THE ROLE OF HIV- AND SIV-SPECIFIC CD8+ T CELLS IN THE
ESTABLISHMENT OF PERSISTENT HIV AND SIV INFECTIONS

A DISSERTATION
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Dr. Pamela J. Skinner, Adviser

December 2009

Acknowledgements

I would like to thank the following people, without whom this journey would not have been possible…

My Lord and Savior Jesus Christ, for never letting me down…

Dr. Pamela J. Skinner, for your infinite patience and understanding, as well as your exemplary teaching skills: you taught me to be a scientist, and in the process, helped me to become a better person as well.

The many Skinner lab team members for providing technical assistance, support, encouragement, and friendship…

My committee members: Dr. Michael Murtaugh, Dr. Stephen Jameson, and Dr. Bruce Walcheck, for providing excellent guidance, feedback, and support…

The Comparative and Molecular Biosciences graduate program for giving me the opportunity to work in a supportive environment…

Juni and Jan, for helping me to find myself and stay sane in the process…

My husband Brad and my children Jake, Carlina, and Robert for their unending love, support, encouragement, advice, sacrifice, and motivation…

My cousin Julie—I couldn't have done this without you.

My mom, my stepfather, my sisters Kelly and Kris, and my dad in heaven—thank you for believing in me.

Dedication

This dissertation is dedicated to my family…past, present, and especially, future.

Abstract

CD8$^+$ T cells are important in controlling viral infections. Although the appearance of HIV-specific CD8$^+$T cells initially correlates with reduced viral load during HIV infection, for unknown reasons HIV is never fully cleared from the body. My central hypothesis is that B cell follicles are sites in which virus-producing cells are protected from virus-specific CD8$^+$T cells. During the chronic stages of infection the majority of HIV-producing cells accumulate in B cell follicles. The localization and abundance of HIV-specific CD8$^+$T cells relative to B cell follicles is not known. For these studies I determined the spatial localization of HIV and SIV-specific CD8$^+$T cells relative to B cell follicles in lymph nodes from HIV-infected humans and SIV-infected rhesus macaques, using immunohistochemistry, in situ tetramer staining, confocal microscopy, and quantitative image analysis. My findings show that most HIV-specific CD8$^+$T cells were concentrated in T cell zones and were largely excluded from areas within B cell follicles where HIV is concentrated. Because many similarities exist between HIV infection in humans and SIV infection in macaques, and SIV model systems are essential tools to understanding HIV/SIV infections and for the development of HIV vaccines, we set out to determine whether the exclusion of virus-specific CD8$^+$T cells from B cell follicles also occurs in the SIV/rhesus macaque model of HIV infection. I found in a small cohort of animals that during the early and late stages of SIV infection, SIV-specific CD8$^+$ T cells were concentrated in T cell zones of lymph nodes, and that within B cell follicles, concentrations of SIV-specific CD8$^+$T cells were significantly lower than in T cell zones. Most B cell follicles showed an absolute exclusion of SIV-specific T cells from more than half of the B cell follicle area where SIV concentrates. These data support the hypothesis that B cell follicles are an immune privileged site in which HIV/SIV-producing cells are protected from HIV/SIV-specific CD8$^+$T cells. These data have important implications for the development of a successful HIV vaccine and treatments to eradicate HIV.

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Chapter 1. Introduction to HIV and AIDS

Human immunodeficiency virus (HIV) has caused one of the worst pandemics in the history of mankind. Over 30 million people have died of HIV/AIDS-related infections since 1981 (1). It is estimated that, currently, more than 33 million people worldwide are infected with HIV (1). Despite intensive efforts on the part of researchers, physicians, and educators, the incidence of HIV infections worldwide continues to climb. It is imperative that efforts to stem the transmission of HIV continue if this pandemic is to be stopped.

HIV infection destroys the human immune system. The virus preferentially replicates in $CD4^+$ T cells within lymphoid tissues (2, 3). $CD4^+$ T cells are a vital component of the adaptive immune response. When $CD4^+$ T cell populations are diminished, the body's capacity to fight off infection is severely compromised. This enables a myriad of pathogens to invade, eventually leading to debilitating infections, and ultimately, the death of the patient.

The Discovery of HIV-1

Kaposi's sarcoma is a form of relatively benign cancer that usually only affects the elderly. However, in early 1981, at least 8 cases of this cancer had been reported in

young homosexual males in New York (4, 5). At around the same time, an increase in a rare form of pneumonia, *Pneumocystis carinii* pneumonia (PCP) was being reported in young homosexual males in both California and New York (4, 6). The Centers for Disease Control and Prevention (CDC) reports that followed are commonly referred to as heralding the beginning of the AIDS epidemic, although it would be more appropriately referred to as the event that brought AIDS into the eyes of the general public.

Because the causative agent was as yet unknown, theories were developed as to what might be causing this increase in formerly rare diseases. Viruses such as cytomegalovirus were suspect (6), as were the ingestion of street drugs known as 'poppers' (7), and a condition known as 'immune overload' (8). Various names were attributed to the syndrome. Most of these, such as gay-related immune deficiency (GRID), gay cancer, gay compromise syndrome, and community-acquired immune dysfunction (8, 9) continued to link the problem to its initial occurrence in gay men. However, as cases of the ailment in non-homosexual intravenous drug users began to surface, it became evident that this was no longer just a gay man's problem (10). The name 'acquired immune deficiency syndrome', or AIDS, was coined in July of 1982. It was deemed appropriate because the immune deficiency is acquired and not hereditary, and consisted of various manifestations and not a single disease (8).

Shortly thereafter, several people, including a 20-month-old child, died from AIDS-related infections after having received blood transfusions (10). The first instance of mother-to-child transmission was also reported at this time (11). It was becoming all

the more evident that whatever was causing AIDS was transmissible in blood as well as sexual fluids.

In May of 1983, doctors at the Institute Pasteur in France announced that they had isolated a virus that they suspected may be the cause of AIDS. The virus was named lymphadenopathy-associated virus (LAV) by scientists at the Centers for Disease Control because of its association with swollen lymph nodes (12).   Samples of the virus were later sent to the National Cancer Institute (NCI). Nearly one year later, scientist Robert Gallo of the NCI announced that he and his colleagues had discovered the cause of AIDS: a virus which was named human T-lymphotropic virus, or HTLV-III (13). It was soon realized that LAV and HTLV-III were one and the same. Many arguments between the two groups ensued over what to officially name the virus. It was finally decided by the International Committee on the Taxonomy of Viruses in 1986 that both LAV and HTLV-III should be dissolved as names, and a new name was instituted: human immunodeficiency virus, or HIV (14).

Origins of HIV-1 and HIV-2

There are two main types of HIV: HIV-1 and HIV-2. HIV-1 is by far the most common type of HIV, and can be further subdivided into groups M, N, and O. Over 95% of HIV infections worldwide are caused by HIV-1 group M (15, 16, 17).   HIV-2 is much more rare, less easily transmissible and less virulent than HIV-1 (17). HIV-2 is confined to small groups of people in West Africa (17).

HIV is a lentivirus, which means 'slow virus'. Lentiviruses are so named because they take a long time to cause disease after infection. Lentiviruses have been found that infect sheep, horses, cattle, and other mammals (18, 19). Of special interest to the present study, however, are the non-human primate lentiviruses: the simian immunodeficiency viruses (SIVs). It is commonly believed that HIV originally came from SIVs. Much evidence led to the conclusion that HIV-2 came from SIV in sooty mangabeys (SIVsm) (20). The origin of HIV-1 was much more difficult to determine. However, in 1999, a group of researchers from the University of Alabama announced that they had isolated a chimpanzee SIV (SIVcpz) that was nearly identical to HIV-1 (21). It has since been determined that HIV-1 groups M and N originated in SIVcpz from Gabon (22) and that HIV-1 group O originated in gorillas (23). It is possible that these viruses were passed from non-human primates to humans via the butchering and consumption of the animals for meat.

The Global Impact of HIV-1 (referred to as HIV from this point on)

More than 30 million people have died of AIDS-related infections since 1981 (1). Currently, it is estimated that approximately 33 million people worldwide are living with HIV infection (1). Perhaps the most disturbing element of this fact is, without some major breakthroughs in prevention or treatment options, the vast majority of those currently infected will be dead within 10 years. And in spite of the education, research,

and medical advances that have been made over the past 27 years, the incidence of HIV infection continues to climb (1).

HIV/AIDS is not evenly spread throughout the world. The majority of HIV/AIDS cases are seen in sub-Saharan Africa, where more than two-thirds of the 33 million cases of HIV/AIDS worldwide are concentrated, even though this region contains less than 10% of the world's population (1). In the African countries of Botswana, Lesotho, Swaziland, and Zimbabwe, >20% of the adult population is HIV+, a higher number than was thought possible (1). Families, the educational system, business and economic industries are all significantly affected by the HIV/AIDS pandemic. HIV/AIDS is literally changing the entire socioeconomic structures of some nations (1).

The Structure of HIV

HIV is a retrovirus (24). Retroviruses are enveloped viruses that use single-stranded RNA as their genetic material (24). The RNA is transcribed to cDNA, inserted into the host cell chromosome, and then replicates as part of the host cell (24). This is the hallmark of the retrovirus (24).

HIV belongs to the genus of retroviruses called lentiviruses, from the Latin word *lentus,* which means 'slow.' This nomenclature reflects the fact that lentiviruses take a long time to produce symptoms of disease once the host is infected (14).

As shown in Figures 1 and 2, each virion contains two copies of an RNA genome protected by a nucleocapsid, along with the viral proteins reverse transcriptase, protease,

and integrase (25). All of this is surrounded by a structural protein called the matrix (p17) protein, which, in turn, surrounds a capsid made of p24 protein (25). This entire structure is wrapped in a lipid envelope, which the virus acquires from the host cell as it buds out (25). Embedded in the envelope are the proteins known as gp120 and gp41. The gp120 and gp41 proteins are associated with one another as dimers, and groups of 3 dimers are assembled as about 72 trimers on the surface of the virion (26) , (Figure 2).

Like all retroviruses, HIV has 3 major genes: *gag, pol*, and *env*. The *gag* gene is translated to produce polyproteins, which are cleaved to components that produce the viral structural proteins: the matrix protein, the capsid protein, and the nucleocapsid protein (27). The *pol* gene is translated into polyproteins, which ultimately produce the viral reverse transcriptase, integrase, and protease. These proteins are vital to the virus making more copies of itself (27). The *env* gene encodes proteins that are found on the surface of the virion: the gp120 and gp41 spike proteins, which enable the virus to bind to and enter a host cell (26). HIV also encodes six smaller genes: *tat, rev, nef, vpr, vpu*, and *vif*. The *tat* and *rev* genes encode the tat and rev proteins. These proteins are important in promoting viral replication in activated CD4$^+$ T cells (27). The *nef* gene encodes the nef protein. Nef promotes several functions which increase viral infectivity. Some of these include mediating the activation of CD4$^+$ T cells, and promoting down-regulation of MHC class I and other molecules on the surface of T cells (28). The *vpr, vpu*, and *vif* genes encode the vpr, vpu, and vif proteins, respectively. These genes are important for, among other things, affecting viral infectivity, enhancing virion production, and releasing new viral particles from the cell membrane (27).

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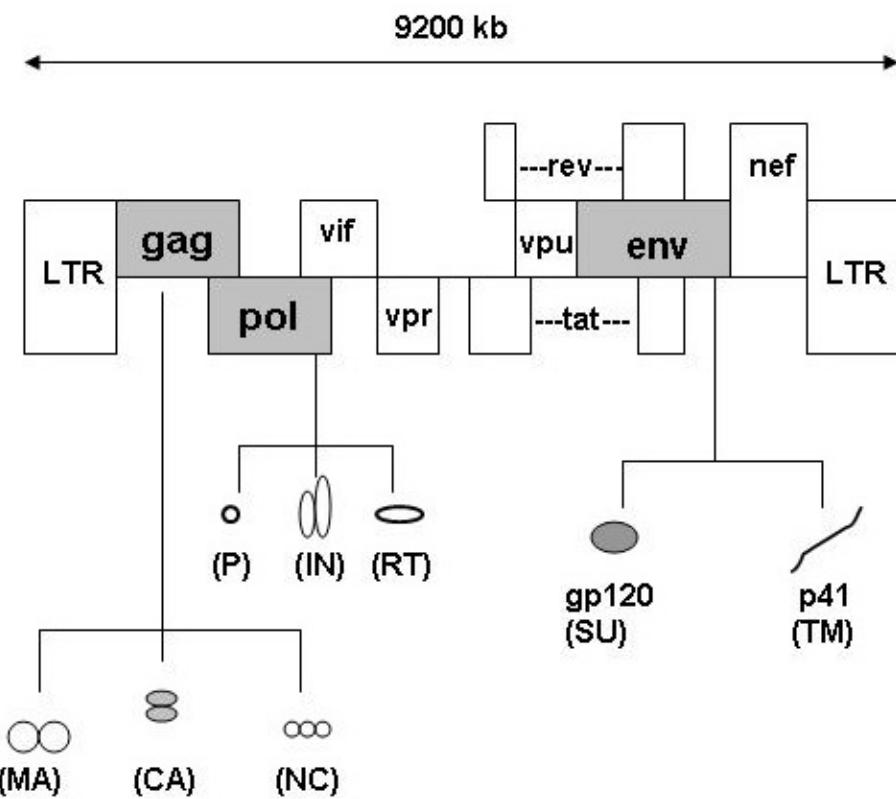
Figure 1. The HIV genome.   The *gag* gene codes for the main structural proteins, which include the capsid (CA), the matrix (MA), and the nucleocapsid (NC). The *pol* gene codes for reverse transcriptase (RT), integrase (IN) and protease (P). The *env* gene codes for the cell surface protein gp120 (SU) and for the transmembrane protein gp41 (TM). The accessory proteins include viral infectivity factor (vif), viral protein U (vpu), viral protein R (vpr), and negative effector (nef). Regulatory proteins include tat and rev. Each of these proteins is encoded by the gene of the same name, respectively. The genome is flanked on either side by long terminal repeats (LTR)**,** which play a role in the

initiation of transcription. Figure adapted from (27).
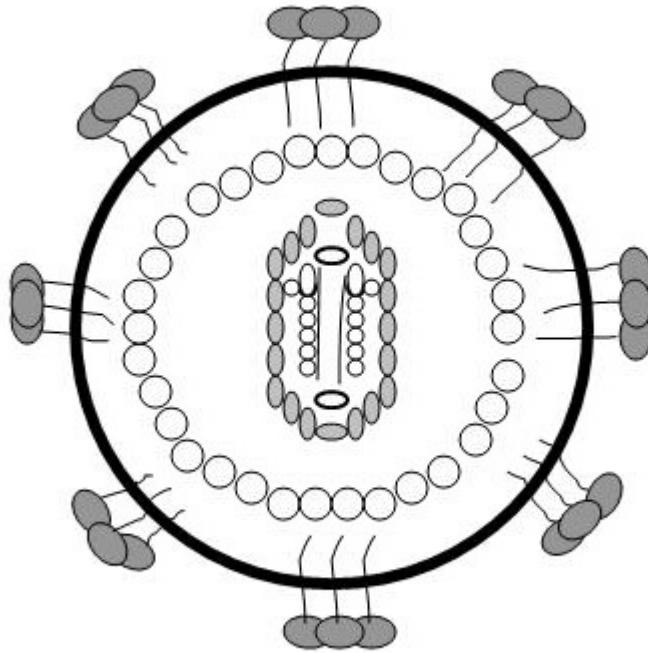
Figure 2. Structure of HIV. Each HIV virion contains integrase, reverse transcriptase, and protease proteins, as well as 2 copies of an RNA genome, which is protected by nucleocapsid proteins.   All of this is surrounded by the capsid protein, which in turn is surrounded by a matrix protein. The entire structure is wrapped in a lipid envelope, which the virus obtains from the host cell as it buds out. Embedded in this envelope are the envelope or 'spike' proteins gp120 and gp41. Key to parts of Figure 2 are shown in Figure 1. Figure adapted from (25, 26).

HIV Life Cycle

The life cycle of HIV begins when the gp120 and gp41 envelope proteins on the

surface of the virion bind to CD4 molecules on the surface of a host cell. While some

macrophages and dendritic cells also express CD4 on their surface, the vast majority of

cells expressing this molecule are, of course, $CD4^+$ T cells (29). Besides the CD4

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molecule, the viral envelope proteins must also bind to a co-receptor, which, depending on the tropism of the specific virion, can be either the chemokine receptor CXCR4 or CCR5 (30). Once the envelope proteins are engaged with the CD4/chemokine receptor complex, a conformational change takes place that enables the viral contents to enter the host cell through its membrane (26).

Once inside the cell, the virus uses its own reverse transcriptase to transcribe its single-stranded RNA genome into double-stranded cDNA (31). The cDNA is inserted into the host cell chromosome by the viral integrase   It is now a part of the host cell chromosome and is known as a provirus (31). Integration can lead to either a latent infection or a transciptionally active infection (27, 32).

When the host cell becomes activated and begins to divide, the viral genetic material is transcribed and translated into viral proteins, using the host cell's machinery (27). Newly formed viral components are gathered and assembled into new virions in areas called lipid rafts, just below the surface of the cell membrane (33). The new virions bud from the surface of the host cell, taking along with them part of the cell membrane, into which gp120 and gp41 envelope proteins have been placed (33). The cell membrane, studded with gp120 and gp41, wraps around the virion as it buds, and becomes the viral envelope (33). The virion is now ready to find a host cell and begin the process anew.

Transmission of Virus Between Hosts

The number of ways that HIV can be transmitted between hosts is very limited, and with precautions, can be quite easily prevented. The most common mode of transmission is across mucosal barriers during vaginal, anal, or oral sex (34). Breaks in the mucosal barrier due to sexually transmitted disease, injury, or female hormonal cycle can greatly increase the chances of infection occurring via sexual activity (35-38). However, condom use can effectively block transmission of HIV (39). HIV can also be transmitted from mother to child during pregnancy, birth, or breastfeeding, however, anti-retroviral drugs given to the mother prenatally, postnatally, or during the time of breastfeeding can reduce this risk tremendously (40-44). The use of infected blood or blood products may spread the virus from person to person, but the increasing use of screening methods in both developed and less-developed countries has greatly reduced this risk (45). The sharing of dirty needles during intravenous (IV) drug use is another means by which virus is transmitted (46). For those drug users unable or unwilling to stop injecting IV drugs, it is advisable to use sterile needles and syringes (46).

Non-human primate models for HIV infection

Non-human primate models for disease pathogenesis are of vital importance to the understanding of human disease. Currently, the most commonly used non-human primate model in HIV research is the SIV-rhesus macaque model, because of its similarities to HIV-mediated pathogenesis (47). SIV is closely related in genetic structure to HIV (48). Disease progression and viral pathogenicity in the SIV-infected

rhesus macaque consists of an increase in viral load and a decline in CD4$^+$ T cell populations, leading to diminished immune function and progression to an AIDS-like illness (48, 49, 50, 51), as well as progressive changes in lymph node structure, which is very similar to that seen in humans (52-54). There are various pathogenic and non-pathogenic strains of SIV that can be used in the rhesus macaque model to answer questions relating to immune correlates of protection and progression of disease (47). The SIV-rhesus macaque model has helped to show that CD8$^+$ T cells are important in the initial control of SIV infection, and that the appearance of CD8$^+$ T cells correlates with a decrease in viral load (55). These findings corroborate what has been found in HIV-infected humans (56, 57-59).

Stages of Infection

The earliest stages of HIV infection have never been elucidated, for practical and ethical reasons. The following descriptions are based on and extrapolated from studies of acute SIV infection in macaques. Infection begins with an acute phase, during which virus disseminates from the portal of entry and rapidly replicates to set up productive infection in the lymphoid tissues. Immediately after infection, virus and virus-infected cells cross the mucosal barrier and infect previously-activated but now resting CD4$^+$ T cells in the mucosal lamina propria. Macrophages and dendritic cells in this area may also become infected (60, 61). Within days, infected cells travel through the bloodstream and lymphatic system and reach first the draining lymph nodes, then distal lymphatic

tissues, including the gut-associated lymphoid tissue (GALT) (62). Upon arriving in the lymphoid tissues, the infected cells have easy access to the large numbers of CD4$^+$ T cells that are gathered there. This association with closely-packed, easily accessible CD4$^+$ T cells causes a large burst of viral replication that reaches its peak at 10 to 14 days after infection (34). CD4$^+$ T cell populations become rapidly depleted (62, 63). By this time, a reservoir of virus has been established within the lymphoid tissues, and the host immune system is unable to clear the established pool of virus, even at this early stage of infection. At this time, the patient may not know he or she is infected with HIV, and may present to a clinician with only mild flu-like symptoms (64-67). This is followed by a latent, asymptomatic phase of disease progression, which can last for many years. Over time, the number of CD4$^+$ T cells in the blood slowly decreases. As AIDS-defining levels are reached (<200 cells/mm^3), opportunistic infections and tumors begin to set in, to which the patient eventually succumbs (34).

Current methods for prevention and treatment of HIV infection

Because HIV is primarily a sexually transmitted virus (34), the best way to avoid infection with HIV is, of course, abstinence from sexual activity. For sexually active individuals, latex condoms, when used properly, are a very effective means by which to prevent transmission of HIV (39). Remaining sexually faithful to one partner helps reduce the risk of becoming infected. Avoiding sexual relations with individuals in 'high risk' groups such as paid sex workers and IV drug users may reduce the risk of becoming infected. For IV drug users, it is important not to share needles with others (46).

Education is also very important in the slowing of HIV transmission. Individuals who are educated about preventive measures are more likely to use precautions against becoming infected, and if already HIV+, against infecting others (68).

Once an individual is infected with HIV, there is no cure. There are many treatment options, however, that can reduce viral load and symptoms of HIV infection. Antiretroviral drugs are so named because of their effects on retroviruses. Several different classes of antiretroviral drugs are available, distinguished according to the part of the retrovirus life cycle they affect. Nucleoside reverse transcriptase inhibitors are base analogs, and stop viral replication by incorporating into the newly synthesizing nucleic acid strand (69-73). Non-nucleoside reverse transcriptase inhibitors stop HIV replication by binding to the reverse transcriptase enzyme itself and interfering with its function (74). Protease inhibitors keep large viral precursor proteins from being cleaved into smaller, functional viral proteins (75, 76). Integrase inhibitors prevent viral replication by inhibiting viral genetic material from integrating into the host cell chromosome (74). Fusion inhibitors and entry inhibitors prevent virus from entering into host cells (74). Maturation inhibitors prevent the maturation of *gag*-encoded proteins from occurring (74). Combination drugs perform a combination of the above functions. Oftentimes, a combination of 3 or more different drugs is most effective at stopping or slowing the effects of HIV infection. This combined treatment regimen is known as highly active antiretroviral therapy, or HAART (74).

There is, of course, a down-side to treatment with antiretroviral drugs. As with any drug, antiretroviral drugs often have side effects, many of which are unpleasant (77-

79). Side effects can be so unpleasant that the patient may prefer to refrain from therapy

and progress to AIDS naturally. Antiretroviral drugs may only be helpful for a period of

several months before viral mutations render the virus drug resistant (80-82). The drugs

can be very expensive and therefore are not available to all who could benefit from their

use (83). And unfortunately, in developing countries where HIV infection is highly

prevalent, antiretroviral drugs are not always accessible (83).

HIV and SIV are highly adaptable

Retroviruses are very adaptable viruses, and HIV is no exception (84). This is

due in large part to their genetic material being inherently replicated by means of reverse

transcriptase. Reverse transcriptase has no proofreading mechanism, therefore its rate of

error is many times greater than those of DNA polymerases (85). Reverse transcriptase

also has the ability to jump between templates during synthesis, so there is a high

incidence of recombination (86). Once the viral genetic material has been integrated into

the host chromosome, it is subject to the same mutations that can occur in the host's own

genetic material (85). It has been estimated that the error rate of HIV is as high as 10^{-4}

errors per base pair (87, 88). The genome of HIV is approximately 10^4 nucleotides long.

So at this rate, it could theoretically be expected that each newly infected cell would

contain a virus that differs from the previous cell by one mutation. Since millions of cells

are infected each day, this makes for an extremely high viral diversity and resultant

adaptability within a single host (85).

HIV and SIV evade host immune response

HIV has an amazing capacity for evading the host immune response. Some of these means of escape come from the aforementioned adaptability. Studies of the neutralizing antibody response to HIV infection indicate antibodies can be evaded by the virus in many ways (85). B cells do produce antibodies that bind to and neutralize HIV. But the virus mutates so quickly within a single host that, by the time antibody neutralization has occurred, virions with antigens not recognized by the present antibody repertoire have arisen, and a sterilizing antibody response does not have an opportunity to occur (89). The envelope proteins on the surface of the virions, and also on the surface of infected cells, are the most likely target for antibody-mediated immune responses (90). However, over time, the forces of selection have patterned these protein complexes in such a way as to make them as non-immunogenic as possible, while still maintaining their ability to allow attachment and entry into cells (85). Often these complexes are put together in such a way as to make epitopes physically inaccessible to antibody binding (85). It has also been hypothesized that the majority of antibodies raised against HIV are not raised against virions or infected cells, but against viral debris (90, 91).

HIV's extreme ability to mutate can also wreak havoc on the cytotoxic CD8$^+$ T cell response to infection (90). HIV forms 'escape mutants', which make recognition of virus by CD8$^+$ T cells a challenge (92, 93). This can occur through mutation of viral genes encoding important CD8 T cell epitopes. The expression of these mutations can lead to altered antigen processing (94, 95), loss of binding of the epitopes to MHC class I, or loss of T cell receptor recognition (84).

The CD4$^+$ T-regulatory cell (Treg), may also play a role in HIV's ability to exploit and outwit the immune system. T-regulatory cells are important for suppressing immune cell activation, thereby maintaining immune system homeostasis and tolerance to self-antigens (96-99). However, studies have found that, during HIV and SIV infections, Tregs may serve to knock down virus-specific CD4$^+$ and CD8$^+$ T cell responses, thereby allowing HIV-infected cells to escape immune surveillance (100-102).

Another way in which HIV may escape from the immune system is by simply being too quick for the immune response to control. Studies using the vaginally inoculated SIV-rhesus macaque system by Miller, at al (103, 104) showed that, while productive SIV infection is found in lymphoid tissues as early as 7 days post-infection, SIV-specific CD8$^+$ T cell response is not seen until 21 days post-infection, and then mainly at the portal of entry and to a lesser degree, in lymphoid tissues (103, 104). Thus, it appears as if the virus is several steps ahead of a specific immune response.

HIV may also escape immune surveillance by sequestering in B cell follicles. It is known that HIV primarily replicates in B cell follicles during the chronic stages of infection (105, 106). I hypothesized that follicles are an immune privileged site from which HIV-specific CD8$^+$ T cells are largely excluded, and this explains, in part, why HIV cannot be cleared by the immune system (107).

CD8 T cells are very important for, but not entirely effective at, controlling HIV and SIV infections

There is much evidence to suggest that CD8$^+$ T cells are important in controlling viral infections, including HIV (108-111). Although the appearance of HIV-specific CD8$^+$ T cells does initially correlate with reduced viral load during HIV infection (56, 59), for unknown reasons this response is ultimately insufficient, and the virus is never cleared from the body. Because the CD8$^+$ T cell response is important in controlling viremia, yet unable to completely eliminate virus from the system, it is important to determine why this vital component of the adaptive immune system fails to completely control HIV infection.

My central hypothesis is that virus-specific CD8$^+$ T cells cannot effectively clear virus-producing cells inside B cell follicles in secondary lymphoid tissues. My studies, done in collaboration with the lab of Dr. Elizabeth Connick, show that HIV-specific CD8$^+$ T cells are largely excluded from B cell follicles during HIV infection. My findings indicate that B cell follicles of HIV-infected human subjects appear to be immune privileged sites from which HIV-specific CD8$^+$ T cells are largely excluded. This can explain why HIV primarily replicates inside B cell follicles after the acute stages of infection and establishes a persistent infection. Because many similarities exist between HIV infection in humans and simian immunodeficiency virus (SIV) infection in macaques, I tested whether the exclusion of virus-specific CD8$^+$ T cells from B cell follicles also holds true in the SIV/rhesus macaque model system. I found that, in the rhesus macaque model as well as in humans, virus-specific CD8$^+$ T cells are largely excluded from lymphatic B cell follicles. These studies have enabled us to gain insights

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into the importance of virus-specific CD8$^+$ T cells in the establishment of persistent HIV/SIV infection.

Chapter 2. Biology of T cells, B cells, and the Lymphatic System

General Overview of T cells and B cells

CD4+ and CD8+ T cells

Naïve T cells are initially formed in the bone marrow, but must migrate to the thymus for maturation (112). As noted by Janeway, et al. and Shortman, et. al. (113, 114), upon arriving in the thymus, T cells express neither CD8 nor CD4 molecules, and are thus termed CD8$^-$CD4$^-$ T cells, or double-negative T cells. After undergoing initial maturation processes in the thymus, double-negative T cells become CD8$^+$CD4$^+$, or double-positive T cells. The double-positive T cells migrate deep into the thymic cortex, where cortical epithelial cells present them with self-derived antigens on MHC molecules. T cells that bind with proper affinity to antigen/MHC complex receive survival signals; this is known as positive selection. Those cells that do not properly bind antigen/MHC complexes die by apoptosis. Positively selected T cells that bind properly to antigen complexed with MHC class I down-regulate the CD4 antigen and become CD8+ T cells. Positively selected T cells that bind properly to antigen complexed with MHC class II down-regulate the CD8 antigen and become CD4+ T cells.   The single-

positive T cells then migrate to the edge of the thymic cortex to the thymic medulla, where they are presented again with self antigen/MHC complexes, this time on the surface of macrophages and dendritic cells. T cells which bind with too strong an affinity to self antigen/MHC complexes die by apoptosis, which helps prevent autoimmunity, in a process known as negative selection. The remaining cells then exit the thymus as naïve, mature T cells (113, 114).

CD4+ T cells

CD4$^+$ T cells play a pivotal role in the generation and maintenance of both the adaptive (cell-mediated) and the humoral (antibody-mediated) immune response. They help to mediate and direct a myriad of immune responses, including those involving CD8$^+$ T cells and B cells (115). CD4$^+$ T cells are generally categorized into T helper 1 (T$_h$1), T helper 2 (T$_h$2) (114, 115), T helper 17 (T$_h$17) (116), and T-regulatory (Treg) cells (117). T$_h$1 cells produce IFN-γ, TNF-α, and interleukin-2 (IL-2). These cytokines are important in the activation and proliferation of CD8$^+$ T cells (114, 115). T$_h$2 cells produce IL-2 as well, and also interleukins 4, 5, 6, 10, and 13. These factors are important in the activation and proliferation of B cells, and also help B cells perform class switching and increase production of antibodies (114, 115). T$_h$17 cells secrete IL-17, and play an important role in immune responses to extracellular bacteria, and are involved in the pathology of autoimmune disease (118-120). Evidence of cytolytic CD4$^+$ T cells has recently been shown, although very little is known about them (121). The CD4$^+$CD25$^+$FOXp3$^+$ T- regulatory cell plays an important role in immune system

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homeostasis, helping to maintain immunological self-tolerance, and also providing

negative control to various non-self antigens (117).

CD8+ T Cells

CD8$^+$ T cells, or cytotoxic T lymphocytes, play a key role in the adaptive (cell-

mediated) immune response against HIV as well as other viral infections. Studies of

HIV-infected human subjects have shown that patients with high numbers of HIV-

specific CD8$^+$ T cells showed slower disease progression than those with fewer numbers

(122). The appearance of HIV-specific CD8$^+$ T cells correlates with a decrease in viral

load (56, 59). This suggests that the CD8$^+$ T cell response is initially controlling virus to

some degree. The same is true in studies using SIV-infected rhesus macaques (123, 124).

Also, antibody depletion of SIV-specific CD8$^+$ T cells from rhesus macaques during SIV-

infection resulted in an increased viral load in plasma, while the reappearance of these

cells resulted in a drop in plasma viremia, indicating that SIV-specific CD8$^+$ T cells helps

control viral load (55, 125).

CD8$^+$ T cells protect the host by using an arsenal of cellular weapons, both lytic

and non-lytic. One means by which CD8$^+$ T cells can cause lysis of infected cells is by

the exocytosis of lytic molecules including perforin and granzymes. These molecules

work as a team to trigger apoptosis of the infected cell (55, 126). Apoptotic death of an

infected cell can also occur upon the ligation of Fas on its surface by Fas ligand (FasL)

on the surface of the CD8$^+$ T cell (127, 128). CD8$^+$ T cells also contribute to host

defense by non-cytolytic methods. This includes the production of molecules such as β–chemokines, macrophage inflammatory protein-1alpha and -1beta (MIP-1α and MIP-1β), and RANTES (**R**eleased on **A**ctivation, **N**ormal **T** cell **E**xpressed and **S**ecreted). These factors competitively inhibit HIV's entry into cells by binding to the CCR5 co-receptor, and also by down-regulating it (108). The release of cytokines such as interferon-gamma (IFN-γ) inhibits viral replication by suppressing transcription (109), and increases the expression of MHC class I, which increases the chance of infected cells being recognized by CD8$^+$ T cells. IFN-γ, in combination with tumor necrosis factor-alpha and beta (TNF-α and -β), activates macrophages and recruits them into sites of infection, where they engulf and destroy infected cells (108, 109). Another molecule termed CAF (**C**D8 T cell **A**ntiviral **F**actor) inhibits HIV replication by interrupting the transcription process (129).

T cell recognition of antigen

In order to perform the function of protecting the body from invading organisms, T cells need to know which cells are harboring the foreign invaders. This is accomplished by means of surface T cell receptors (TCR) and their ability to recognize peptide antigens held within MHC class I or II molecules and expressed on the surface of infected cells. These MHC-peptide complexes are an infected cell's way of announcing to the surrounding environment what is present within its cellular contents (130, 131).

Antigens, depending on the characteristics of the pathogen from which they are derived, may be presented utilizing either of two pathways. Viruses and bacteria that

replicate within the cellular cytosol are processed by a structure called a proteasome, which chops the viral or bacterial proteins into short peptides 8 – 10 amino acids in length. The peptides enter the endoplasmic reticulum by means of a sort of 'doorway' called the tap transporter. Once inside, the peptides meet up with partially folded MHC class I molecules which have been produced there. Chaperone proteins work to correctly fold the peptides and MHC molecules into a stable complex. Once the folding is complete, the MHC-peptide complexes are brought to the cell surface, where they are displayed to the surrounding environment. CD8$^+$ T cells recognize and bind only to the peptide/MHC class I complexes for which they are specifically generated. The T cell receptor forms a complex with the CD8 molecules on the surface of the T cell. This binding of the TCR/CD8 complex on a CD8$^+$ T cell with a peptide/MHC complex on the surface of an antigen-presenting cell begins a signaling process that results in the eventual activation, differentiation, and proliferation of cytotoxic CD8$^+$ T cells that are specifically programmed to destroy infected cells and interfere with replication of pathogens by various means (see CD8$^+$ T cells section above).

The second type of antigen presentation is utilized by CD4$^+$ T cells. Several types of pathogens, mainly bacteria, replicate outside of cells. These pathogens are engulfed by phagocytic cells, such as macrophages and dendritic cells. Pathogens are degraded to components within vesicles inside phagocytic cells. Since the components are inside a membrane-bound vesicle, they are not accessible to the proteasome in the cytosol for processing. Instead, these proteins are degraded into peptides by proteases within the vesicles themselves. Peptides of about 12-14 amino acids then form complexes with

MHC class II molecules. The peptide/MHC complexes are brought to the cell surface, where they are recognized by CD4$^+$ T cells. Subsequent activation, differentiation, and proliferation follow in a manner similar to that of CD8$^+$ T cells (113, 130-132).

B cells

B cells are the key players in the humoral (antibody-mediated) immune response. B cells are formed in the bone marrow, and remain there for early maturation processes. B cell maturation occurs through a complex process involving several stages, each of which is represented by a change in the antibody genome loci. Failure to progress beyond any of the maturation stages, or exhibiting too strong an affinity for self-antigen results in apoptosis of the cell, also known as clonal deletion.

The role of B cells is to produce antibodies that bind to specific parts of pathogens or molecules displayed on infected cells. This binding of antibodies to antigens contributes to immunity in three main ways. In order to enter and infect cells, viruses and intracellular bacteria need to use their surface receptors to bind to other receptors on the surface of the cell. Antibodies can bind to viral and bacterial surface receptors, thereby blocking the pathogen's mode of entry into a potential host cell and preventing infection. Antibodies can also protect against pathogens that multiply outside of a host cell. One way of doing this is by coating the outside of the pathogen with antibody. This coating is recognized by certain phagocytic cells, and increases the ability of the phagocyte to

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ingest and destroy the pathogen. This is called opsonization of the pathogen. Related to this is complement-mediated destruction of pathogens. As in opsonization, antibodies coat the outer surface of the pathogen. Components of the complement system, whose proteins are present throughout the body, then bind to the antibodies, enhancing phagocytosis (113, 133-136).

General Overview of the Lymphatic System

As discussed by Janeway, et al. (113), the lymphatic system comprises a series of vessels and organs that serve to bring antigens, antigen-presenting cells (APC), and lymphocytes (T cells and B cells) together to initiate immune responses. Lymphatic vessels channel a clear fluid called lymph, in which antigen-presenting cells travel. The lymph circulates throughout the body, flowing through specialized lymphoid organs along the way. These organs consist of the primary lymphoid organs (thymus and bone marrow) where lymphocytes are generated and become mature, and the secondary lymphoid organs (spleen, lymph nodes, tonsils, adenoids, intestinal Peyer's patches, and others), where immune responses are initiated.

Although there are various types of secondary lymphoid tissues, I will from here forward focus on events that take place in lymph nodes.

Organization of Lymph Nodes

Lymph nodes are small, bean-shaped organs grouped in clusters at various locations throughout the body, in association with the lymphatic vessels. A typical lymph node is surrounded by a protective, fibrous capsule. Directly beneath the capsule is the cortex of the node, which mainly houses B cells. Located toward the center of the lymph node from the cortex is the paracortex, an area which consists mainly of T cells. In the central-most portion of the lymph node is the medulla, to which memory B cells and antibody-producing B cells migrate, and in which antibody-producing B cells develop into plasma cells. Lymphatic fluid, with its cargo of APCs, enters the lymph node through a series of afferent lymphatic vessels. The lymphatic fluid and APCs travel through a system of channels or sinuses which intersperse the compartments of the lymph node. They eventually filter through to the medulla, along the way encountering T cells and B cells in the cortex and paracortex, and possibly leading to their activation and initiation of an immune response. Lymphatic fluid and associated cells then exit the lymph node through the efferent lymphatic vessel (137) as shown in Figure 3.

Besides T cells and B cells, several other cell types populate the lymph nodes. Fibroblastic reticular cells (FRCs) are a population of fixed (stromal) cells that give the node its architecture, and form a sort of scaffolding that supports the node (138, 139). In addition to providing structural support, the FRC network also forms channels which act as 'roadways' upon which other cells and soluble molecules travel (137, 140-143). Utilizing collagen, elastin, and laminin fibers, FRCs form a complex system of interconnected channels, referred to as the FRC conduit system. This system aids in the

transfer of soluble materials from one part of the lymph node to another (140, 143).

FRCs also produce various chemokines and express surface molecules that help direct the

trafficking of T cells, B cells, and APCs (144, 145).

Several types of dendritic cells (DCs) are resident in lymph nodes (146). Two of

these are the CD11c+ and CD11c- dendritic cells that occupy the T cell zones (147, 148).

CD11c+ DCs have long, delicate processes that extend out into the surrounding area.

Because of this, CD11c+ DCs are often called interdigitating dendritic cells (149, 150).

The processes hold antigen on their surface and bind together with T cells to form

clusters and initiate T cell activation (149, 150). CD11c- DCs are also known as

plasmacytoid DCs. These are typically found near the high endothelial venules, and also

form clusters with and help to activate T cells (151).

A distinct type of DC resides in the B cell follicles of lymph nodes. These are the

follicular dendritic cells (FDCs). FDCs possess long processes, which bind with those of

other FDCs to form a sponge-like network (139). FDCs hold antigen in the form of

immune complexes on their processes. The presentation of these immune complexes to

B cells is necessary for B cell differentiation and proliferation (152). FDCs are also vital

in the formation of primary lymphoid follicles (145).

As reviewed by Price, et al., naïve T cells, which have been positively selected in

the thymus (see T cell section above), leave the thymus and circulate through the blood.

Upon reaching a lymph node, T cells migrate through the high endothelial venules into

the T cell zone of the lymph node. Upon encountering and binding with a DC bearing

cognate antigen, T cells become activated and begin to proliferate, resulting in the

generation of approximately 1000 descendents of identical specificity. Specialized CD8^{+} effector T cells migrate out through the efferent lymphatic vessels and into the periphery (153). Specialized CD4^{+} T cells begin expressing chemokine receptors, such as CXCR5, that bring them into close proximity with B cells at the edges of the B cell zone (154).

Naïve B cells are formed in the bone marrow, and remain there to undergo their maturation process. B cells enter the lymph node through the high endothelial venules and emerge into the T cell zone (136). They are guided through the T cell zone to the B cell zone in response to chemoattractants such as CXCL13, which is produced by FDCs in the B cell follicles (155). B cells migrate to the edge of a primary follicle. Here they encounter CD4^{+} T cells and FDCs, which signal the B cells to begin proliferating and forming a germinal center (GC) (137, 156). Germinal center B cells go through rounds of clonal expansion (157), isotype switching (158), positive or negative selection (159), and differentiation into antibody-secreting plasma cells or memory B cells (160-162). The GC consists of a dark zone, which contains closely packed, rapidly dividing B cells known as centroblasts; and a light zone, which contains more loosely packed B cells called centrocytes. While GC processes are underway, the B cells not going through maturation and differentiation processes remain along the outer edge of the follicle and form the follicular mantle zone. Therefore, a fully formed follicle consists of a GC containing light and dark zones, surrounded by a mantle zone (156). Once B cells have been positively selected and have differentiated into plasma cells or memory cells in the medulla, they travel through the medullary sinus, exit the lymph node through the efferent lymphatic vessel, and go out into the periphery (137) (Figure 3).
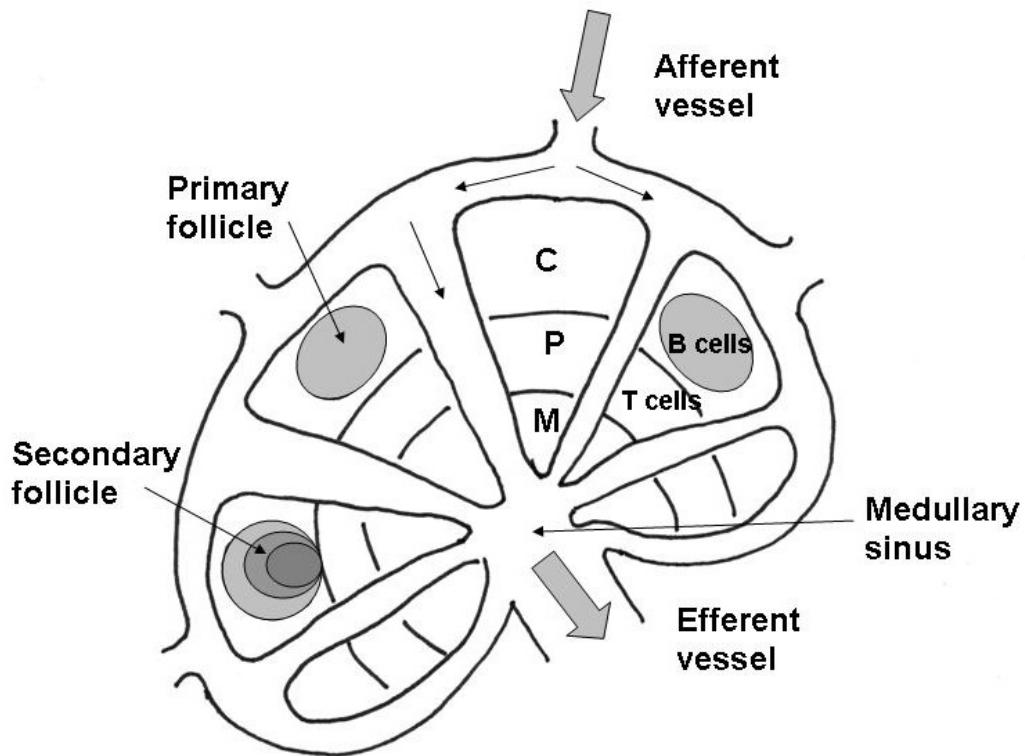
Figure 3. Schematic diagram of typical lymph node structure. C, P, and M represent cortex, paracortex, and medulla, respectively. Figure adapted from (137).

HIV infection leads to destruction of immune function

As I mentioned previously, the hallmark of HIV infection is a severely deficient and compromised immune system. This is due to an intricate web of cause-and-effect events, as one immune function becomes compromised and leads to the downfall of another, and so on.

The main target cell for HIV infection is the CD4$^+$ T cell. CD4$^+$ T cells can be destroyed by HIV in three ways. First, the infected cell may lyse when there gets to be

simply too many virions produced inside the cell. This cellular 'burst size' for SIV and, by extension, HIV, is estimated to be about 4×10^4 to 5.5×10^4 viral copies per cell (163). Second, infection with HIV can lead to destruction of infected cells by cytotoxic $CD8^+$ T cells (164). Third, infection with HIV can lead to increased levels of apoptosis of uninfected cells (164).

Over the course of time, an HIV+ person's immune system deteriorates to the point such that few CD4+ T cells remain. At this point, the individual is left with virtually no defense left to fight off any bacteria or viruses to which he or she is exposed. At this stage, 'opportunistic infections' begin to set in (34). Some of the most common opportunistic infections that affect HIV+ individuals are, as previously mentioned, Kaposi's sarcoma and pneumocystis carinii pneumonia. Others include cytomegalovirus, Epstein Barr virus, herpes simplex virus, *Candida albicans* infections, toxoplasmosis, tuberculosis, and many others (165). A clinical diagnosis of AIDS is in order when the infected person's $CD4^+$ T cell number drops below 200 cells/ml of blood, *and* when 2 or more HIV-associated opportunistic infections have set in (68). Patients sometimes develop AIDS dementia, which is characterized by ataxia and loss of cognitive function, due to direct effects of virus on the brain or toxic effects of antiretroviral drugs (166). It is only a matter of time before the body can no longer cope, and death ensues.

Chapter 3: HIV-specific CD8+ T cells are largely excluded from B cell follicles during HIV infection

Introduction and Rationale

CD8$^+$ T cells are very important when dealing with viral pathogens, including HIV. CD8$^+$ T cells can destroy HIV-infected cells by use of lytic molecules such as perforin and granzymes (126), or by induction of the Fas/Fas ligand pathway (127, 128). HIV infectivity can be reduced by competitive inhibition of viral binding to receptors on the surface of host cells by MIP 1-alpha, MIP 1-beta, and RANTES (108). HIV replication can be reduced by the release of molecules such as IFN-γ and TNF-α (109). HIV transcription can be inhibited by the production of CAF (129). (See also CD8$^+$ T cells section in Introduction).

Studies of HIV-infected human subjects have shown that some patients with high numbers of HIV-specific CD8$^+$ T cells showed slower disease progression than those with fewer numbers (122). During the initial stages of infection, the appearance of HIV-specific CD8$^+$ T cells correlates with a decrease in viral load (56, 59). This suggests that the CD8$^+$ T cell response is initially controlling virus to some degree. The same is true in studies using SIV-infected rhesus macaques (123, 124). Also, antibody depletion of SIV-specific CD8$^+$ T cells from rhesus macaques during SIV-infection resulted in an increased viral load in plasma, demonstrating that SIV-specific CD8+ T cells had been suppressing viral replication (55, 125).

In spite of the CD8$^+$ T cell response, however, HIV replication continues to the point where the immune system can no longer control it. In untreated patients, CD4$^+$ T cells become depleted, the immune system is destroyed, AIDS develops, and the patient encounters various opportunistic infections and, eventually, death.

It is not clear why CD8$^+$ T cells cannot control HIV, although many hypotheses have been developed to answer this enigmatic question. It has been proposed that one reason why CD8$^+$ T cells cannot defeat HIV infection is because they are present in insufficient numbers to be efficient in clearing infected cells. However, there is evidence indicating that there are significant numbers of HIV-specific CD8$^+$ T cells in HIV-infected patients (167-170). It has also been suggested that perhaps CD8$^+$ T cells are deficient in effector function during HIV infection. Although there are some lines of evidence to support this (171-173), there are also data that suggest that HIV-specific CD8$^+$ T cells are functional against infected cells in cytolytic assays (107, 174).

We postulate that CD8$^+$ T cells are unable to control HIV because there is an immune privileged area in lymphoid tissues in which virus resides and replicates, where HIV-specific CD8$^+$ T cells are not effective at entering to destroy infected cells. This hypothesis is the basis of my thesis and will be discussed in detail in the remainder of this thesis.

Lymphoid tissues are the primary site of HIV infection (2, 3, 65, 106). There are 5 – 10 times more virus-producing CD4$^+$ cells in lymphoid tissues than in peripheral blood (3, 175). HIV replication in lymphoid tissues is not evenly distributed, and mainly

occurs within B cell follicles of secondary lymphoid tissue, even though there are significantly fewer CD4$^+$ T cells in follicles than in extrafollicular areas (105-107, 176). CD4$^+$ T cells within lymphoid follicles are about 31 times more likely to be infected than are CD4$^+$ T cells in extrafollicular areas (106).   The reason for this preferential replication of HIV in B cell follicles has not been adequately explained.

Studies by Connick, et al. showed that, although HIV mainly replicates within B cell follicles of secondary lymphoid tissues, many antiretroviral proteins commonly produced by CD8$^+$ T cells, such as α-defensins 1, 2, and 3, RANTES, and granzyme A, were mainly found outside of follicles (106). This led us to speculate that B cell follicles are immune privileged sites during HIV infection, and that HIV-specific CD8$^+$ T cells are largely excluded from these areas.

In normal conditions, significantly more CD8$^+$ T cells are located outside B cell follicles in secondary lymphoid tissues than inside. This makes sense from a physiological standpoint. Cytotoxic CD8$^+$ T cells are highly destructive, and setting them loose inside an important site of B cell activation and antibody production could be disastrous in terms of immune response. So the disparity in numbers of CD8$^+$ T cells inside vs. outside of follicles is not something that is specific to HIV or other viral infections. It is more likely that this is a naturally-occurring mechanism that the body may use as protection from immunopathology. It has been shown that in HIV-infected human subjects, more CD8$^+$ T cells are found inside lymph nodes than in uninfected subjects (107). This difference may be due to increased numbers of CD8$^+$ T cells arriving

in lymph nodes to fight off virus. Yet the increased numbers of CD8$^+$ T cells are unable to completely clear HIV from the lymphoid tissues. Although CD8$^+$ T cells encounter antigen in the T cell zones of secondary lymphoid tissues and, for the most part reside there, it is not unusual to find small populations in B cell follicles. CD8$^+$ cells infiltrate B cell follicles in human tonsils (177), inguinal lymph nodes (106), and mouse spleen (178). It has been shown that CD8$^+$ T cells that localize in B cell follicles in humans usually express the chemokine receptor CXCR5, and to some degree are able to provide survival signals to B cells (177).

It is not known whether HIV-specific CD8 T cells enter B cell follicles. This is an important question to answer because most HIV is located inside B cell follicles. I postulated that HIV-specific CD8$^+$ T cells are largely excluded from B cell follicles during HIV infection, and that the inability of HIV-specific CD8 T cells to kill infected cells inside follicles is a mechanism which prevents CD8$^+$ T cells from completely controlling virus replication, and a mechanism that contributes to viral persistence. If CD8$^+$ T cells that function to destroy HIV-infected cells are kept away from the main sites of viral replication, this would cause the establishment of a reservoir of virus that could continue to seed infection throughout the remainder of the patient's life.

MHC Tetramers

The invention of MHC tetramer technology to label antigen-specific cells has led to a greatly enhanced understanding of the immune system in general, and especially CD8$^+$ T cell biology (167). Initially, these studies were limited to staining antigen-

specific cells that had been isolated from blood or tissues, and analyzed using a flow

cytometer. Subsequently, methods were developed that enable MHC tetramers to be used

to stain antigen-specific CD8$^+$ T cells in tissue sections (179). This method, termed in

situ tetramer staining, enables visualization of antigen-specific CD8$^+$ T cells with their

spatial localization to other cells of interest still intact, as well as the determination of the

quantity and protein expression patterns of antigen-specific CD8 T cells in situ (Figure

4).

MHC Class I Tetramer Staining

Anti-CD8
antibody

MHC Class I
Tetramer

CD8
molecule

T cell receptor
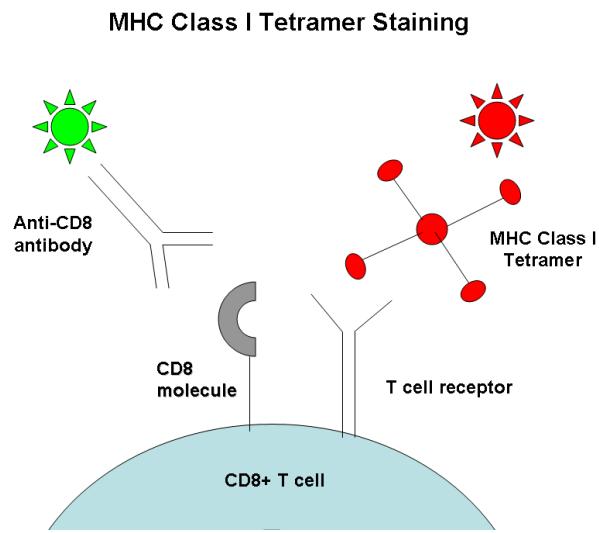
CD8+ T cell

Figure 4: Model of the interactions between a CD8+ T cell, MHC class I tetramer, and anti-CD8 antibody

I set out to visualize HIV-specific CD8$^+$ T cell localization inside and outside of B

cell follicles in lymph nodes from HIV-infected humans using in situ tetramer staining

combined with immunohistochemical staining of CD20. CD20 antibodies label B cells

and can be used to delineate B cell follicles. I hypothesized that HIV-specific CD8$^+$ T

cells would mainly be concentrated in extrafollicular areas of lymphoid tissues, and not in

the follicles. This is the first time such studies have been performed. To this end, I

worked with Dr. Connick's group to test this hypothesis. Dr. Connick's laboratory

performed lymph node biopsies on HIV-infected human subjects and used a portion of

the biopsies to visualize viral RNA in situ and determine numbers of HIV-specific CD8^{+}

T cells in disaggregated tissue. I used the lymph node biopsies to determine spatial

localization of HIV-specific CD8^{+} T cells and B cell follicles in situ.

Materials and Methods

Tetramers and Antibodies

 Primary antibodies: Anti-CD8 antibody clone DK25, directed against the human CD8-

alpha chain, was purchased from Dako CytoMation. Anti-CD20 antibody clone L26,

directed against the transmembrane domain of human CD20, was purchased from Nova

Castra.

 Secondary antibodies: Cy-3- conjugated goat-anti-rabbit IgG was purchased from

Jackson ImmunoResearch. Alexa 488-conjugated goat-anti-mouse IgG was purchased

from Invitrogen.

 MHC class I tetramers: Biotinylated HIV A2 *gag*, A2 *pol*, or an irrelevant melanoma

(ELAGIGLTV) peptides were synthesized at Beckman Coulter Immunomics.

Biotinylated HLA A3 *gag*, A3 *nef*, B8 *gag*, B8 *nef*, and B7 *nef* tetramers were produced

at the NIAID Tetramer Facility (Table 1). HLA/peptide tetramers were produced by

adding six aliquots of FITC-labeled ExtraAvidin (Sigma Aldrich) to biotinylated

HLA/β_2-microglobulin/peptide monomers over the course of 8 hours to a final molar ratio of 4.5:1.

Fresh tissue specimens from HIV-infected human subjects

Fourteen HIV-infected human subjects who were not receiving antiretroviral therapy donated an inguinal lymph node to the study. Plasma viral load, CD4$^+$ T cell numbers, and HLA typing were performed at the University of Colorado Hospital (Table 1). Subjects underwent excisional biopsies under local anesthesia. A portion of each node was placed in RPMI containing heparin and shipped to our lab overnight on freezer blocks. Upon arrival, the piece of lymph node was cut into 0.5 cm x 0.5 cm chunks and embedded in low melt agarose. The agarose-embedded tissue blocks were glued to a chuck using Loctite glue, and placed into a Vibratome. 200 micron thick sections were cut and kept in cold phosphate-buffered saline with heparin (PBS-H) until the staining process was begun.

In situ tetramer staining

Fresh tissue sections were stained free floating in 1 ml of solution, with 1 – 4 sections per well in 24-well tissue culture plates. Tetramers at a concentration of 0.5 ug/ml and antibodies at a concentration of 1:200 (CD8) or 1:100 (CD20) were diluted in PBS-H containing 2% normal goat serum (PBS-H/NGS). Incubations were done at 4*C overnight. Sections were washed with cold PBS-H and fixed at room temperature for 2 hours with 4% paraformaldehyde. Sections were again washed in cold PBS-H, then

incubated for 1 – 3 days in rabbit-anti-FITC antibodies diluted 1:10,000 in PBS-H/NGS.

Sections were washed 3 x 20 minutes or more in cold PBS-H, then incubated in

secondary antibodies diluted 1:1000 in PBS-H/NGS for 1 – 3 days. Sections were again

washed 3 x 20 minutes or more in cold PBS-H, fixed in 4% paraformaldehyde at room

temperature for 1 hour, and mounted onto microscope slides using warm glycerol gelatin

containing 4mg/ml N-propyl gallate (a fluorophore preservative).

Image collection, processing, and analysis

Images of the stained tissue sections were collected using a Biorad confocal laser

scanning microscope. Grayscale image files collected using the confocal microscope

were opened in the Confocal Assistant computer program. Images of tetramer staining

were artificially colorized red, and images of CD8 or CD20 staining were colorized

green. A merged red and green image was also created.

For sections stained with CD20 antibody, observations were made to determine the

localization of HIV tetramer+ cells relative to B cell follicles and to see whether HIV

tetramer+ cells were excluded from the follicles. CD20-stained sections were examined

with a confocal microscope to determine if follicles were present. If so, images were

collected of the follicles and adjacent extrafollicular areas. Colorized images were

opened in Adobe Photoshop and a line was drawn to demarcate the follicle. The follicle

was defined visually as the area in which CD20-stained (green) cells were most closely

clustered together, such that the cells were merged into a solid green mass with individual cells not clearly distinguishable. The line was then copied onto the red tetramer image. Tetramer^{+} cells inside the line were ruled as being inside the follicle, and those outside the line were ruled as being outside the follicle.

Results

I examined lymph node biopsies from 14 HIV-infected human subjects for the spatial localization of HIV-specific CD8^{+} T cells in relation to B cell follicles to determine whether HIV-specific CD8^{+} T cells are excluded from or allowed into B cell follicles where HIV concentrates. Table 1 presents a detailed list of HLA molecules and viral epitopes that are recognized by HIV-specific CD8^{+} T cells during HIV infection that were examined via MHC class I tetramers in this study.

HLA Restriction	Epitope Location	Amino Acid Sequence	Abbreviation
HLA-A*0201 (A2)	HIVp17.77-85	SLYNTVATL	A2 *gag*
HLA-A*0201 (A2)	HIVrt.476-484	ILKEPVHGV	A2 *pol*
HLA-A3	HIVp17.18-26	KIRLRPGGK	A3 *gag*
HLA-A3	HIV*nef*71-80	QVPLRPMTYK	A3 *nef*
HLA-B7	HIV*nef*128-137	TPGPGVRYPL	B7 *nef*
HLA-B8	HIVp24.259-267	EIYKRWII	B8 *gag*
HLA-B8	HIV*nef.*89-97	FLKEKGGL	B8 *nef*

Table 1: HIV CD8+ T cell epitopes evaluated by in situ tetramer staining.

The age, sex, number of HIV copies per ml of plasma, number of CD4+ T cells/mm^3, and

HLA alleles for the subjects in this study are shown in Table 2. All but one of the

subjects (Subject 64) had been infected with HIV for more than one year (Table 2). None

of the subjects were on antiretroviral therapy at the time of lymph node biopsy.

Patient #	Age	Sex	Duration of infection at LN biopsy	Plasma HIV copies RNA/ml log 10	CD4 T cells/mm3	HLA type
32	48	F	5 years	3.71	775	A3, B7
61	42	M	2 years	3.29	821	A3, B7
62	32	M	3.5 years	4.35	305	A2
64	25	F	4 months	3.44	467	A2
67	45	F	chronic	4.37	406	A2, B27, B7
79	42	F	chronic	4.74	653	A2, A3
80	43	M	> 1 year	3.94	628	B8
81	29	M	chronic	4.23	697	B8, B7
82	41	F	> 1 year	3.57	835	A2
84	39	F	2 years	3.83	358	B7
86	31	F	chronic	4.01	460	A3, B7
87	26	F	chronic	4.36	256	A2
90	34	F	chronic	4.86	332	B7, B8
92	40	F	not given	4.02	671	A2, A3

Table 2. HIV-infected human subjects included in these studies.

My results showed HIV *gag*- and/or *nef*-specific CD8 T cells in lymph nodes from 6

of 13 subjects examined (Table 3). Tetramer staining of disaggregated cells analyzed by

flow cytometry and viral load analysis was done by Liz Connick's group at the

University of Colorado. Tetramer staining with a broader panel of tetramers performed

on disaggregated cells from a portion of the same lymph nodes that I examined, as well

as on peripheral blood revealed *gag*, *nef*, and *pol* tetramer$^+$ cells in 11 of 14 subjects

(107) as shown in Table 3. In every instance in which tetramer staining of disaggregated

lymph nodes via flow cytometry showed that >1% of CD8$^+$ T cells were also tetramer+,

in situ tetramer staining showed tetramer+ cells as well (Table 3), indicating that there is

a good correlation between flow cytometry and in situ tetramer staining when

concentrations of tetramer binding cells are greater or equal to 1% of the CD8 T cell

population. However, in instances in which tetramer staining of disaggregated lymph

node cells via flow cytometry showed that <1% of CD8$^+$ cells were tetramer$^+$, in situ

tetramer staining results were negative (Table 3). This indicates that although in situ

tetramer staining detects cells as well as does flow cytometry when concentrations of

tetramer$^+$CD8$^+$ cells are >1%, it is less sensitive a method when concentrations of

tetramer$^+$CD8$^+$ cells are <1%.

Patient #	HIV epitope	% Tet+CD8+ Cells in PBMC	% Tet+CD8+ cells in LN	In Situ Tetramer Staining Result
32	A3/gag	Negative	Negative	Negative
	A3/nef	Negative	Negative	Not done
	B7/nef	not done	**0.2**	Negative
61	A3/gag	Negative	Negative	Negative
	A3/nef	Negative	Negative	Negative
62	A2/gag	**4.1**	**5.3**	**Positive**
	A2/pol	Negative	Negative	Negative
64	A2/gag	**2.7**	**4.8**	**Positive**
	A2/pol	Negative	Negative	Negative
67	A2/gag	Negative	Negative	Negative
	A2/pol	Negative	Negative	Negative
	B7/nef	**0.4**	**1.9**	**Positive**
79	A2/gag	**0.4**	**1.2**	**Positive**
	A2/pol	**0.1**	**0.4**	Not done
	A3/gag	Negative	Negative	Negative
	A3/nef	Negative	Negative	Negative
80	B8/gag	**0.4**	**1.4**	Not done
	B8/nef	Negative	Negative	Negative
81	B7/nef	Negative	Negative	Negative
	B8/gag	**0.8**	**1**	**Positive**
	B8/nef	**1.9**	**3.2**	**Positive**
82	A2/gag	Negative	Negative	Negative
	A2/pol	**0.4**	**0.8**	Negative
84	B7/nef	Negative	Negative	Negative
86	A3/gag	Negative	Negative	Negative
	A3/nef	**2.6**	**3.6**	Not done
	B7/nef	**1**	**2.1**	**Positive**
87	A2/gag	Negative	Negative	Negative
	A2/pol	Negative	Negative	Negative
90	B7/nef	Negative	Negative	Not done
	B8/gag	**0.7**	**0.7**	Not done
	B8/nef	**7**	**5.1**	Not done
92	A2/gag	Negative	Negative	Negative
	A2/pol	**0.2**	**0.4**	Negative
	A3/gag	Negative	Negative	Negative
	A3/nef	**0.1**	**0.2**	Not done
	B7/nef	Negative	Negative	Negative

Table 3. Tetramer staining of peripheral blood, disaggregated lymph node, and lymph node sections

Tissue sections that were stained with tetramers and anti-CD8 antibodies indicated

that most of the tetramer$^+$ cells were also CD8$^+$, indicating that the tetramer staining was

specific for a subset of CD8$^+$ T cells (Figure 5). Negative control sections stained with

CD8 antibodies and tetramers containing an irrelevant peptide, or tetramers that were

HLA-mismatched to the subject's genotype showed staining similar to that of subjects

that did not show any tetramer staining, further indicating that tetramer staining with

HLA tetramers loaded with peptides from HIV encoded proteins was specific (Figure 5).
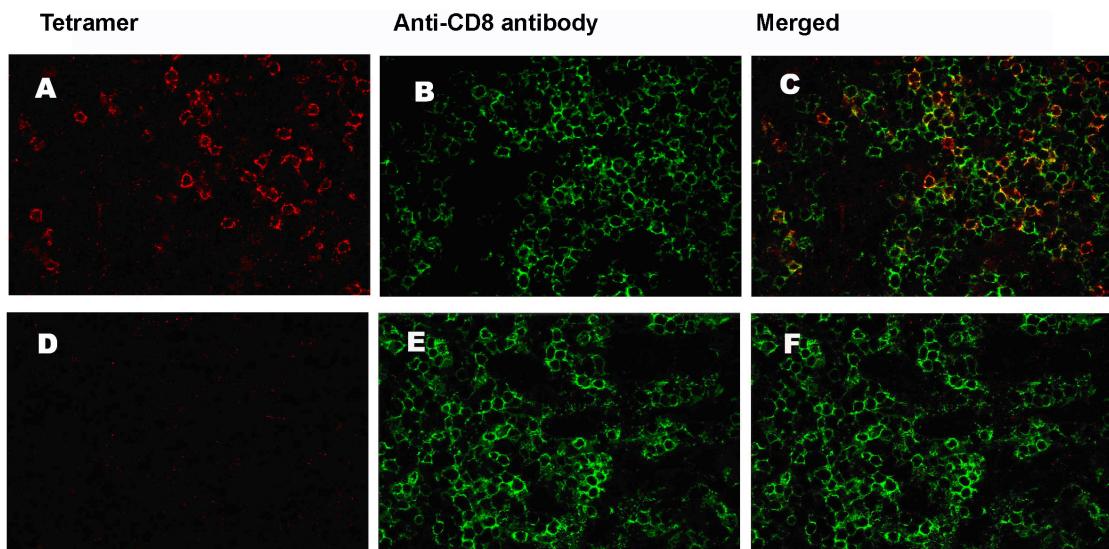
Figure 5. MHC class I tetramers (left panels) and CD8 (middle panels) and merged images (right panels) in lymph node sections from HIV-infected human subjects. Lymph node from Subject 62 (A-C) was stained with A2 gag tetramers and CD8 antibody. The majority of the tetramer-staining cells also stained with CD8, as shown in the merged image (C). Lymph node from subject 67 (D-F) stained with A2 gag tetramers and CD8 antibody did not reveal tetramer staining cells, even though an abundance of CD8+ cells were evident. Images are z-scans collected at 9um into the tissue, using a 60X objective.

To analyze the localization of HIV-specific CD8$^+$ T cells inside vs. outside of B cell follicles, tissue sections were stained with MHC tetramers and CD20 antibodies. Microscopic examination revealed that of the 14 subjects studied, only three of the subjects had abundant tetramer-stained cells as well as multiple defined follicles. Visual analysis of stained sections from these three subjects revealed that considerably more tetramer-stained cells were located outside B cell follicles compared to inside. Many follicles were nearly devoid of tetramer-stained cells, while others had some cells located mainly along the edges of the follicle (Figures 6 - 8). In all follicles examined, there was an exclusive region in which tetramer$^+$ cells were not present (Figures 6 - 8).
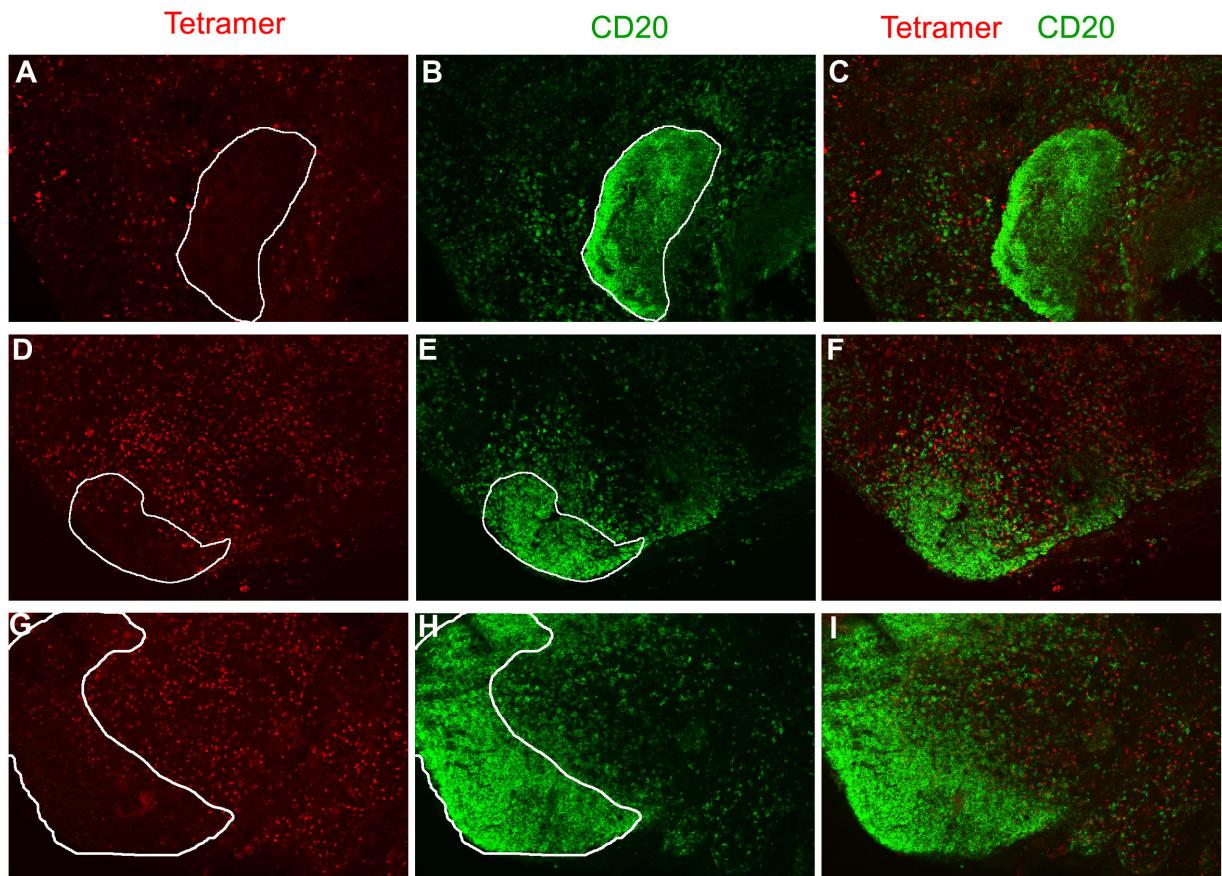
FIgure 6. MHC class I tetramers (left panels) and CD20 antibody (middle panels) staining and merged images (right panels) in representative lymph node sections from 3 HIV-infected human subjects. Lymphoid follicles, identified by CD20 staining, are outlined in white. Lymph node sections from Subject 64 (A-C) and Subject 62 (D-F) were stained with A2 gag tetramer. Lymph node sections from Subject 81 (G-I) were stained with B8 nef tetramer. Lymph node sections from each subject showed fewer tetramer stained cells localized within follicles compared to extrafollicular regions. Images are projected series of confocal z-scans collected using a 10X objective.
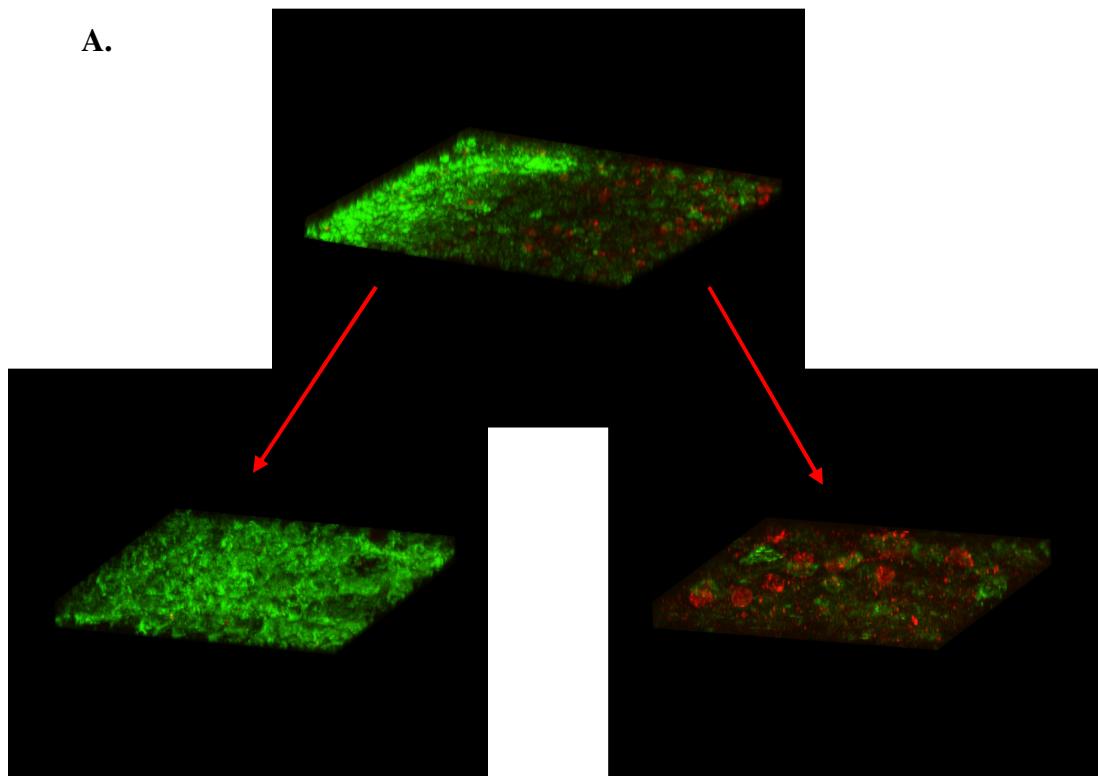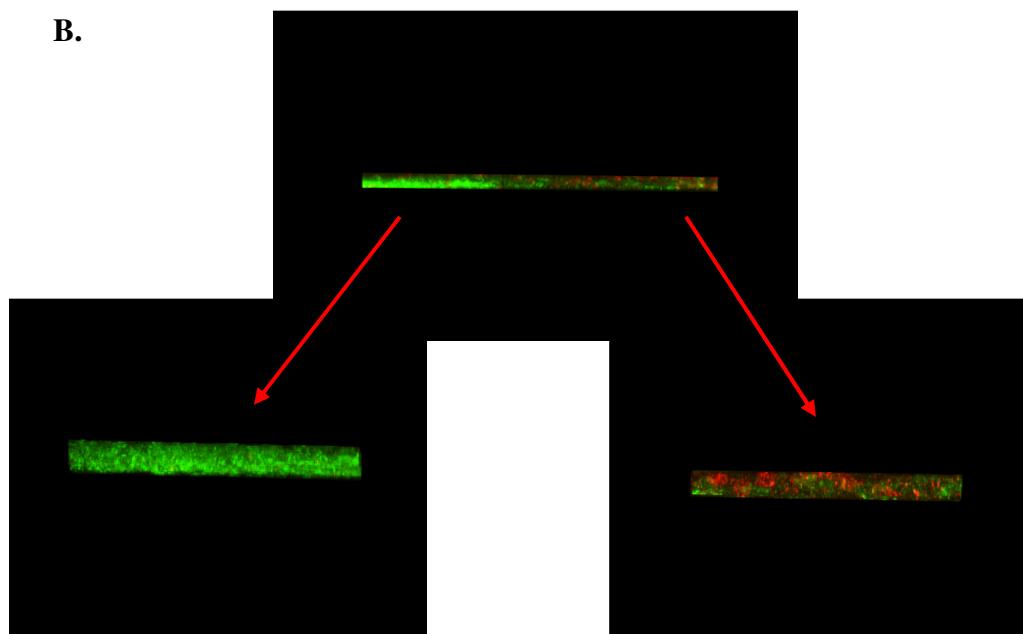
Figure 7. 3-dimensional rendering of a confocal z-series shows distinct areas where tetramer+ cells are excluded from B cell follicle in lymph node of HIV-infected human subject. Panel A shows a confocal z-series collected using a 20x objective, projected into 3 dimensions and rotated to show an angled view. B shows the same confocal z-series, projected into 3 dimensions and rotated to show an edge-on view. Red arrows indicate images collected using a 60x objective, and illustrate the nearly exclusive localization of tetramer+ cells (right half of image, red) to areas outside of the B cell follicle (left half of image, green). Images were collected from lymph node section of Subject 64.
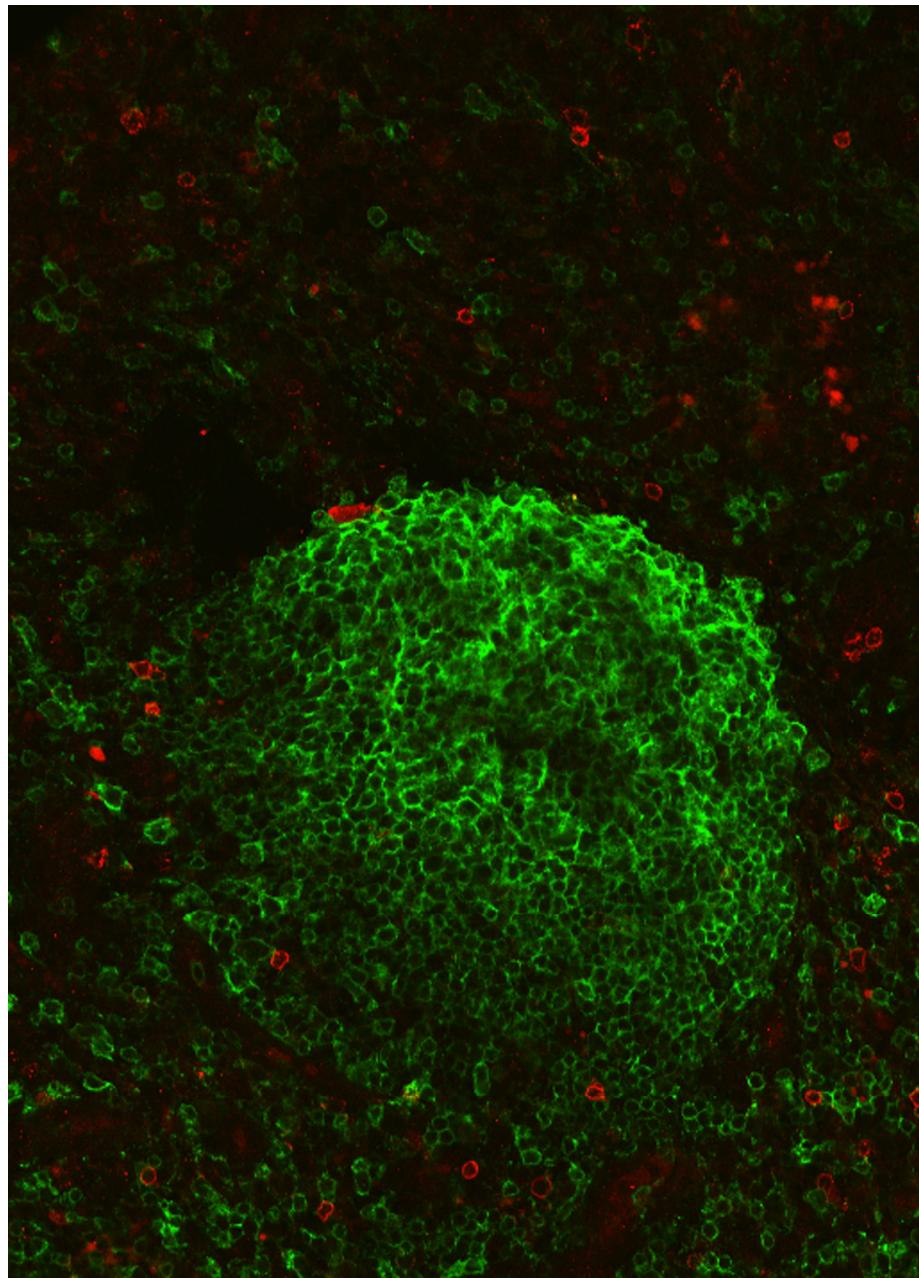
Figure 8. MHC Class I tetramers (red) and CD20 antibody (green) staining in a lymph node section from HIV-infected human Subject 64 illustrates the localization of tetramer+ cells outside of the B cell follicle. Image is a single z-scan collected using a 10X objective.

Discussion

The inability of CD8^{+} T cells to control the replication of HIV has never been satisfactorily explained. Many ideas have been generated as to why this might be. It has been hypothesized that virus-specific CD8^{+} T cells are not present in great enough numbers to control virus, and that virus-specific CD8^{+} cells are incapable of performing effector functions during HIV infection (171-173). However, these hypotheses do not satisfactorily explain viral persistence. There is evidence indicating that there are significant numbers of HIV-specific CD8^{+} T cells in HIV-infected patients (167-170). There are also data that suggest that HIV-specific CD8^{+} T cells are functional against infected cells in cytolytic assays (107, 174).

Our results indicate that B cell follicles in secondary lymphoid tissues are immune privileged sites during HIV infection. Our collaborator, Liz Connick, found that HIV primarily replicates inside lymphoid B cell follicles, and my studies revealed that HIV-specific CD8^{+} T cells are largely excluded from B cell follicles. From my data, we conclude that HIV-specific CD8^{+} T cells are mostly found in extrafollicular areas, while few are found inside the follicles (107) and Figures 6-8. The tetramer^{+} cells localized inside follicles tended to be located along the very edge of the follicle (Figures 6 - 8). Each follicle examined had an exclusive region that was completely devoid of tetramer^{+} cells (Figures 6 – 8).

The exclusion of HIV-specific CD8^{+} T cells from B cell follicles has important implications in the establishment of persistent HIV infection. HIV primarily replicates

within B cell follicles (2, 106, 180). The exclusion of HIV-specific CD8 [+] T cells from the primary site of viral replication likely enhances the formation of a reservoir of virus that propagates infection throughout the remainder of the patient's life. This helps explain why HIV-specific CD8^{+} T cells are not successful in controlling viral replication and contributes to viral persistence.

Of course, it is plausible to speculate that other mechanisms are responsible for the inability of the immune response to eliminate the viral reservoir within the B cell follicles of HIV-infected subjects. It is possible that the relative scarcity of CD8+ T cells within B cell follicles is due to an increased level of apoptosis of CD8+ T cells upon entry into the follicle. It is also possible that CD4+ T cells in B cell follicles, the preferred site of HIV replication, are more amenable to infection by HIV. These possible mechanisms for viral persistence will be discussed in Chapter 5.

It is not likely that the exclusion of CD8^{+} T cells is something that occurs only during HIV infection, as it is known that CD8^{+} T cells are generally localized to the paracortical area of lymph nodes, while B cells are generally found in the cortex (137). This particular localization of different cell types to specific areas of the lymph node facilitates antigen presentation, activation, differentiation, clonal expansion, and other events necessary to mount an effective immune response (141). CD8^{+} T cells have an impressive repertoire of cellular 'weapons' with which they protect the body from invaders (see CD8^{+} T cell section of Introduction). If turned loose within an important site of immune activation, it is possible that cytotoxic T cells could do more harm than

good. In light of this, it is likely that HIV exploits the reality that CD8$^+$ T cells are excluded from most areas inside B cell follicles.

Recently, some phenotypic analyses of those CD8$^+$ T cells that do enter B cell follicles have been accomplished. Most CD8$^+$ T cells that enter B cell follicles in human tonsils express the surface molecule CXCR5 (177). This is not surprising, as this chemokine receptor is also expressed on other cells that enter B cell follicles, namely, B cells themselves, and also on CD4$^+$ T cells that must enter the B cell areas to help promote B cell activation (181). The 'homing molecule' toward which CXCR5$^+$ cells migrate is CXCL13, which is expressed by follicular dendritic cells within B cell follicles (152, 181-183). CXCR5$^+$CD8$^+$ T cells within B cell follicles do not express perforin, and to some degree, help promote and enhance B cell survival (177).

Further studies need to be done to elucidate why some CD8$^+$ T cells, but not others, enter B cell follicles. Studies to compare the repertoire of surface receptors, especially chemokine receptors, of CD8$^+$ T cells found inside vs. outside of B cell follicles are in order. Once it has been established what enables CD8$^+$ T cells to enter B cell follicles, studies involving the manipulation of virus-specific CD8$^+$ T cells to express these trafficking receptors could be implemented. Using the rhesus macaque/simian immunodeficiency virus model for HIV infection, I stained fresh macaque lymph node section with three different clones of anti-human and anti-mouse CXCR5 antibodies. Unfortunately, these antibody clones did not cross react with rhesus macaque CXCR5 and staining results were negative (data not shown). Thus, there is a need to create

macaque reactive CXCR5 antibodies for in situ analysis in order begin determining which trafficking receptors are most important for enabling cells to enter B cell follicles. Once that goal is accomplished, studies in which virus-specific CD8^{+} T cells are manipulated to enter B cell follicles during SIV infection could be implemented.

It is interesting to speculate on the outcome of disease progression if CD8^{+} T cells with full effector function were manipulated to express CXCR5 and enter B cell follicles during HIV infection. Of course it is possible that, with a full armament of cellular weapons, virus-specific CD8^{+} T cells would be like loaded guns in the hands of an inexperienced shooter and cause more harm than good. It is more hopeful and optimistic, however, to conjecture that CD8^{+} T cells inside B cell follicles would destroy infected cells in large numbers, thereby reducing the number of infected cells and slowing propagation of virus. After a sufficient time, it may be possible that eventually the number of infected cells destroyed would outweigh number of cells becoming newly infected, and this could deplete the viral reservoir. With this in mind, it is possible that a new means of treatment for HIV[+] patients, outside the current realm of antiretroviral therapy, could be developed. The reduction of viral burden by the dissolution of the B cell follicle viral reservoir may give the immune system a fighting chance to actually rid the body of virus once and for all.

Chapter 4: SIV-producing cells concentrate inside B cell follicles, while SIV-specific CD8+ T cells are largely excluded from follicles during SIV infection

Introduction and Rationale

Non-human primates are often used as models for human disease because the anatomy and physiology of these animals is similar to that of humans (103, 184-186). Of particular interest to the present work is the use of the simian immunodeficiency virus (SIV)-rhesus macaque model for HIV infection. This system is extremely useful as a model for HIV infection in humans because of its great similarities to HIV-mediated human disease. SIV is genetically similar to HIV and is a CD4$^+$ T cell tropic virus (187, 188). SIV infection of rhesus macaques manifests as an increase in viral load, a decrease in CD4$^+$ T cell numbers, and a progression to simian AIDS (SAIDS) (49). The usefulness of this model is also evident in that studies can be performed on monkeys that cannot be performed on humans, for reasons both ethical and practical. For example, studies of the events that occur at very early time points after SIV infection can be performed in monkeys, while at these early time points, humans are often unaware that they are even infected with HIV (104).

Several different types of SIV, both pathogenic and non-pathogenic, are used in the rhesus macaque model for various challenge and correlate of protection studies (49). The strain of virus used in the studies in which our group participated was SIVmac239. As does HIV, SIVmac239 preferentially replicates in lymphoid tissues, making the SIVmac239/rhesus macaque system a suitable model for these studies (3, 60, 189, 190).

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I have shown that B cell follicles are relatively immune privileged sites from which HIV-specific CD8$^+$ T cells are largely excluded (107) (Chapter 3). Because the SIV-rhesus macaque model is a well-described model with which to study HIV infection, we set out to determine whether the same holds true in the macaque model as well as in humans. If this was indeed the case, this information could set the stage for future rhesus macaque model studies, focusing on the events that take place within the lymphoid tissues during HIV infection. This knowledge may make possible the type of studies mentioned in the Discussion section of Chapter 3, namely, those in which virus-specific effector CD8$^+$ T cells could be manipulated to enter B cell follicles and, perhaps, help to deplete the reservoir of virus.

In light of this, we set out to determine if the exclusion of virus-specific CD8$^+$ T cells from B cell follicles occurs in SIV-infected rhesus macaques, as we showed in humans. We hypothesized that, as in humans, virus-specific CD8+ T cells would be found mainly in the extrafollicular regions of the lymph nodes of infected rhesus macaques.

Materials and Methods

Tetramers and Antibodies

Primary antibodies: Anti-CD8 antibody clone DK25, directed against the human CD8-alpha chain, was purchased from Dako CytoMation. Anti-CD20 antibody clone L26, directed against the transmembrane domain of human CD20, was purchased from Nova

Castra. Previous experiments by the Skinner lab indicated that these antibodies work well in fresh tissue immunohistochemistry of rhesus macaques (104).

Secondary antibodies: Cy-3 conjugated goat-anti-rabbit IgG was purchased from Jackson ImmunoResearch. Alexa 488-conjugated goat-anti-mouse IgG was purchased from Invitrogen.

MHC class I tetramers: Biotinylated Mamu A*01/β_2m/peptide molecules were produced with Gag (CTPYDINQM) or irrelevant (FLPSDYFPSV) peptide at the National Institute for Allergy and Infectious Disease tetramer facility. Tetramers were produced by adding six aliquots of FITC-labeled ExtraAvidin (Sigma Aldrich) to biotinylated Mamu A*01/β_2-microglobulin/peptide monomers over the course of 8 hours to a final molar ratio of 4.5:1.

SIVmac239-infected rhesus macaques

Rhesus macaques of with a MHC class I genotype including Mamu A*01 were inoculated with SIVmac239 and sacrificed at various days after infection. Viral load in peripheral blood was determined by our collaborating laboratories at the University of California, Davis (animals 26222, 24818, 28630, 30551, and 24225) (103, 104), or the University of Wisconsin, Madison (animal Rh2123) (Reynolds, personal communication). Animals were necropsied and various tissues, including lymph nodes, spleen, cervix, vagina, and gut were placed in RPMI containing heparin and shipped to our lab overnight on freezer blocks.

In situ tetramer staining

Upon arrival, tissues were cut into 0.5 cm x 0.5 cm chunks using a scalpel and embedded in low melt agarose. The agarose-embedded tissue blocks were glued to a chuck using Loctite glue, and placed into a Vibratome bath containing cold phosphate-buffered saline with heparin (PBS-H). 200 micron thick sections were cut and kept in cold PBS-H until the staining process was begun. Fresh tissue sections were stained free floating in 1 ml of solution, with 1 – 4 sections per well in 24-well tissue culture plates. Tetramers at a concentration of 0.5 ug/ml and antibodies at a concentration of 1:200 (CD8) or 1:100 (CD20) were diluted in PBS-H containing 2% normal goat serum (PBS-H/NGS). Incubations were done at 4*C overnight. Sections were washed with cold PBS-H and fixed at room temperature for 2 hours with 4% paraformaldehyde. Sections were again washed in cold PBS-H, then incubated for 1 – 3 days in rabbit-anti-FITC antibodies diluted 1:10,000 in PBS-H/NGS. Sections were washed 3 x 20 minutes or more in cold PBS-H, then incubated in secondary antibodies diluted 1:1000 in PBS-H/NGS for 1 – 3 days. Sections were again washed 3 x 20 minutes or more in cold PBS-H, fixed in 4% paraformaldehyde at room temperature for 1 hour, and mounted onto microscope slides using warm glycerol gelatin containing 4mg/ml N-propyl gallate (a fluorophore preservative). The in situ tetramer staining of animals 30551 and 24225 were performed in 2001 by others in the Skinner lab and the sections were archived at -20 degrees Celsius since processing. I performed the in situ tetramer staining on animals 28630 and Rh2123 in April 2007 and November 2007, respectively.

Image collection, processing, and quantitative analysis

Images of the stained tissue sections were collected using a Fluoview confocal laser scanning microscope. During image collection, images of tetramer staining were artificially colorized red, and images of CD8 or CD20 staining were colorized green. A merged red and green image was also created.

For sections stained with CD8 antibody, determination of whether CD8$^+$ cells were also tetramer$^+$ was performed using the following method: with the colorized image files open in Adobe Photoshop, the merged red and green image was viewed. It was determined whether the tetramer$^+$ cells were also CD8$^+$ by observing if the cells were double stained both red and green. For sections stained with CD20 antibody, observations were made to determine if SIV-specific CD8$^+$ T cells were excluded from the follicles. CD20-stained sections were examined with a confocal microscope to determine if follicles and tetramer$^+$ cells were present. If so, images were collected of the follicles and adjacent extrafollicular areas. Colorized images were opened in Image J software. Using the green (CD20) image, a line was drawn around the follicle and the area was measured in mm^2. The same was done for extrafollicular area. In cases in which it was not clear if a region was follicle or extrafollicular, this area was excluded from the measurement. The lines of demarcation were copied onto the red (tetramer) image, and the number of red-stained cells within each follicle or extrafollicular region was hand counted. These data were entered into a Microsoft Excel spreadsheet, and numbers of tetramer$^+$ cells/mm^2 of tissue were calculated using Excel functions. Statistical significance of the values were determined using GraphPad Software

QuickCalcs for Scientists one-sample *t* test.

(www.graphpad.com/quickcalcs/OneSampleT1.cfm).

Results

Dr. Skinner's lab has performed in situ tetramer staining on tissues from many SIV-infected rhesus macaque tissues since 2000, through several collaborative efforts, and has maintained the slides at -20 degrees Celsius. The present studies were initiated by examining records of all archived slides kept in the laboratory and focusing the analysis on those slides containing tissue samples that had been stained with A*01 gag tetramers and anti-CD20 antibodies, and showed substantial numbers of tetramer staining cells. Archived tissue samples from 2 rhesus macaques infected with SIVmac239, sacrificed after 14 days post-infection, and stained with Mamu-A*01 tetramers and anti-CD20 antibodies were identified (animals number 24225 and 30551). Microscopic examination of the stained tissue sections revealed that sections from these animals had plentiful tetramer staining and some B cell follicles to analyze. In addition, during my tenure in the lab, fresh tissues from 7 rhesus macaques infected with SIVmac239 and sacrificed at or after 14 days post-infection were stained with Mamu-A*01 tetramers and anti-CD20 antibodies. Of these 7 animals, 2 (animals number 28630 and Rh2123) were found to have abundant Mamu A*01 tetramer staining and B cell follicles. This resulted in a cohort of 4 rhesus macaques which had been infected with SIVmac239 and sacrificed at 14, 21, 28, and 420 days after infection.

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Because it is known that HIV primarily replicates in B cell follicles (105-107, 176), and my previous work showed that virus-specific CD8$^+$ T cells are largely excluded from B cell follicles in the lymph nodes of HIV-infected human subjects during the chronic stages of infection (107), I set out to determine if the same exclusion holds true in the SIV-infected rhesus macaque model system. Unfortunately, I did not have access to any tissues from chronically SIV-infected macaques, however I did have access to and examined tissue sections from lymph nodes of SIVmac239-infected rhesus macaques, sacrificed at very early times 14, 21, 28 days post-infection, and at a very late time point at ~420 days after infection (Table 4). Viral loads from this cohort of animals are also presented in Table 4 (103, 104, and Dr. Matt Reynolds, personal communication).

I determined the localization and abundance of SIV-specific CD8$^+$ T cells inside vs. outside of B cell follicles, in tissue sections stained with MHC tetramers and anti-CD20 antibodies (animals 28630, 30551, 24225, and Rh2123, Table 5 and Figure 9).

Animal ID	Duration of Infection at Time of Sacrifice	Plasma SIV copies RNA/ml
28630	14 days	7.5 x 10^6
30551	21 days	3.6 x 10^7
24225	28 days	1.5 x 10^6
Rh2123	~420 days	5 x 10^5*

Table 4: SIVmac239-infected rhesus macaques used in these studies. *Viral load for Rh2123 was measured one year prior to sacrifice.

Confocal microscopy of tissue sections stained with tetramers and anti-CD8 antibodies revealed that most tetramer$^+$ cells were also CD8$^+$ (Figure 10), and negative

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control staining with MHC tetramers loaded with an irrelevant peptide showed that staining with tetramers loaded with SIV peptides was specific (not shown).

Confocal microscopic imaging followed by quantitative image analysis revealed that significantly more tetramer-stained cells were located outside B cell follicles compared to inside (p-value=0.03), (Table 5 and Figure 11). Although some tetramer^{+} cells localized inside B cell follicles, in most follicles (16 out of 19) tetramer binding cells were excluded from greater than half the B cell follicle area; and several follicles were nearly entirely devoid of tetramer-binding cells (Figure 11). Tetramer-binding cells within B cell follicles were often located along the edges of the follicle (Figure 11). Thus, in most follicles, the majority of the area within the follicle was devoid of tetramer^{+} cells (Figures 9 and 11).

Animal ID	Duration of infection at time of sacrifice	# of fields examined	Tetramer+ cells/mm2 in follicle	Tetramer+ cells/mm2 outside follicle	# and % of follicles in which more than half the area devoid of tetramer+ cells
28630	14 days	9	43.33	55.36	(2/4) 50%
30551	21 days	8	15.33	41.00	(4/4) 100%
24225	28 days	2	55.56	94.65	(1/1) 100%
Rh2123	~420 days	23	30.83	60.06	(9/10) 90%

Table 5. Tetramer+ cells inside vs. outside of B cell follicles in lymph nodes of SIV-infected rhesus macaques.
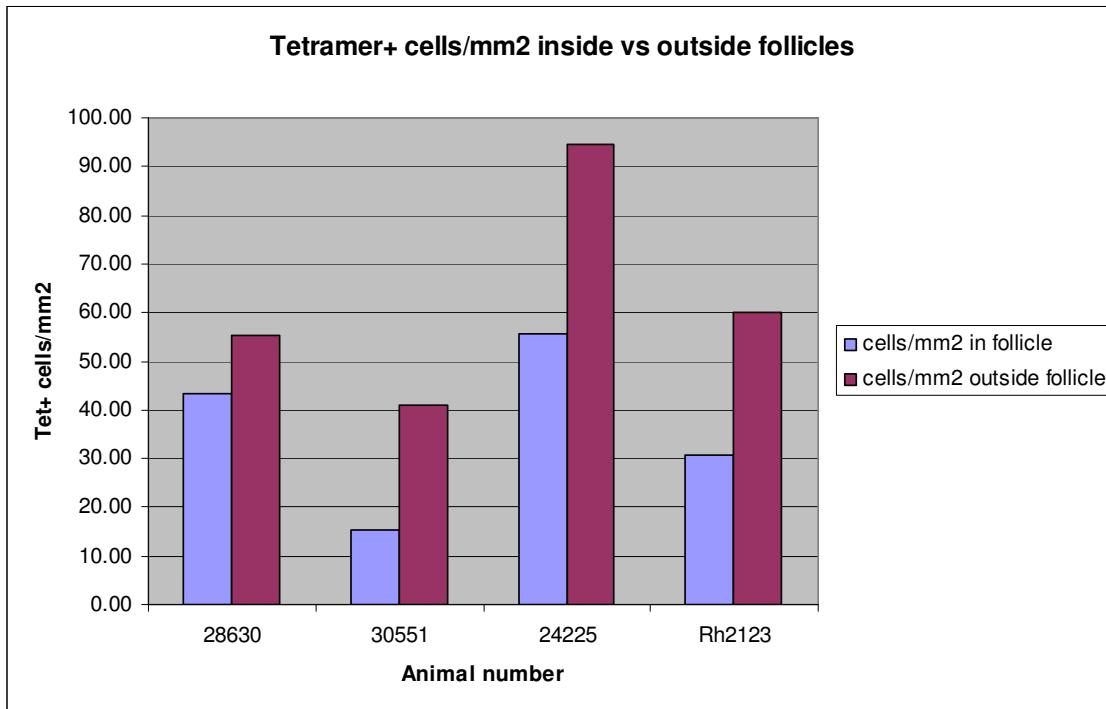
Figure 9. Tetramer+ cells/mm2 inside vs. outside follicles of SIVmac239-infected rhesus macaques. Differences between follicular and extrafollicular tissues were statistically significant (p = 0.03).
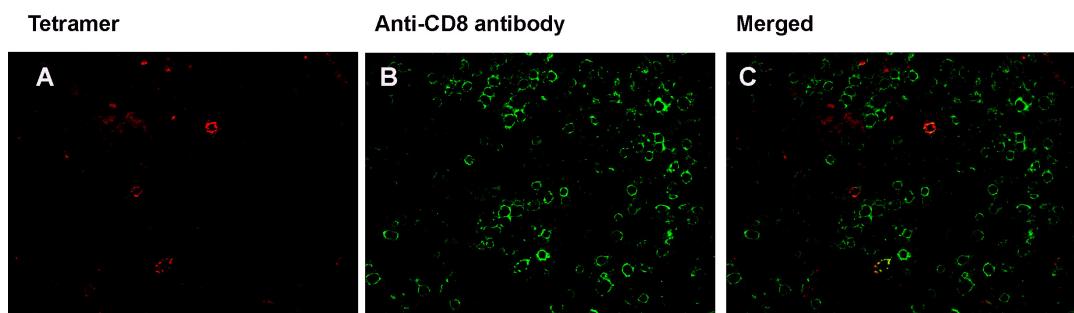
Figure 10. MHC class I tetramers (A) and CD8 antibodies (B) and merged images (C) in lymph node section from SIVmac239-infected rhesus macaque. Lymph node from animal 24225 was stained with A*01 gag tetramers and CD8 antibody. The majority of the tetramer-staining cells also stained with CD8, as shown in the merged image (C). Images are z-scans collected at 15um into the tissue, using a 60X objective.
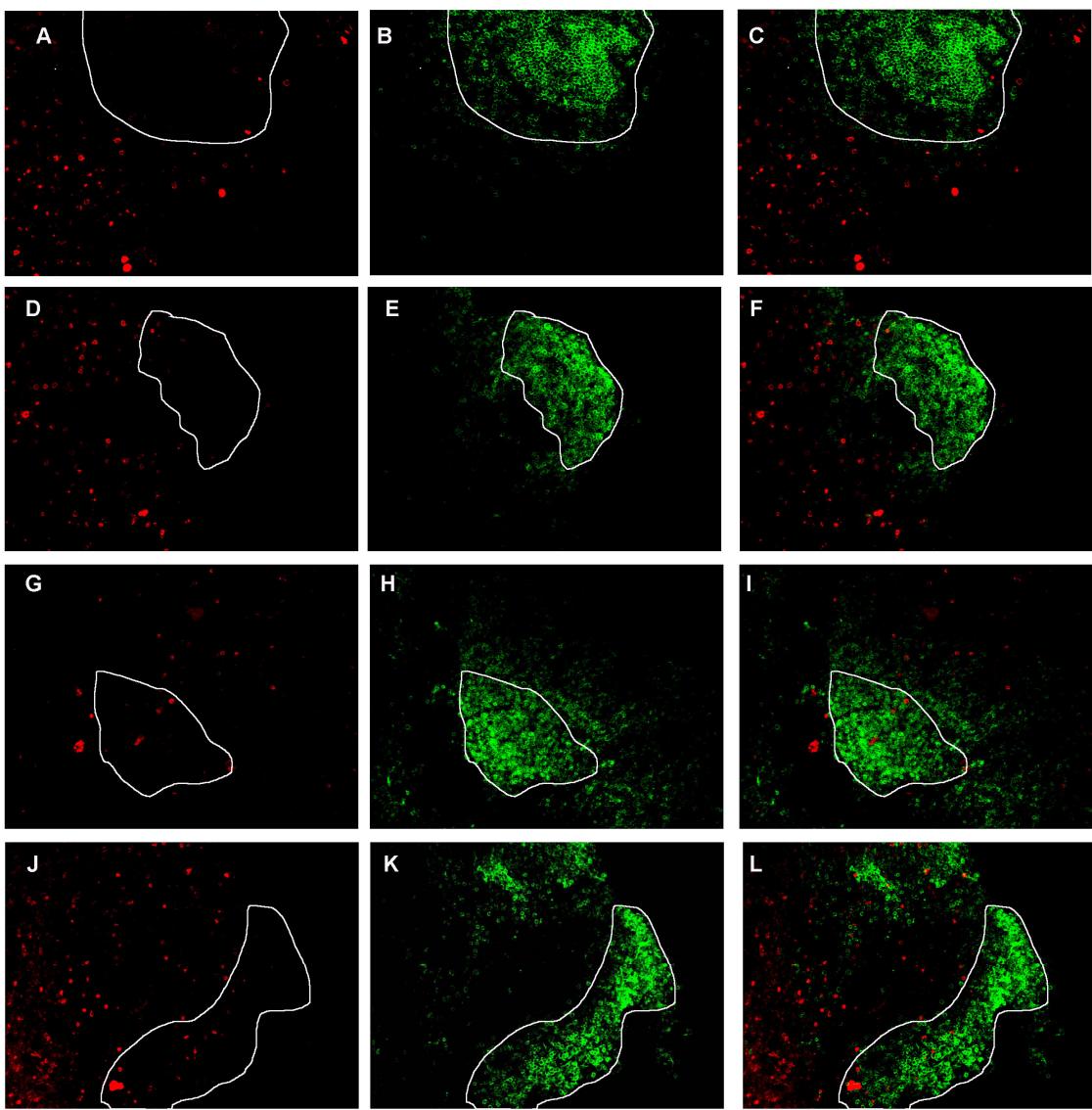
Figure 11. MHC class I tetramers (left panels) and anti-CD20 antibodies (middle panels) staining, and merged images (right panels) in representative lymph node sections from 4 SIVmac239-infected rhesus macaques. A-C shows tissue from animal # 28630 (14 dpi), D-F shows animal #30551 (21 dpi), G-I shows animal #24225 (28 dpi), and J-L shows animal Rh2123 (~420 dpi). Lymphoid follicles, identified by CD20 staining, are outlined in white. All sections were stained with A*01 gag tetramers.   Images are confocal z-scans collected using a 20X objective.

Discussion

The inability of CD8$^+$ T cells to control HIV infection has never been adequately explained. My studies show that HIV-specific CD8$^+$ T cells are largely excluded from B cell follicles in secondary lymphoid tissues during HIV infection, the site in which viral replication is most actively occurring (107), (Chapter 3). This knowledge has strong implications for explaining viral persistence.

Because the SIV-rhesus macaque model is an excellent model system for HIV infection in humans, we evaluated whether the exclusion of virus-specific CD8$^+$ T cells from B cell follicles also holds true in the rhesus macaque model system, using in situ tetramer staining combined with immunohistochemistry. We hypothesized that more SIV-specific CD8+ T cells would be found outside lymphoid follicles than in follicular regions in SIV-infected rhesus macaques. From our preliminary studies of SIV-infected rhesus macaques, we conclude that, as predicted, significantly more SIV-specific CD8$^+$ T cells were outside B cell follicles than inside (Table 5). Although based on a small sample size, these preliminary results imply that the relative inability of virus-specific CD8$^+$ T cells to enter an active site of viral replication may play a role in the establishment of a follicular reservoir of virus that cannot be cleared. Further, these preliminary studies indicate that the exclusion of SIV-specific CD8$^+$ T cells from the follicles holds true in the rhesus macaque model as well as in humans. This indicates that the SIV-rhesus macaque system could be utilized for future studies of the events that occur in the lymphoid tissues during SIV and, by extension, HIV infections.

Admittedly, the present work is a small study based on relatively few animals, and small numbers of fields were available to examine, as shown in Table 5 and Figure 10. In addition, the tissues that I examined were not from animals sacrificed during the chronic stages of infection, but were rather from three animals sacrificed during the acute stages of infection (14, 21, and 28 dpi) and one animal sacrificed during the very late stages of infection (420 dpi). Ideally in the future greater numbers of animals and animals from the chronic stages of infection will be examined. Nonetheless, the small cohort of animals showed significantly greater numbers of tetramer-binding cells outside of B cell follicles than inside and importantly that in the majority of follicles examined (16 out of 19), there was a large area of the follicle (>50% of the total follicular area) from which tetramer-staining cells appeared to be completely excluded. These data indicate that B cell follicles are immune privileged sites in SIV-infected rhesus macaques, similar to that found in the lymph nodes of HIV-infected humans, in which virus can replicate relatively unchecked by virus-specific CD8$^+$ T cell surveillance.

The animals in our in situ tetramer staining study cohort were sacrificed at largely disparate times post-infection, ranging from 14 days to ~420 days. I found in the 14 day post-infection animal (28630), that the numbers of tetramer$^+$ cells inside vs. outside follicles were quite similar to one another (43.33 cells/mm^2 inside vs. 55.36 cells/mm^2 outside). At the later time points, somewhat larger differences were seen in the numbers of tetramer$^+$ cells inside vs. outside follicles (Table 5 and Figure 9). In addition, only 2 of the 4 follicles from the 14 dpi animal showed regions from which tetramer$^+$ cells were clearly excluded, while all or nearly all of the follicles from the remaining 3 animals

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showed regions of clear exclusion. The reasons for these discrepancies are not clear. It could be that, at very early time points after infection, the differences in proportions of SIV-specific CD8$^+$ T cells inside vs. outside B cell follicles are typically not as pronounced as at later time points. It could also be possible that this characteristic is specific to this particular animal. Other work of this sort has, to our knowledge, never before been performed, so there is no body of knowledge with which to compare it. Also, the small sample size used in our studies makes it difficult to compare such findings between animals or between different animals at various time points after infection. Further studies using larger sample sizes need to be done in order to determine the extent to which it is typical for there to be similar numbers of tetramer$^+$ cells inside vs. outside of B cell follicles at acute time points after infection.

It should also be pointed out that animal Rh2123 was sacrificed very late after infection (~420 days). It is possible that this animal had begun to progress to simian AIDS (SAIDS). We cannot be sure that the results of either the in situ tetramer staining or in situ hybridization are typical or comparable to what would be seen in an animal that had not yet progressed to SAIDS. However, the animal appeared to have many intact B cell follicles, and large numbers of SIV-specific CD8$^+$ T cells, and the data gained is good preliminary data on which to base further studies of the localization of SIV-specific CD8+ T cells in lymphoid tissues during SIV-infection at very late stages.

As mentioned previously, further studies involving more animals at various time points after infection need to be performed to validate and extend the results of the

present study. More lymph node samples from each animal would also benefit the studies. Because often there are not many B cell follicles present in a given tissue section, having many sections available could theoretically increase the number of fields available for analysis.

We can also conclude from these preliminary studies that the differences in numbers of virus-specific CD8+ T cells inside vs. outside B cell follicles during the acute stages of infection in macaques were not as pronounced as those seen in humans during the chronic stages of infection. This could be a species-specific phenomenon. As noted above, there is no body of knowledge with which to compare these results, so it is not yet known if it is typical for the numbers of virus-specific CD8+ T cells inside vs. outside of B cell follicles to differ in macaques vs. humans. It is also plausible that more virus-specific Cd8+ T cells are allowed inside follicles during the acute stages of infection than during chronic stages of infection. As noted previously, the majority of animals examined were at early time points after infection (14 – 28 days), while the human subjects examined were at much later time points after infection (4 months to chronic stage infection). Again, performing these studies on a larger cohort of animals during chronic stages of infection, in correlation with the human time points, is needed to clarify these findings.

Studies such as those mentioned in Chapter 3 Discussion in which experiments are performed to determine what mechanisms enable some virus-specific T cells to enter B cell follicles will help elucidate why some virus-specific CD8^{+} T cells enter follicles

while others do not, and what effects would result if CD8$^+$ effector T cells were manipulated to enter B cell follicles where virus is highly concentrated. Because studies of this sort may not be practical or ethical using humans, it is helpful to know that such studies could be performed using a monkey model. Although the present study is a good start, continued work to discover the similarities between viral pathogenesis and immune response in humans and macaques will lead to a greater potential for valuable and relevant model systems for future studies.

Chapter 5: Significance of Findings

Since its emergence in the early 1980's, HIV/AIDS has caused a pandemic of catastrophic proportions. Millions of people have died from AIDS-related infections. Millions of children have been orphaned. Entire societies are disintegrating, all due to the effects of HIV/AIDS. Yet, in spite of education and ongoing research, the incidence of HIV infection climbs ever higher. A definitive prevention method or cure for HIV infection has yet to be developed.   Without a major breakthrough in treatment methods, the majority of the 33 million people currently infected with HIV will be dead within the next 10 years. It is imperative that a preventive method or cure for HIV infection be developed sooner rather than later.

HIV infection destroys the human immune system. The virus preferentially replicates in CD4$^+$ T cells (2, 3), which are a vital component of the adaptive immune response (115). When CD4$^+$ T cell populations are diminished, the body's capacity to fight off infection is severely compromised. This enables a myriad of pathogens to invade, eventually leading to debilitating infections, and ultimately, the death of the patient.

There is much evidence to suggest that CD8$^+$ T cells are important in controlling viral infections, including HIV (108-111). Although the appearance of HIV-specific CD8$^+$ T cells does initially correlate with reduced viral load during HIV infection (56, 59, 122), for unknown reasons this response is ultimately insufficient, and the virus is never cleared from the body. Because the CD8$^+$ T cell response is important in controlling

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viremia, yet unable to completely eliminate virus from the system, it is important to determine why this vital component of the adaptive immune system fails to completely control HIV infection.

My data supports our central hypothesis that virus-specific CD8$^+$ T cells are largely excluded from B cell follicles in secondary lymphoid tissues of HIV-infected human subjects. This may help to explain why virus-specific CD8+ T cells cannot effectively clear virus-producing cells inside B cell follicles in secondary lymphoid tissues, and as a result, HIV-producing cells concentrate within B cell follicles. Our findings indicate that B cell follicles of HIV-infected human subjects appear to be immune privileged sites from which HIV-specific CD8$^+$ T cells are largely excluded. This can explain why HIV primarily replicates inside B cell follicles after the acute stages of infection, establishing a persistent reservoir of virus that seeds infection throughout the remainder of the patient's life.

My data also supports the hypothesis that SIV-specific CD8+ T cells are largely excluded from B cell follicles of SIV-infected rhesus macaques. In collaboration with Dr. Elizabeth Connick and colleagues, we conjectured that, at time points after which CD8$^+$ T cell response had been initiated (14 days post-infection or later) (103, 104), SIV-producing cells would be found primarily inside lymphoid B cell follicles. In addition, we speculated that, at early time points prior to the emergence of CD8$^+$ T cell response (earlier than 14 days after infection) (103, 104) there would be little difference in the amount of virus inside vs. outside the B cell follicles. These speculations are based on the thought that, at early time points before a virus-specific CD8$^+$ T cell response has

developed, virus can replicate relatively unchecked either inside or outside the follicles. The emergence of SIV-specific CD8$^+$ T cells at later time points would then clear infected cells from the extrafollicular regions, while virus would continue to replicate inside B cell follicles, from which the virus-specific CD8$^+$ T cells are largely excluded.

To determine the localization of viral replication at time points both before and after the appearance of SIV-specific CD8$^+$ T cells, in situ hybridization for SIV RNA was performed by our collaborator, Dr. Liz Connick, at the University of Colorado. Animals 26222 and 24818, both of which were sacrificed at time points before the development of SIV-specific CD8$^+$ T cell response, showed similar amounts of virus inside and outside of B cell follicles. Conversely, in animal 301, which was sacrificed 4 months after infection, significantly more virus was found inside B cell follicles than outside (unpublshed data). These data may indicate that, once a T cell response to infection has developed, virus-specific CD8+ T cells are able to clear virus from extra-follicular areas, but are unable to clear virus that is sequestered inside follicles. These data suggest that the location and concentration of SIV-producing cells may be related to the timing and location of SIV-specific CD8$^+$ T cells. This leads us to further speculate that virus-specific CD8$^+$ T cells are able to effectively clear virus-infected cells from extrafollicular regions, but not inside follicles, and this may cause virus-infected cells to concentrate within B cell follicles. This may help to explain, in part, the persistence of SIV and HIV infections (Figure 12).
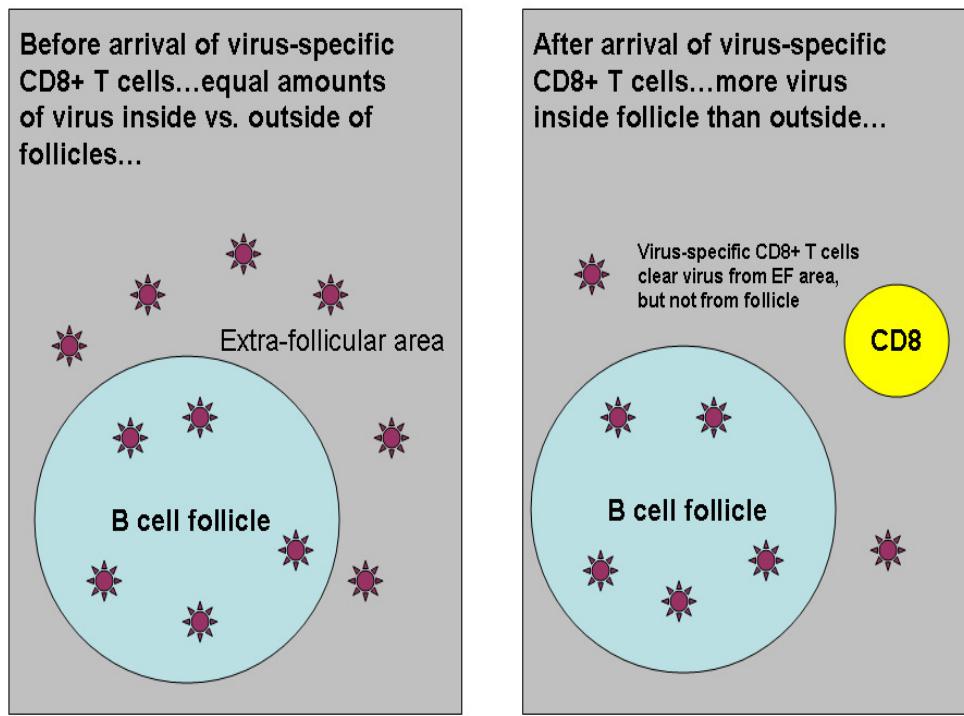
Figure 12. Model of the localization of virus-infected cells inside and outside B cell follicles, before and after the development of a virus-specific CD8+ T cell response.

More definitive information could perhaps be drawn from future in situ hybridization studies performed using animals depleted of CD8$^+$ T cells. With no repertoire of CD8$^+$ T cells to destroy infected cells, it would be expected that the amount of virus inside vs. outside B cell follicles would be relatively similar, analogous to the results seen at early time points after infection, before the development of a CD8$^+$ T cell response. It would also be interesting to note what would happen to the localization of virus upon reappearance of CD8$^+$ T cells in the same system. In this model (Figure 12), infected cells in extrafollicular areas would be destroyed after the emergence of CD8$^+$ T cells, with the end result being a concentration of virus within the follicles. Such CD8$^-$

depletion studies would show more conclusively that CD8$^+$ T cells do indeed play a role in the localization of virus, and the establishment of persistent infection.

Of course, it is possible that other mechanisms contribute to the inability of CD8+ T cells to destroy the reservoir of virus inside B cell follicles. One possible explanation for the relatively low levels of CD8+ T cells in B cell follicles is that CD8+ T cells that enter follicles undergo heightened levels of apoptosis compared to other CD8+ T cells. Since the molecules BCL-2 and caspase 3 (1-3) are both shown to be up-regulated on cells undergoing apoptosis, we tested whether follicular CD8+ T cells show higher levels of these apoptotic molecules than do extrafollicular CD8+ T cells. Preliminary data shows that follicular CXCR5+ CD8+ T cells do not show significantly higher levels of the apoptosis-associated molecules BCL-2 and caspase 3 than do non-follicular CXCR5-CD8+ T cells (unpublished data).

Most HIV in vivo is produced by Ki67+CD4+ T cells (4). HIV mainly replicates in Ki67+CD4+ T cells, therefore, it is reasonable to speculate that one reason HIV is contained mainly within B cell follicles is that there are more Ki67+CD4+ T cells within follicles, thereby making follicular CD4+ T cells more amenable to infection than are CD4+ T cells outside the follicle. To test whether this was indeed the case, we stained lymph node sections of HIV-infected humans and SIV-infected rhesus macaques with CD4, CD20, and Ki67 antibodies, and analyzed them using a fluorescent microscope. As it turns out, concentrations of Ki67+CD4+ cells were not significantly different inside vs. outside of follicles of either humans or macaques, suggesting that the preferential

replication of HIV and SIV inside follicles is not due to more amenable targets inside follicles (unpublished data).

.   Recent studies from our laboratory have shown that cells that enter B cell follicles in the lymph nodes of SIV-infected rhesus macaques are CD8 low or CD8- (191). This could be indicative of a down-regulation of cell surface CD8 molecules upon entry of the cell into the B cell follicle, or could suggest that only cells with low levels of or no CD8 molecules displayed on the cell surface are able to enter B cell follicles. Further studies are needed to elucidate this phenomenon, however, low or no CD8 on the surface of a cell may indicate that lesser degrees of activation and functionality are present (192). This would be consistent with the findings of Quigley, et al. (177), which showed that CD8$^+$ T cells that enter B cell follicles in the tonsils of human subjects express a non-cytolytic phenotype.

This is the first study to visualize the spatial localization of HIV-specific CD8$^+$ T cells in their relationship to B cell follicles. Until our studies were performed, it was not known that virus-specific CD8$^+$ T cells are largely excluded from B cell follicles, where virus replication is most highly concentrated. These findings have important implications in the eventual development of a treatment strategy for HIV infection. Although CD8$^+$ T cells are most often found in the paracortical regions of lymph nodes, they are, on occasion, found inside B cell follicles. Tonsillar CD8$^+$ T cells that enter B cell follicles express the chemokine receptor CXCR5 on the cell surface (177). Further studies will elucidate any other mechanisms by which CD8$^+$ T cells enter B cell follicles. Once it has been determined how and why CD8$^+$ T cells are able to enter follicles, it may then be

possible to manipulate populations of HIV-specific CD8^{+} T cells to enter follicles. Equipped with their stores of cellular weapons, it may be possible for follicle-entering cytotoxic T cells to destroy infected cells in those areas, thereby depleting long-lasting reservoirs of virus. Even though it will not likely be possible for every follicle within a patient's body to be targeted by HIV-specific CD8^{+} T cells, even destroying some or most of the reservoirs may give the immune system a chance to stave off the development of AIDS. With a reduced viral reservoir, fewer CD4^{+} T cells would be infected, thereby leaving a larger population of cells to direct the activities of the immune response. The result of this may be a slower progression to AIDS. It is even plausible to speculate that, depending on the state of the patient's immune system at the outset of therapy, treatment of this sort could prolong life as antiretroviral therapy does, but without the toxic and unpleasant side effects caused by antiretroviral drugs.

Of course, it could be reasoned that artificially induced effector CD8^{+} T cells that are manipulated into entering B cell follicles to destroy reservoirs of virus might destroy the follicular architecture itself, thereby eliminating a vital source of immune function. Effector CD8^{+} T cells carry with them a variety of weapons with which to destroy infected cells, including perforin and granzymes. It is known that human patients suffering from Crohn's disease and other inflammatory bowel diseases have increased levels of perforin in the digestive tract, whereas in normal humans, little perforin is expressed in these areas (126, 193). Therefore, it is thought that a low level of perforin in the gut is an implicit means by which the body protects itself from the potentially harmful effects of perforin expression. With this in mind, it is plausible to consider that CD8^{+} T

cells sent into B cell follicles may very well secrete high levels of perforin as they perform their task of destroying infected cells, creating mass destruction of the follicular architecture as well. If effector CD8$^+$ T cells are manipulated to enter B cell follicles an effect might be seen in which reservoirs of virus are cleared from the follicles. Regardless of the above-mentioned or other speculated detrimental or beneficial effects of CD8$^+$ T cells entering an area from which they are teleologically excluded, it is still well worth performing studies of this kind in an animal model. To our knowledge, studies of this sort have never before been performed. The possibility of manipulating effector CD8+ T cells into lymphoid follicles to destroy reservoirs of virus which were previously out of reach will be a huge step toward a possible cure for persistent HIV infection.

In summary, we conclude that HIV-specific CD8+ T cells are largely excluded from the B cell follicles of HIV-infected humans and SIV-infected rhesus macaques. These studies were the first to generate preliminary data indicating that the exclusion of virus-specific CD8$^+$ T cells holds true in these systems. In addition to this, we have seen that, before the development of a CD8$^+$ T cell response, SIV is localized both inside and outside B cell follicles. However, at time points after CD8$^+$ T cells have appeared, virus is more concentrated inside B cell follicles than outside. This may indicate that CD8$^+$ T cells are able to clear virus from extrafollicular areas, while follicles remain 'off limits' to their cytotoxic effects. This could indicate that virus-specific CD8$^+$ T cells do indeed play a role in viral localization and persistence. Because studies of this sort may not be

practical or ethical using humans, it is helpful to know that such studies could be performed using a monkey model. Discoveries of the similarities between viral pathogenesis and immune response in humans and macaques will lead to further possibilities for valuable and relevant model systems for future studies.

Additional Material

The following publications are included as supplementary documents in the online submission of this dissertation, and in pages following the References section of the printed document:

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