus) down-regulation of CD83 expression resulted in the reduction of the capacity for the human DCs to stimulate T cells (236, 237). The direct interference of CD83 molecules using Ig fusion proteins resulted in the inhibition of antigen-specific T cell activation in mice (238). In addition, down-regulation of CD83 expression by CD83 siRNA transfection showed impaired stimulation of $CD8^+$ T cell lines, while CD83 overexpression by CD83 mRNA transfection showed better stimulation of CD8 T cell lines (239). Taken together, these in vitro findings indicate that CD83-expressed on cells may modulate T cell activation including memory T cells during virus infection. It also suggests that CD83-expressing cells could be T cell stimulators in SIVΔnef -immunized rhesus macaques after challenge with pathogenic SIV.

Many imaging studies have shown that physical contacts of DCs, particularly mature DCs, with $CD8^+$ T cells in lymphoid tissues (231, 240, 241). Recent evidence has shown that mature DCs stimulate proliferation and activation of HIV/SIV specific $CD8^+$ T cells (217, 242), suggesting the important role of antigen presentation to CD8 T cells by mature DCs in the protection against HIV/SIV infection. In the present study, I quantified the physical interactions between CD83-expressing cells and SIV-specific T

cells in the spleen of animals immunized with SIVΔnef before or after the pathogenic SIV challenge. I found that three vaccinees challenged with pathogenic SIV showed higher concentrations of SIV-specific T cells interacting with $CD83^+$ cells with DCs morphology compared to an immunized animal that was not challenged with pathogenic SIV. This indicates that vaccine-induced protection may be influence by the interaction between DCs and SIV-specific T cells. However, interestingly, we found that there was no substantial expansion of SIV-specific T cells in SIVΔnef -immunized and SIVmac251-challenged animals (Figure 12). This finding is in agreement with our recent data (174) (Figure 9) showing that protection afforded by SHIV89.6 immunization does not require the robust expansion of SIV-specific T cells in lymphoid tissues as well as genital tissues. Our previous findings showed that SHIV89.6-induced SIV-specific CD8 T cells were polyfunctional CD8 T cells with the ability to secrete more than one immune mediators such as IFN-r, IL-2, and TNF (174, 175) and suggests the possibility that mature DCs stimulate SIV-specific T cells to differentiation into polyfunctional CD8 T cells without extensive proliferation of SIV-specific CD8 T cells.

In summary, I have quantified interactions of $CD83^+$ DCs with SIV-specific T cells in SIVΔnef-immunized rhesus macaques before or after the challenge of pathogenic SIV. I found increased numbers of interactions of mature DCs with SIV-specific T cells in T cell rich areas of lymphoid tissues. Although the small sample size in this study does not allow a conclusive statement on this change, my preliminary results imply that these interactions may modulate SIV-specific T cell function that contributes to protection against pathogenic SIV. The further study on the function of CD83 expressed cells

interacting with T cells will provide a better understanding of immune correlates involved in the protection afforded by HIV/SIV future vaccines.

Conclusions

Here I performed vaccine studies with two live-attenuated lentiviruses to indentify immunologic correlates of protection in order to understand what is needed to develop a successful HIV vaccine. In my thesis studies, I have investigated the localization, abundance, and phenotype of virus-specific CD8 T cells in tissues from rhesus macaques vaccinated with SHIV89.6 and SIVΔnef and challenged with pathogenic SIV using in situ tetramer staining, confocal microscopy and quantitative image analysis. First, I found that early localization of virus-specific CD8 T cells was observed in lymph node and genital tract of vaccinated but not unvaccinated macaques and that vaccine-mediated protection against pathogenic SIV was CD8 T cell dependent. Further, the majority of early localized SIV-specific CD8 T cells in vaccinated animals challenged with pathogenic SIV showed little to no perforin expression, compared to non-vaccinated animals infected with pathogenic SIV. Finally, I found that vaccinated animals showed an increase in the interaction of vaccine-induced SIV-specific T cells with $CD83^+$ dendritic cells after pathogenic SIV challenge. Genesca *et al.* recently found that SHIV immunized animals elicited and maintained populations of central and effector memory T cell subsets after challenge with pathogenic SIV (174, 205). These vaccinated animals showed more polyfuntional virus-specific CD8 T cells, especially with the ability to secrete multiple cytokines such as interleukin-2, interferon-γ, and TNF-α, leading to better control of pathogenic SIV replication than unimmunized SIV control animals (205). These results

together with my findings indicate that a successful HIV vaccine needs to induce a sustained population of polyfunctional memory HIV-specific CD8 T cells with little to intracellular perforin localized at the portal of viral entry and in lymph nodes. Additionally my studies suggest that a successful HIV vaccine will induce increased interactions of HIV-specific CD8 T cells with mature dendritic cells after infection with HIV. Future studies further characterizing the memory T cell population induced by live attenuated lentivirus vaccines may provide a better understanding of protective mechanism of SIV-specific T cells in controlling infection with pathogenic SIV and what is needed to create a successful HIV vaccine. The model presented below in Figure 25 illustrates these points.

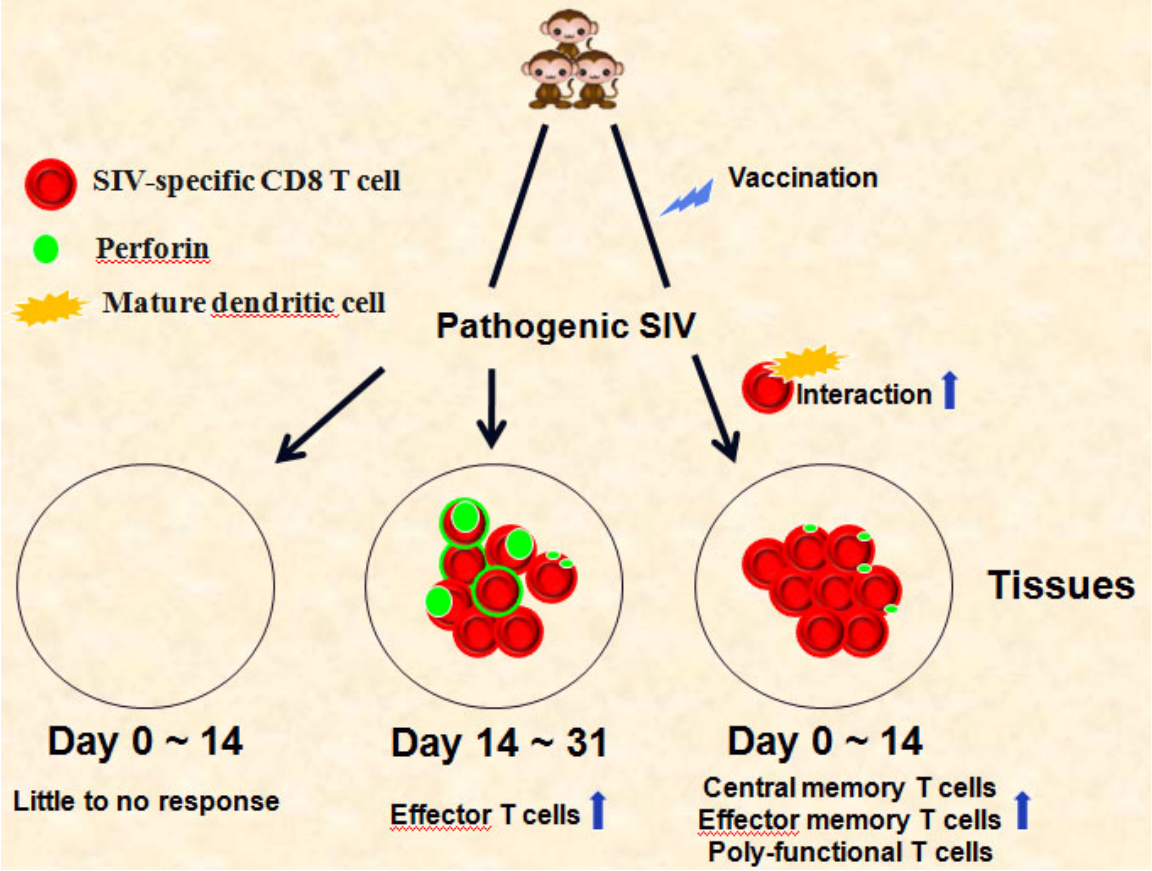


Figure 25. SIV-specific CD8 T cells response in tissues correlates with protection from challenge with pathogenic SIV. Unimmunized control animals do not develop an SIV-specific CD8 T cells response that is detectable in situ until after 14 days post-infection which is after SIV has spread systemically. In contrast, live-attenuated SIV-vaccinated animals show SIV-specific CD8 T cells localized to the portal of viral entry and in lymph nodes at the time of challenge with pathogenic SIV. Most SIV-specific T cells in genital and lymphoid tissues in vaccinated animal are likely memory T cells including central memory T cells and effector memory T cells that have the ability to secrete multiple cytokines (polyfunctional T cells) and show little to no intracellular perforin. In addition, it is possible that vaccine-induced memory T cells may be stimulated by the interaction with mature dendritic cells after SIV challenge.

Chapter 6: Publications

The following publications are included as supplementary documents in the online submission of this dissertation and are included in the printed version of this dissertation.

Greene, J.M.; Burwitz, B.J.; Blasky, A.J.; Mattila, T.L.; **Hong, J.J**.; Rakasz, E.G.; Wiseman, R.W.; Hasenkrug, K.J.; Skinner. P.J.; O'Connor, S.L.; O'Connor, D.H. 2008. Allogeneic lymphocytes persist and traffic in feral MHC-matched Mauritian cynomolgus macaques. *PLoS ONE*, 2008, 6, e2384.

Genescà, M.; Skinner, P.J.; **Hong, J.J**.; Li, J.; Lu, D.; McChesney, M.B.; Miller, C.J. 2008. With minimal systemic T cell expansion, CD8+ T cells mediate protection from vaginal SIV challenge in rhesus macaques immunized with attenuated SHIV89.6. *The Journal of Virology*, 2008, 82(22):11181-96

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