LOCATION, ABUNDANCE AND PHENOTYPE OF FOLLICULAR SIMIAN IMMUNODEFICIENCY VIRUS-SPECIFIC CD8 T LYMPHOCYTES

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

Shengbin Li

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

Dr. Pamela J. Skinner, Adviser

May 2018

Acknowledgements

I would like to thank my Ph.D. advisor, Dr. Pam Skinner, for guiding me in the past four years. Pam, thank you for your patience and effort in teaching me how to be a good scientist. Your continuous encouragement made me a lot more confident. Actually, I received your help even before I came here. If you didn't forward my email to Kate, I might not be able to complete the online application which was a precondition for everything ensued. Thank you very much.

I would also like to thank the Skinner lab team members for providing technical support, intellect and friendship. Including past and present members: Heyon Kim, Reece Wagstaff, Preethi Haran, Gwantwa Mwakalundwa, Ryoko Hatsugai, Hadia Mohammad, Katie Kovacs, Mary Pampusch, Chi Phan, Renu Pandy, Mattew Well and Mary Wuollet. They are not just good team members in the lab work, but also good friends in my life.

I would like to thank my thesis committee members: Dr. Bruce Walcheck, Dr. Yuying Liang and Dr. Steve Jameson. Thanks a lot for their valuable advice, excellent guidance and precious time. Special thank you to Dr. Liang and Dr. Hinh Ly for the invitation to their Thanksgiving Party every year.

I next want to thank Dr. Michael Murtaugh and Dr. Mark Rutherford for their great help since my application in 2012. Without their help, my life would be totally different. I also want to say thanks to Lisa Hubinger and Kate Barry. Without their great help during the application and PhD study here, my life would be a lot harder.

I would like to thank all of my friends and everyone who has ever helped me. Your presence in my life supported me to get through the difficult times and made my life better and more colorful. Thanks for sharing my happiness, sadness and accomplishments.

Finally, I would like to most sincerely thank my parents and younger sister. My parents give me the best of everything they have. My sister did a lot to share my responsibility for the family to let me focus on my work. Without their true love, unending encouragement and unconditional support, this exciting and wonderful journey would not have been possible.

Dedication

This dissertation is dedicated to my parents and my younger sister. Because my parents truly believe education is the best way to change my life, they did their best to teach me everything they know and help me pursue as much knowledge as possible. Without their constant and unreserved support, I couldn't even start my PhD study. After I came to US in 2013, my younger sister took my responsibility in the family. Without her indispensable role in the family, I couldn't focus on my PhD study and reach where I am now. Thank you so very much for everything you have done for me!

Abstract

CD8 T cells play a critical role in controlling and eradicating virus-infected cells. Although many studies demonstrated the notable anti-viral effect of HIV-specific CD8 T cells during HIV infection, these cells fail to fully eliminate viral replication. The phenomenon that only a small population of HIV-specific CD8 T cells migrate into B cell follicles where HIV-producing cells are most highly concentrated during chronic infection is one major mechanism account for the failure of these cells to fully suppress HIV replication. It is not known whether this phenomenon also occurs during early infection. Moreover, whether follicular HIV-specific CD8 T cells are functional in suppressing viral replication is not fully understood. Simian immunodeficiency virus (SIV)-infected rhesus macaques are a good animal model for HIV research. In the present study, we determined the location, abundance and phenotype of follicular SIV-specific CD8 T cells in lymph nodes from SIV-infected rhesus macaques using *in situ* tetramer staining combined immunohistochemistry, confocal microscopy and quantitative image analysis. We found that during chronic SIV infection, despite high levels of exhaustion and likely inhibition by Foxp3+ cells, a subset of follicular SIV-specific CD8 T cells are functional and suppress viral replication *in vivo*. Similar to chronic infection, low levels of SIV-specific CD8 T cells migrate into B cell follicles during early stages of infection and a subset of these cells likely possess cytolytic function and suppress viral replication. In addition, low levels of follicular SIV-specific CD8 T cells from GCs during early infection may set the stage for the establishment of persistent chronic infection. These findings provide important insights into HIV immunopathogenesis and support HIV cure strategies that augment functional follicular virus-specific CD8 T cells to enhance viral control. We also evaluated the effect of ALT-803, a novel human IL-15 superagonist and potent immunostimulatory molecule, on SIV-specific CD8 T cells in chronically SIV-infected rhesus macaques. We found that ALT-803 drives dramatic expansion of SIV-specific CD8 T cells in lymphoid tissues and, importantly, induces significant

accumulation of SIV-specific CD8 T cells in B cell follicles, reducing the number of SIV-producing cells within B cell follicles. These data justify the further evaluation of ALT-803 for eradication of HIV-infected cells.

Table of contents

List of Tables

List of Figures

Chapter 1

Introduction

HIV Background

Human immunodeficiency virus type 1 (HIV-1) is the cause of acquired immune deficiency syndrome (AIDS) which continues to be a severe global pandemic. Since the 1980s, HIV has infected over 53 million people and caused more than 20 million deaths (1). Currently, more than 36 million people worldwide are living with HIV and there are still around 1.8 million new infections annually ("Fact sheet – Latest statistics on the status of the AIDS epidemic". UNAIDS.org. 2016). Although great effort has been invested in the development of effective antiretroviral therapy (ART) to limit HIV-1 replication and progression to AIDS, a protective vaccine and a sterilizing cure are still unavailable.

After infection, HIV preferentially infects CD4+ T cells, a key component of the human immune system, and results in a progressive loss of CD4+ T cells (2-4). In the absence of treatment, continuous loss of CD4+ T cells will ultimately severely damage most patients' immune system and compromise the host's capacity to fight against infection. This leave the patients unprotected to a myriad of pathogens in the natural environment. Any infection, normally insignificant to healthy individuals, may lead to severe a health crisis or even death, of HIV infected patients.

The discovery and global impact of HIV

In 1981, AIDS was first observed in clinic in a cluster of young homosexual males with pneumocystis pneumonia (PCP) - a rare opportunistic infection that almost only occurred in severely immunocompromised patients (5). During the same time period, a relatively benign form of skin cancer called Kaposi's sarcoma, which usually affected elderly men, was reported in young homosexual men in the United States (6, 7). Around two years later, Dr. Luc Montagnier's group declared that they isolated a virus that may be the causative agent of AIDS and named it lymphadenopathy-associated virus (LAV) (8). In the same year, another group led by Dr. Robert

Gallo announced that their isolated virus named human T-lymphotropic viruses-III, or HTLV-III was the cause of AIDS (9). In addition, Dr. Jay Levy and his colleagues independently published report about discovery of the AIDS virus and named it the AIDS associated retrovirus (ARV) (10). These viruses were soon discovered to be the same and arguments about the official name of this virus ensued. Finally, the International Committee on the Taxonomy of Viruses decided to use human immunodeficiency virus (HIV) to name the newly discovered virus in 1986 (11).

Currently, it is estimated that approximately 36.7 million people worldwide are living with HIV-1 and the number of new infections in 2016 was about 1.8 million which is lower than 3.1 million in 2001 ("Fact sheet – Latest statistics on the status of the AIDS epidemic". UNAIDS.org. 2016). The distribution of HIV-1 infection in the world is uneven. Sub-Saharan African populations, which make up only 10% of the world's population, contain approximately 68% of the world's HIV-1 infected individuals. In addition, around 70% of new infections each year occur in this region (1). HIV-1 infection remains a lifelong physical and psychological torment for infected individuals as well as an enormous economic burden for society. Globally, most HIV transmission is occurring by unprotected intercourse where girls and young women represent a particularly vulnerable group. HIV can be transmitted by blood or blood products where routine donor testing is not available. HIV infection is also prevalent among intravenous drug abusers through the use of contaminated needles.

Origins and structure of HIV

HIV belongs to the genus Lentivirus, family Retroviridae. Lentivirus, which means "slow virus", takes a long time to cause serious diseases after infection in human and other mammalian species (11). There are two major types of HIV: HIV-1 and HIV-2. HIV-1 has higher virulence and infectivity, and can be further classified into groups M, N and O. Group M is responsible for

the majority of HIV infections worldwide (12-14). In contrast to HIV-1, HIV-2 is much rarer, less virulent and largely restricted to West Africa (14). (HIV-1 is referred to as HIV from this point on.)

The HIV virion is about 120nm in diameter with 2 identical copies of a positive polarity, single-stranded RNA genome that encodes the nine genes of the virus. The HIV RNA genomes, along with the nucleocapsid proteins, reverse transcriptase, integrase and protease, are enclosed by a conical capsid which consists of p24 protein. The capsid is, in turn, protected by the matrix which is made of p17 protein. This entire structure is surrounded by the viral envelope which is comprised of viral glycoproteins (gp) and components acquired from the infected host cell membrane when new viral particles bud out from the cell (15, 16). Heterodimers consisting of viral proteins gp120 and gp41, are embedded in the envelope as homo-trimers, and play a critical role in attachment and initiation of the infectious cycle (17) (Fig. 1).

The HIV genome has 9 genes that encode 19 proteins (three major genes: *gag*, *pol* and *env*; six regulatory genes: *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu*) (Fig. 2). The *gag* gene is translated to generate precursor polyproteins which in turn are cleaved to produce the viral structural proteins (the matrix protein, the capsid protein and the nucleocapsid protein) (18). The *pol* gene produces the viral enzymes including reverse transcriptase, integrase and protease which are critical for viral replication. The *env* gene encodes precursor proteins to produce viral surface proteins such as gp120 and gp41 that are key components for HIV to bind and enter the host cell (19). The proteins encoded by *tat* and *rev* genes are important to enhance the viral gene expression and promote viral replication (20). The functions of *nef* protein include enhancement of viral infectivity, down-regulation of HLA/MHC class I and class II molecules and stimulation of viral replication (21-23). The *vif* protein helps neutralize the action of host antiviral proteins such as APOBEC3G (24, 25). The *vpu* protein functions to efficiently release new viral particles from the

infected cells (26). The *vpr* protein promotes HIV pathogenesis by arresting the host cell cycle in G2 phase to stimulate virus transcription (27).

The life cycle of HIV

The life cycle of HIV starts with the interaction of the envelope glycoprotein, gp120, and CD4 molecules on the surface of target cells such as CD4+ T cells, macrophages and dendritic cells (28). Following on from the interaction between gp120 and CD4, there are conformational changes that allow gp41 to interact with HIV co-receptors, either CXCR4 or CCR5, to complete membrane fusion and viral entry (29-32). After the fusion and entry are completed, core structures containing viral RNA genomes are released into the cytoplasm and the genomic RNA is converted into double-stranded viral DNA by the combined enzymatic activities of viral reverse transcriptase. After translocation into the cell nucleus, the viral DNA is inserted into the host cell genome by the viral integrase to create a provirus (33). Once the integration is completed, the provirus can lead to either a latent infection by producing few or no copies of new virus, or an active infection (20, 34). Under conditions of little or no provirus transcription, progeny virion formation does not occur and the infected cell could be considered to be in a quiescent but chronically-infected state. The provirus can be recognized and transcribed by host RNA polymerase II and that active transcription is necessary for a productive infection. When the provirus is activated, materials required for producing new viral particles start to be synthesized by using the host machinery (20). Newly formed viral cores assemble below the cell membrane where the envelope proteins gp120 and gp41 have accumulated. During budding, viral particles incorporate substantial portions of the cell membrane along with gp120 and gp41 to form a viral envelope (35). During the releasing process, the cleavage of Gag and GagPol polyproteins by

protease triggers the maturation of infectious viral particles. Finally, the newly generated mature viral particles are ready to infect new target cells (36).

Non-human primate model for HIV infection

Non-human primate models have become a critical resource for better understanding of human disease progression and development of vaccines and effective therapeutic strategies. For quite a long time, simian immunodeficiency virus (SIV)-infected rhesus macaques have been commonly regarded as the best animal model for HIV research (37). Like HIV, SIV also belongs to the genus of lentivirus and is closely related to HIV in genetic structure (38, 39). SIV-infected rhesus macaques develop clinical symptoms of disease and show progressive changes in lymph node structure which are very similar to disease progression of HIV infection in humans (38, 40-44). Different strains of SIV, either pathogenic or non-pathogenic, can be used in rhesus macaque models to help improve understanding of HIV pathogenesis, and develop an effective vaccine and functional cure (37). Generally, SIV-infected rhesus macaques, an indispensable animal model for HIV infection in humans, has been widely used in both basic and applied research.

Stages of HIV infection

There are multiple stages of HIV infection. It is extremely hard to study the earliest stages of HIV infection in human patients because, ethically, treatment must be offered immediately when infection is suspected or confirmed and, in many patients at all stages of infection, it is impossible to define the exposure event leading to primary HIV infection. Therefore, SIV-infected rhesus macaques have been used to study the early stages of HIV infection. After experimental intravaginal infection, the virus crosses the mucosal barrier, infects

and replicates predominantly in CD4+ T cells close to the portal of entry. In this process, not only activated and proliferating CD4+ T cells, but also resting CD4+ T cells are infected. Among the infected CD4+ T cells, proliferating cells serve as a short-lived population that generates many viral particles, while resting cells may contribute to the latent and chronic infection. Other susceptible cells in this area such as macrophages and dendritic cells may also be infected (45, 46). Sometime within the first 5-7 days of infection, virus infected cells migrate to the draining lymph node and distal lymphatic tissues including the gut-associated lymphoid tissue (GLAT) through the bloodstream and lymphatic system (47). Once virus-infected cells reach the lymphoid tissues, where many CD4+ T cells are located, easy access to target cells leads to high level viral replication and a peak viral load occurs between 10 to 14 days post infection (48). CD4+ T cell numbers decrease rapidly (49). During this process, a stable viral reservoir of chronically infected cells becomes established in lymphoid tissues. The large pools of virus and virus-infected cells cannot be fully eradicated naturally by this time even though the host immune system is still functional and has been primed to recognize HIV-specific antigens. In this stage of infection, many infected individuals only develop mild influenza-like symptoms and may not know that they are infected with HIV (50-53). Early infection is followed by an asymptomatic phase during which the patients typically show no clinical disease. This stage, which is called clinical latency or chronic infection, can last anywhere from three to twenty years without treatment (54-56). Progressive loss of CD4+ T cells also occurs in this phase. When the CD4+ T cell count is lower than 200 cells/μL, which meets the definition of AIDS, most patients start to develop opportunistic infections and viral-induced cancers. In the absence of specific treatment, the majority of patients succumb to the complexities of immunodeficiency syndrome and die within 1-2 years (48).

Mechanisms of HIV and SIV to evade host immune response

Both HIV and SIV have multiple mechanisms to evade attack and elimination by the host immune system. After infection, antibodies are developed within a few weeks of detectable viremia. However, these early antibodies don't have detectable effect on controlling viremia. Neutralizing antibodies which have the potential to protect against HIV develop months later (57). Studies showed that HIV can evade vigorous neutralizing antibody response in many ways. The envelope proteins complexes on the surface of virion can serve as a direct antibody target (57, 58). Nonetheless, extensive glycosylation of the envelope proteins results in masking key epitopes. The modified surface proteins further act as a shield to protect other more vulnerable eptitopes by minimizing the physical access of antibody to these viral epitopes (59, 60). Moreover, quick generation of virions with unrecognizable antigens to the present antibody repertoire by rapid mutation makes a sterilizing antibody response impossible (60, 61). HIV mutation also seriously weakens CD8+ T cells immune response. HIV is able to change the main epitopes recognized by CD8+ T cells by mutating corresponding genes to form escape variants. These variants make recognition of the virus difficult for CD8+ T cells (62, 63).

T regulatory cells (Treg) play a pivotal role in maintaining immunological self-tolerance, controlling autoimmune disease (64, 65), and suppressing immune activation (66, 67). This cell population may also be utilized by HIV to promote its immune evasion. Previous studies showed that Treg may downregulate T cells response to facilitate the virus-infected cells to escape immune surveillance during HIV and SIV infection (68-70).

Quick establishment of productive infection is another way that HIV may use to escape the host immune response. Previous research showed that productive systemic infection of SIV and peak virus production in tissues were established between 7 to 10 days post infection. While virus-specific CD8+ T cell immune response typically was not fully developed until 21 days post infection (71, 72). Therefore, the relatively late specific immune response is unable to fully control the established productive systemic virus infection.

HIV may also escape immune surveillance by hiding in an anatomical sanctuary in the host. During chronic HIV and SIV infections, virus-producing cells are concentrated within B cell follicles in secondary lymphoid tissues (73-77). In rhesus macaques undergoing long-term, fully suppressive combined antiretroviral therapy (cART), residual SIV infection is also preferentially localized in B cell follicles (78). A number of studies showed that HIV- and SIV-specific CD8+ T cells are typically most concentrated in T cell zones outside B cell follicles in lymph node and spleen tissues and, therefore, are largely excluded from follicles (73, 74, 79, 80). Thus, B cell follicles appear to be somewhat of an immunoprivileged site in which virus-specific CD8+ T cells are unable to clear all virus-producing cells.

Current methods to treat HIV infection

Currently, there is no cure for HIV infection. However, building on decades of basic HIV research, effective antiretroviral drugs have been developed to target different steps of the HIV life cycle. Drugs, that bind to the chemokine receptor CCR5 or viral envelope protein gp41, serve as fusion and entry inhibitors are able to prevent HIV from entering into target cells (81, 82). Moreover, inhibitors that target viral reverse transcriptase, integrase and protease are available (83). Nucleoside reverse transcriptase inhibitors consist of chain-terminator deoxynucleotide analogues that lack the 3'-hydroxyl group on the deoxyribose moiety which is required for synthesis of the viral DNA. They impair viral replication by competing with the natural deoxynucleotides. The newly synthesized viral DNA chain cannot further elongate once nucleoside reverse transcriptase inhibitors have been incorporated (84). On the other hand, non-nucleoside reverse transcriptase inhibitors are able to bind to the viral reverse transcriptase enzyme directly to induce conformational changes, thus interfering with active site function and independently disrupting the viral replication (85). Protease inhibitors prevent cleavage of the *gag*

precursor polyprotein into mature viral structural proteins (86). Integrase inhibitors interfere with viral replication by preventing the double-stranded proviral DNA from integrating into the host cell genome (87). Combinations of multiple antiretroviral drugs have been widely applied and current standard of care therapy in the USA is based on three drug combinations. This combined treatment regimen is now known as antiretroviral therapy (ART) and has significantly decreased the morbidity and mortality of HIV infection, and prolonged the longevity of HIV infected patients (88). Studies have shown that a combination of different drugs that target 3 or more steps of HIV replication cycle has best curative effect in suppressing HIV infection (88-91).

Although antiretroviral therapies have many benefits for HIV patients, they also come with unpleasant side effects, such as diarrhea, nausea and vomiting, hepatotoxicity, lipodystrophy and skin rash (92, 93). Also, HIV is able to obtain resistance to antiretroviral drugs several months post treatment through mutations (92, 93). Furthermore, the drugs can be very expensive and are often unavailable for patients in underdeveloped countries where the disease is prevalent. Therefore, development of an effective HIV vaccine or a functional cure to fully eradicate HIV is still in urgent need.

Host immune response to HIV infection

The immune system plays an indispensable role in controlling and eradicating invading pathogens in order to protect the host against diseases. The innate and adaptive immune systems are two separate but interrelated arms with the combined function of host protection. Once pathogens breach one of the hosts' physical barriers, non-specific innate immune responses are triggered immediately. However, if the pathogen successfully evades innate immune responses, the adaptive immune system becomes activated producing responses specifically targeting the pathogen. Here I will mainly concentrate on the biology of T cells and their role in immune

responses against viral infection. This is important background directly related to my thesis project which mainly focuses on HIV/SIV-specific CD8 T cell immune responses post infection.

CD4+ T cells

CD4+ T cells are a key cell population in development and maintenance of both humoral and cellular immune responses (94). Naive CD4+ T cells can be induced to become Type 1 helper T cells (Th1), Type 2 helper T cells (Th2), Type 17 helper T cells (Th17) and Tregs under stimulation of different cytokines (94-96). Th1 cells play an important role in immunity against intracellular pathogens. The key transcription factors of Th1 cells are STAT4 and T-bet. Th1 cells produce interleukin-2 (IL-2), interferon-γ (INF-γ) and tumor necrosis factor-α (TNF-α) which are of great importance in activation and proliferation of CD8+ T cells (94, 95). Th2 cells are important against extracellular pathogens including parasites. The key Th2 cells transcription factors are STAT6 and GATA3 (95). Th2 cells produce cytokines such as IL-4, IL-5, IL-6, IL-10 and IL-13 which play a pivotal role in promoting antibody-mediated immunity involving B cells. Cytokines produced by Th2 cells and direct contacts with Th2 cells stimulate B cell proliferation, induce antibody class switching, direct affinity maturation and differentiation of B cells into plasma cells that are the major cell source of antibodies (94, 95). Th17 cells are defined by their production of IL-17. They contribute to pathogen clearance at mucosal surfaces. The dysregulation of these distinct types of CD4 T cells is associated with autoimmune disease and inflammatory disorders (97-99). The loss of Th17 cells leaves the intestinal barrier disrupted which in turn contributes to chronic HIV infection and disease progression via increased movement of bacteria out of the gut (100). In contrast to the positive regulation of immune response, Tregs are a subpopulation of CD4+ T cells are able to modulate overactive immune

response, maintain immunological self-tolerance and control autoimmune disease. These cells express CD4, CD25 and Foxp3 (64-67, 101).

CD8+ T cells

CD8+ T cells, also known as cytotoxic T lymphocytes (CTLs), play a critical role in the cellular adaptive immune response against viral infections and cancer. There are two main mechanisms that CD8+ T cells use to trigger apoptosis of target cells. Firstly, CD8+ T cells can secrete lytic molecules such as perforin and granzymes which in turn work together to kill virus infected cells (75, 102). Perforin is released by CD8+ cytotoxic T cells by exocytosis and is able to make pores in the membrane of target cells recognized by CD8+ T cells (103). Granzymes, include granzymes A, B, H and M, belong to serine proteases family (104). Granzyme B possesses the strongest ability to induce target cell apoptosis in granzyme family (105). It can cleave and activate caspases which in turn trigger cell apoptosis (106). After the pores are formed by perforin in target cells membrane, granzmyes secreted by CD8+ T cells enter the target cells via these pores and induce cell death subsequently via different pathways (105, 107). Secondly, CD8+ T cells are able to induce apoptosis of target cells through cell surface interactions. Interaction between Fas ligand (FasL or CD95L) on the surface of CD8+ T cells and Fas (CD95) drives apoptotic death of target cell (108, 109). Moreover, CD8+ T cells are able to produce IFN-γ which can inhibit viral replication (110). It not only activates and recruits macrophages to the sites of infection, but also induces increased expression of MHC class I molecules as well as MHC class II molecules to facilitate antigen presentation (111).

In addition to the killing mechanisms mentioned above, CD8+ T cells also have other non-cytolytic mechanisms to contribute to host immune defense (112, 113). Anti-viral factors produced by CD8+ T cells such as β-chemokines, macrophage inflammatory protein-1 alpha and

-1 beta (MIP-1α and MIP-1β) and stromal cell-derived factor-1 (SDF-1) are able to competitively bind to co-receptors, necessary for HIV attachment, thus inhibiting viral entry (114, 115). Furthermore, CD8+ T cells are also able to suppress HIV replication by inhibiting its transcription process through CD8+ T cells antiviral factor (CAF) (116).

T cells recognition of antigen

The first step of T cell immune response against infection is recognition of invading pathogens. This is accomplished by the ability of the T cell receptor (TCR) to recognize complexes that are comprised of peptide antigens and MHC class I and II molecules expressed on the surface of antigen presenting cells (APCs) or infected cells via surface T cell receptors (TCR). Two signals are required for activation of both CD4+ and CD8+ T cells. Signal 1 is triggered by the interaction between TCR-CD3 complexes on the T cell surface and peptide-MHC complexes on the surface of APC or infected cells with the assistance of co-receptors (CD4 or CD8 molecules) (117). Signal 2 is provided by the interaction between co-stimulatory molecules on the surface of T cells (CD28) and APC (CD80 or CD86). There are two distinct pathways for antigen presentation.

First, intracellular pathogens including virus are degraded by proteasome into short peptides of 8-10 amino acids. Then these peptides are transported to the endoplasmic reticulum by transporters associated with antigen processing (TAP) where peptide-MHC class I complexes form. After loading of peptides on MHC class I molecules, the assembled complexes are brought to the cell surface via Golgi apparatus where they can be recognized by cytotoxic MHC class I restricted CD8+ T cells (118). Antigen recognition further results in CD8+ T cells activation, proliferation and differentiation. Once activated, cytotoxic CD8+ T cells play an indispensable role in eliminating viral infection.

Second, extracellular pathogens including bacteria can be internalized by APC such as dendritic cells and macrophages. Subsequently, antigens are degraded into peptides are 13-18 amino acids long in vesicles within APC. These peptides bind to MHC class II molecules to form complexes which are brought to the cell surface for recognition of CD4+ T cells. After antigen recognition and activation, CD4+ T cells start to proliferate and differentiate, and perform their functions in inducing and maintaining adaptive immune response such as antibody generation and promote CD8+ T cell activation (119-121).

Structure of lymph nodes

The lymphatic system, consisting of a series of organs and vessels which carry lymph, serves as a vital part in initiating adaptive immune responses. Primary lymphoid organs (bone marrow and thymus) support production and maturation of lymphocytes whereas secondary lymphoid organs (including spleen, lymph nodes, tonsils and Peyer's patches) comprise functional sites where immune responses are initiated. After HIV/SIV infection, the vast majority of virus-producing cells are CD4+ T cells located in secondary lymphoid tissues (2, 122, 123). Within secondary lymphoid tissues, virus-producing cells are most concentrated within B cell follicles during asymptomatic chronic infection (73-77). Here, we mainly focus on the events occur in lymph nodes.

Lymph nodes are small lymphoid organs, widely distributed throughout the body. They are connected through the lymphatic vessels to form a network as part of the circulatory system. A large number of lymphocytes, macrophages and APCs such as dendritic cells (DCs) located in lymph nodes. Typically, lymph nodes are protected by a fibrous capsule. The part underneath the capsule is cortex. The outer superficial part of cortex mainly houses B cells. The deep part of cortex, also known as paracortex, consists mainly of T cells. The inner portion of the lymph node

is the medulla which includes many memory B cells, plasma cells and macrophages (Fig. 3). Plasma cell precursors migrate from cortex to medulla, mature into plasma cells and secrete antibodies there as well (124). APCs travel through the lymphatic system within lymph. APC enter the lymph nodes through a series of afferent lymphatic vessels and pass through different compartments via a system of sinuses and eventually exit the lymph node through efferent lymphatic vessels. In this process, APC expressing foreign peptides on surface MHC molecules encounter the B and T lymphocytes in the cortex and paracortex. APC bearing foreign antigens will activate lymphocytes with surface receptors capable of recognizing specific peptide-MHC complexes and initiate specific immune response. Activated and memory B and T lymphocytes also circulate through the body and continuously traffic between blood stream and secondary lymphoid organs to search for invading pathogens.

B lymphocytes enter the lymph node through high endothelial venules and home to follicles in superficial cortex. Chemoattractant CXCL13 produced by follicular dendritic cells (FDC) drives migration of B cells into follicles by interacting with CXCR5, the receptor for CXCL13, on the B cell surface (125). Follicles can be further categorized into primary lymphoid follicles without germinal center (GC) and secondary lymphoid follicle with GC. Upon receiving antigen stimulation, B cells in follicles start to proliferate and ultimately form a GC (126-128). The GC consists of dark zone and light zone. In the dark zone, B cells go through clonal expansion, somatic hypermutation and isotype switching. During this time, rapidly proliferating B cells are also known as centroblasts (129). As time goes on, some centroblasts reduce their rate of division and begin to express higher levels of surface immunoglobulin. These cells, termed centrocytes, migrate from dark zone to light zone for selection. After selection, surviving cells further differentiate into plasma cells or memory B cells and exit the lymph node via efferent lymphatic vessels (130). During the GC reaction, resting B cells not experiencing this process are displaced to the edge of follicles and form the follicular mantle zone. Therefore, a fully formed

secondary lymphoid follicle includes both a GC and a mantle zone. T follicular helper cells (T_{FH}) and FDC are very important for the GC reaction. T_{FH}, a specialized subset of CD4+ helper T cells that express CXCR5 which is required for migration into B cell follicles, play a critical role in facilitating selection, survival, differentiation and antibody production of B cells (131, 132). FDC can capture large numbers of antigens in the form of immune complexes. Binding to these immune complexes presented by FDC provides an indispensable stimulus for B cell expansion and differentiation (133).

Role of B cell follicles in HIV and SIV infection

Virus-producing cells are highly concentrated within B cell follicles in both asymptomatic chronic HIV and SIV infections (73-75, 77). Furthermore, many studies have identified B cell follicles in secondary lymphoid organs as important anatomical reservoirs of residual productive HIV/SIV infection in controllers who can suppress viral replication either spontaneously or passively (74, 75, 77, 78, 134-136). CD4+ T cells located within B cell follicle are around 40 times more likely to be productively infected by HIV than CD4+ T cells outside B cell follicles (132). Recently, T_{FH} have been implicated as the primary target and main site of HIV/SIV infection and replication inside B cell follicles (73-76, 78, 137). T_{FH} expansion has been reported during chronic HIV and SIV infection, providing increased availability of target cells for virus replication (138, 139). In the context of *ex vivo* CCR5-tropic GFP reporter virus infection, Bcl-2, an antiapoptotic protein, is upregulated in CXCR5+ productively infected cells (140). This suggested that infected T_{FH} cells may have increased survival during HIV infection with the consequence of prolonged virus production. Moreover, T_{FH} function is severely impaired in untreated HIV infection which further damages the capability of host immune responses to control HIV infection (132). During HIV infection, FDC hold many virions as immune

complexes close to T_{FH} in B cell follicles (141-143). This extracellular accumulation of virions is potently infectious to CD4+ T cells, even in the presence of neutralizing antibodies (143). Tumor necrosis factor-α (TNF-α) released by FDC further promotes HIV replication in T_{FH} cells via upreguation of viral transcription (144). Therefore, despite the important positive roles in host immune responses by facilitating the GC reactions mentioned above, T_{FH} and FDC also contribute to persistent HIV and SIV infection. Taken together, these findings provide strong evidence that the B cell follicle is a significant anatomical reservoir for HIV and SIV infection and impedes complete virus eradication.

CD8 T cells are indispensable, but not fully effective for controlling HIV and SIV infection

Numerous studies strongly suggest that CD8+ cytotoxic T lymphocytes (CTL) play an indispensable role in controlling HIV and SIV infection both *in vitro* and *in vivo*. CD8+ T lymphocytes were demonstrated to be able to suppress HIV and SIV replication *in vitro* (145, 146). We previously showed that HIV-specific CD8 T cells are capable of inducing lysis of infected cells in ^{51}Cr release assays (74). The development of HIV-specific CD8 T cells coincides with a decline in plasma viremia during acute infection (147, 148). This suggests that CTL are critical determinants of the initial control of HIV replication. Effector CD8 T cells to virus-infected cell ratios in lymphoid and genital tissues *in vivo* correlate with reductions in viral load (149), and levels of SIV-specific CD8 T cells in lymphoid compartments predict levels of SIV-producing cells in those compartments (73). Further, disease progression is associated with diminished HIV- and SIV-specific CD8 T cells responses (150-152) and with mutations in HIV and SIV at epitopes recognized by HIV- and SIV-specific CD8 T cells (153-156). Elite control of HIV, defined as rare HIV-positive individuals who have very low or undetectable HIV plasma viral load in the absence of any HIV medications (157), is associated with specific MHC-class I

alleles and with polyfunctional CTL responses (158-163). Selective pressure from strong HIV/SIV-specific CD8 T cell responses in elite controllers is reflected by viruses carrying CTL escape mutations (164-166). Perhaps the most powerful evidence that CTL are important in controlling HIV and SIV infections comes from experiments in which CD8 cells were temporarily depleted in rhesus macaques during chronic SIV infection (167-170), which led to as much as 1000-fold increases in plasma viremia, and the subsequent recovery of CD8 cells led to decreased viremia (170). Additionally, another recent study in SIV-infected rhesus macaques showed that CD8+ lymphocyte depletion in elite controllers led to dramatic redistribution of productive SIV infection from T_{FH} within the follicles into other non-T_{FH} CD4+ T cells outside the follicle. The productive SIV infection was pushed back into follicles upon recovery of CD8 T cells (78). Nonetheless, despite the sufficient evidence showing that HIV- and SIV-specific CD8 T cells are abundant and functional in controlling virus replication, they are unable to fully suppress all viral replication or prevent disease progression. Moreover, even the novel immune therapy of infusing exogenous virus-specific CTL into the patient still failed to significantly reduce HIV RNA level in plasma (171-173). It is not clear why HIV/SIV-specific CD8 T cells are unable to fully suppress all virus replication.

In contrast to concentrated HIV/SIV replication in B cell follicles, we and others previously observed that, typically, lower level of virus-specific CD8 T cells are located in B cell follicles compared to extrafollicular regions during both HIV and SIV chronic infection (73, 74, 79, 80). Similarly, paucity of CD8 T cells in follicles was also reported in mice. In contrast to the deficiency in number, recent studies demonstrated that follicular CD8 T cells in mice are actually able to curtail viral infection (174). However, whether follicular HIV- and SIV-specific CD8 T cells are functional in killing virus-producing cells has not been fully understood. Further, whether SIV-specific CD8 T cells are able to migrate into GC where FDCs hold large amounts of infectious extracellular virions (2, 175) has not been tested yet. Tregs impede and impair the

proliferation and effector function of CTL (176, 177) and the programmed cell death protein 1 (PD-1) is strongly related to the functional exhaustion of virus-specific CD8 T cells (178-180). Whether PD-1 expression and Tregs influence follicular virus-specific CD8 T cells function remain to be elucidated. CXCR5 is an important follicular homing molecule and all mature B cells express this molecule (181, 182). CXCR5 is also necessary for follicular CD4+ T cell localization (183). However, only a small fraction of virus-specific CTL within secondary lymphoid tissues express CXCR5 (73). Whether the lack of CXCR5+ CD8+ T cells is responsible for the low level of HIV/SIV-specific CD8 T cells within B cell follicles and failure in suppressing viral replication inside follicle is not clear. These unanswered questions require further investigations about virus-specific CD8 T cells in the follicular area during HIV/SIV infection. By using SIV-infected rhesus macaques, I studied the location, abundance and phenotype of follicular virus-specific CD8 T cells.

Introduction of ALT-803, a novel IL-15 superagonist complex

Interleukin-15 (IL-15), a 14-15 kDa glycoprotein, was simultaneously discovered by two independent groups in 1994 and characterized as a T cell growth factor (184, 185). It belongs to common γ-chain cytokines which also include IL-2, IL-4, IL-7, IL-9 and IL-21 (186, 187). Previous research showed that IL-15 is a critical mediator of immunoglobulin synthesis in B cells, survival, activation and proliferation of T cells, and generation and function of (nature killer) NK cells (188). Moreover, IL-15 is required for long-term maintenance of CD8+CD44^{high} memory T cells (188). IL-15 knockout mice showed markedly reduced numbers of memory CD8 T cells and NKT cells, and deficiency of NK cells in both number and function (189). Administration of recombinant IL-15 in non-human primates drives significant expansion of memory CD8 T cells and NK cells in peripheral blood without preferentially increasing Tregs

which can down modulate immune responses by inhibiting effector CD8 T cells (190). IL-15 also prevents apoptosis of T cells (191). Importantly, IL-15 induces NK cell expansion and preferential proliferation of effector memory T cells, both CD4+ and CD8+, in SIV-infected non-human primates (192, 193). Taken together, IL-15 is a good candidate for clinical immunotherapy.

Unlike to other γ-chain cytokines that circulate as soluble proteins until they bind their receptors directly on target immune cells, IL-15 is delivered through a novel mechanism termed trans-presentation. In this process, IL-15 first binds the high affinity IL-15 receptor alpha chain (IL-15Rα) in the endoplasmic reticulum (ER) to form an IL-15: IL-15Rα complex. Then the complexes are transported to the cell surface where they can stimulate the responding cell through the IL-2Rβ/γC receptor complex (194, 195). In addition, the IL-15: IL-15Rα complex can be internalized via endosomes (194). This process likely extends the half-life of the cytokine and reduces the demand of cytokine production. Like IL-15, IL-15Rα is also very important in maintaining lymphoid homeostasis as IL-15Rα knockout mice are significantly deficient in NK cells, NKT cells and CD8 T cells (196). Together with the short half-life and limited bioavailability, the unique trans-presentation mechanism, at least partially, restricts the therapeutic application of free IL-15. Development of an IL-15 superagonist complex, ALT-803, ameliorates these limitations and promotes clinical applications of IL-15.

A novel IL-15 mutant (N72D) which contains an asparagine to aspartic acid mutation at position 72 showed 5-fold higher biological activity than wild-type IL-15 (197, 198). This mutant can form a stable heterodimeric complex with IL-15Rα. The complex further exhibits enhanced biological activity compared to free IL-15 (197). ALT-803 consists of two IL-15N72D:IL-15Rα complexes fused onto a human IgG1 Fc fragment. Together, these modifications give ALT-803 25-fold higher biological activity and a 35-fold longer half-life in serum than soluble IL-15, resulting in potent stimulation of NK and memory T cells (199). A recent study demonstrated that

ALT-803 is well-tolerated in both mice and cynomolgus macaques at doses up to 100 µg/kg and does not induce a cytokine storm despite potent activation of NK and memory T cells (198). ALT-803 is now being explored as a means to directly enhance anti-viral immune responses in chronic infection such as HIV. For example, in a humanized mice model of HIV, early administration of ALT-803 induced NK cell cytotoxicity and inhibited acute HIV replication (200). More recently, ALT-803 demonstrated the remarkable ability to both reverse HIV latency and enhance CD8 T cell recognition of HIV-infected cells in a primary *in vitro* cell culture model (201). Here, I studied the *in vivo* effects of ALT-803 on SIV-specific CD8 T cells in the context of chronic SIV infection in rhesus macaques.

Figure 1

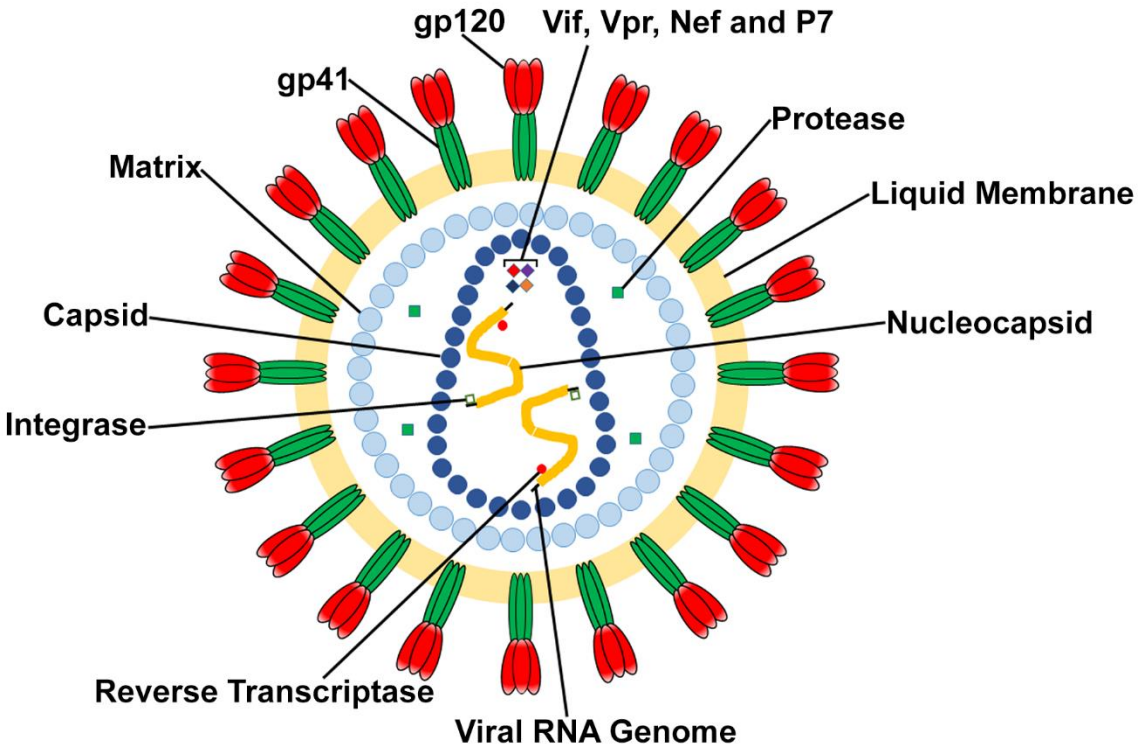

FIG 1 Structure of HIV virion. Figure adapted from National Institute of Allergy and Infectious Disease. How HIV Causes AIDS. National Institutes Health; Bethesda, MD: 2004.

Figure 2

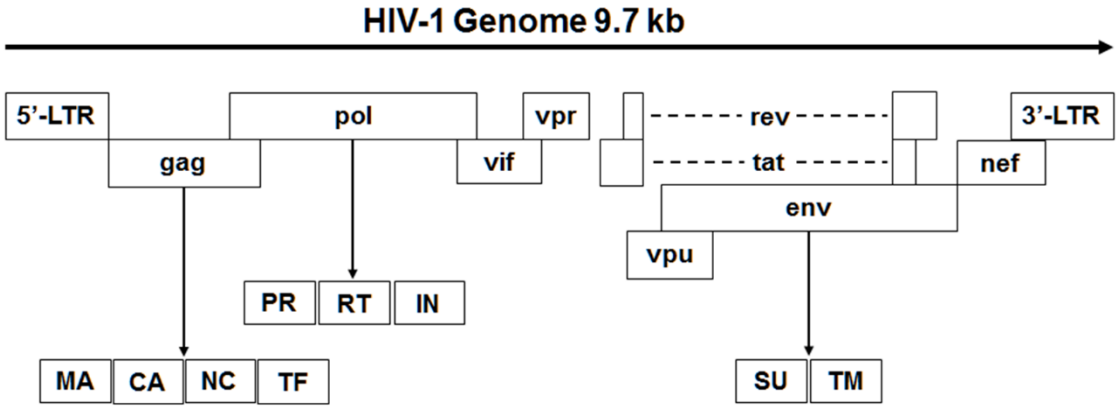

FIG 2 Schematic diagram of HIV-1 genome. The total size of HIV-1 genome is approximately 9.7 kb. Each of the viral genes is drawn based on the relative orientation in the entire RNA genome. Arrows point to cleaved protein products. Dashed lines represent RNA splicing. The number in parenthesis is molecular weight of each protein. *LTR* long-term repeat, *Gag* group-specific antigen, *MA* matrix protein, *CA* capsid domain, *NC* nucleocapsid, *TF* trans-frame protein, *Pol* polymerases, *PR* protease, *RT* reverse transcriptase, *IN* integrase, *Env* envelope protein, *SU* surface membrane protein, *TM* trans-membrane protein, *Vif* viral infectivity factor, *Vpr* viral protein R, *Vpu* viral protein U, *Nef* negative regulatory factor, *Rev* regulator of expression of viral proteins, *Tat* trans-activator of transcription. Figure adapted from Nkeze J. *et al*. Molecular characterization of HIV-1 genome in fission yeast *Schizosaccharomyces pombe*. Cell & Bioscience, 2015, 5:47.

Figure 3

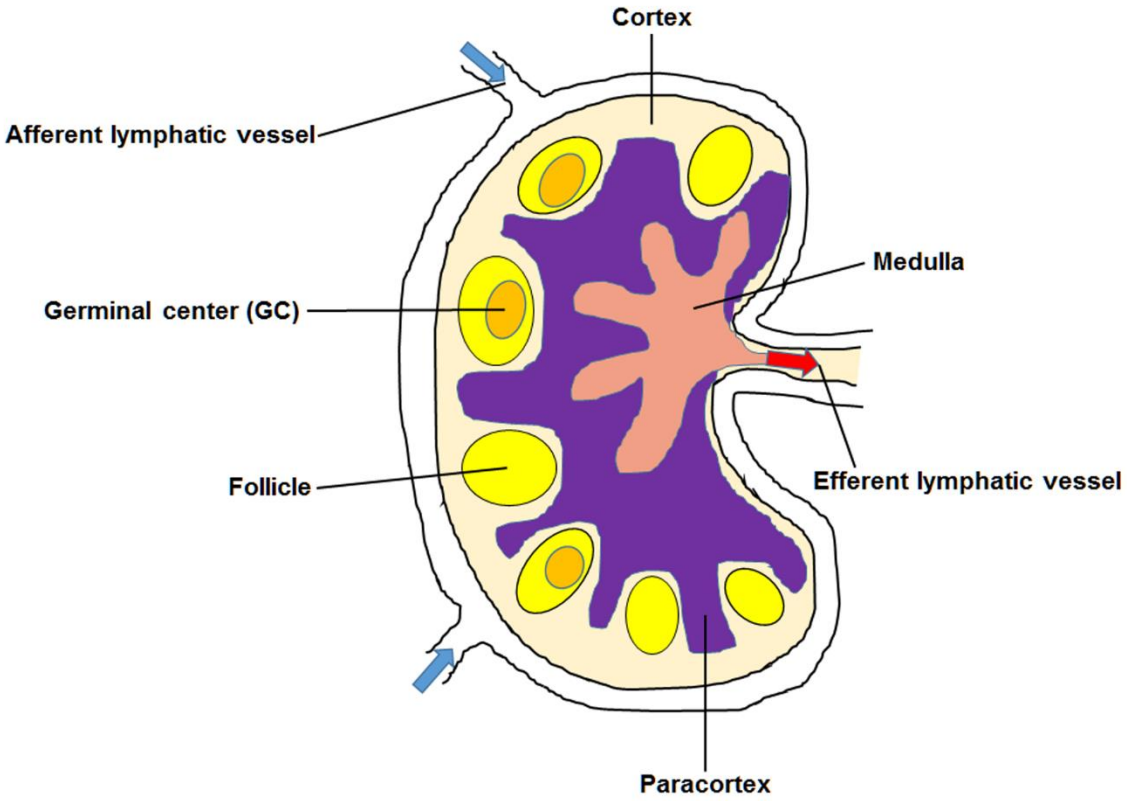

FIG 3 Schematic diagram of lymph node structure. Figure adapted from Janeway's Immunobiology (8th Edition).

Chapter 2

SIV-producing cells in follicles are partially suppressed by $CD8^+$ cells *in vivo*[1]

[1] Reprinted from the Journal of Virology. **Li S**, Folkvord JM, Rakasz EG, Abdelaal HM, Wagstaff RK, Kovacs KJ, Kim HO, Sawahata R, MaWhinney S, Masopust D, Connick E, Skinner PJ. "Simian immunodeficiency virus-producing cells in follicles are partially suppressed by $CD8^+$ cells *in vivo*". Volume 90, pp. 11168-11180, 2016.

Synopsis

HIV- and simian immunodeficiency virus (SIV)-specific $CD8^+$ T cells are typically largely excluded from lymphoid B cell follicles where HIV- and SIV-producing cells are most highly concentrated, indicating that B cell follicles are somewhat of an immune privileged site. To gain insights into virus-specific follicular $CD8^+$ T cells, we determined the location and phenotype of follicular SIV-specific $CD8^+$ T cells *in situ*, the local relationship of these cells to $Foxp3^+$ cells, and effects of CD8 depletion on levels of follicular SIV-producing cells in chronically SIV infected rhesus macaques. We found that follicular SIV-specific $CD8^+$ T cells were able to migrate throughout follicular areas including germinal centers. Many expressed PD-1, indicating they may have been exhausted. A small subset was in direct contact with and likely inhibited by $Foxp3^+$ cells and a few were themselves $Foxp3^+$. In addition, subsets of follicular SIV-specific $CD8^+$ T cells expressed low to medium levels of perforin and subsets were activated and proliferating. Importantly, after CD8 depletion, SIV-producing cells increased in B cell follicles and extrafollicular areas, suggesting that follicular and extrafollicular $CD8^+$ T cells have a suppressive effect on SIV replication. Taken together, these results suggest that during chronic SIV infection, despite high levels of exhaustion and likely inhibition by $Foxp3^+$ cells, a subset of follicular SIV-specific $CD8^+$ T cells are functional and suppress viral replication *in vivo*. These findings support HIV cure strategies that augment functional follicular virus-specific $CD8^+$ T cells to enhance viral control.

Introduction

In the absence of combination anti-retroviral therapy (cART), the majority of HIV infected individuals experience persistent high-level viral replication that results in progressive loss of $CD4^+$ T cells, AIDS and death eventually. During chronic HIV and SIV infections, the vast majority of virus-producing cells are $CD4^+$ T cells located in secondary lymphoid tissues (2, 122, 123). Within secondary lymphoid tissues, virus-producing cells are most concentrated within B cell follicles (73-77). When the frequency of virus-producing cells within follicles and extrafollicular compartments was adjusted by the frequency of target cells (i.e., either $CD4^+$ or $CD4^+$ $Ki67^+$ cells), there was still significantly higher concentration of SIV-producing cells in B cell follicles compared to extrafollicular regions of spleen, lymph nodes, and gut-associated lymphoid tissues (73). In rhesus macaques undergoing long-term, fully suppressive cART, residual SIV infection is also preferentially localized in B cell follicles (78). In addition, follicular dendritic cells (FDCs) within mature B cell follicles hold onto extracellular virions (2, 202), and FDC bound virions are potently infectious to $CD4^+$ T cells (143).

Numerous studies indicate that $CD8^+$ cytotoxic T lymphocytes (CTL) play a key role in controlling HIV and SIV infections both *in vitro* (145, 146) and *in vivo*. For example, the development of HIV-specific $CD8^+$ T cells during acute infection coincides with a decrease in plasma viremia (147, 148). Effector $CD8^+$ T cells to virus-infected cell ratios in lymphoid and genital tissues *in vivo* correlate with reductions in viral load (149), and levels of SIV-specific $CD8^+$ T cells in lymphoid compartments predict levels of SIV-producing cells in those compartments (73). Furthermore, disease progression is associated with diminished HIV- and SIV-specific $CD8^+$ T cells responses (150, 151, 203). Elite control of HIV is associated with specific MHC-class I alleles and polyfunctional CTL responses (158, 161, 162, 204). In addition, HIV and SIV mutate virally encoded CTL epitopes to evade HIV- and SIV-specific $CD8^+$ T cells responses (165, 166). Perhaps the most powerful evidence that CTL are important in controlling

HIV and SIV infections comes from experiments in which $CD8^+$ cells were temporarily depleted in rhesus macaques during chronic SIV infection (167-170), which lead to as much as 1000-fold increases in plasma viremia, and the subsequent recovery of $CD8^+$ cells led to decreased viremia (170). Nevertheless, HIV- and SIV-specific $CD8^+$ T cells are not able to fully suppress all virus replication and prevent disease progression.

We and others previously showed that HIV- and SIV-specific $CD8^+$ T cells are typically most concentrated in T cell zones outside of B cell follicles in lymph node and spleen tissues, and are largely excluded from follicles (73, 74, 79, 80). Thus, B cell follicles appear to be somewhat of an immune privileged site in which virus-specific $CD8^+$ T cells are not able to clear all virus-producing cells. The relatively low levels of follicular virus-specific $CD8^+$ T cells can be explained by a lack of expression of the follicular homing molecule CXCR5 on most lymphoid $CD8^+$ T cells (73). In addition to numerical deficiencies of follicular virus-specific $CD8^+$ T cells, there likely exist other factors that may inhibit follicular virus-specific $CD8^+$ T cells function.

Evolutionarily it makes sense for B cell follicles to be immune privileged sites in order to prevent unwanted $CD8^+$ T cell cytolytic activity within follicles, which might lead to decreased ability of B cells to make antibodies. Follicular $CD8^+$ T cells might primarily serve to provide help to $CD4^+$ T follicular helper cells (T_{FH} cells) or B cells. In support of this thesis, we previously reported that many SIV-specific $CD8^+$ T cells down-modulate CD8 upon entering B cell follicles (205) and Xu et al. found that $CD8^{low}$ SIV-specific T cells show impaired function (206). In addition, we frequently observe SIV-specific $CD8^+$ T cells in contact with B cells, with their cell membranes intertwined (unpublished data), and Quigley et al., showed that isolated follicular $CD8^+$ T cells, to some extent, supported IgG production in tonsillar B cells (207). Thus, many follicular SIV-specific $CD8^+$ T cells may down-modulate cytolytic function in favor of providing help to B cells to produce SIV-specific antibodies.

There is also evidence that at least some follicular $CD8^+$ T cells likely maintain cytolytic function. For example, we found that subsets of follicular SIV-specific $CD8^+$ T cells express the cytolytic enzymes granzyme B and perforin, indicating that some follicular $CD8^+$ T cells have the capacity for cytolytic function (73). Furthermore, we found that levels of SIV-specific $CD8^+$ T cells inversely correlated with levels of SIV RNA^+ cells in follicular and extrafollicular compartments of lymph nodes, suggesting suppression of follicular virus-producing cells by virus-specific $CD8^+$ T cells *in vivo* (73).

In this study, to gain further insights into follicular virus-specific $CD8^+$ T cells, we determined the location and phenotype of follicular SIV-specific $CD8^+$ T cells *in situ*, the local relationship of these cells to $Foxp3^+$ cells, and effects of CD8 depletion on levels of follicular SIV-producing cells in SIV infected rhesus macaques. We hypothesize that subsets of follicular SIV-specific $CD8^+$ T cells evade suppression by T regulatory cells (Tregs), evade functional exhaustion, display an effector memory phenotype, and can suppress follicular viral replication *in vivo*. Our findings support this hypothesis, and further support HIV cure strategies that increase frequencies of functional follicular virus-specific $CD8^+$ T cells.

Materials and Methods

Tissues from chronically SIV-infected animals

Lymph nodes and spleen were obtained from captive-bred rhesus macaques of Indian origin chronically infected with either SIVmac239 or SIVmac251. Five animals were inoculated with SIVmac251 intravaginally, six were inoculated with SIVmac239 rectally and four intravenously. Two animals (R03094 and R01106) had <200 $CD4^+$ T cells/mm^3 (Table 1). All animals were housed and cared for according to American Association for Accreditation of Laboratory Animal Care standards in accredited facilities. All animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committees of the Wisconsin National Primate Research Center and the University of Minnesota. Portions of fresh lymphoid tissues were immediately snap frozen in OCT and/or formalin fixed and embedded in paraffin. In animals with MHC-class I alleles known to restrict SIV-specific $CD8^+$ T cells, portions of fresh lymphoid tissue were also collected in RPMI 1640 medium with sodium heparin (18.7 U/ml) and shipped overnight to the University of Minnesota for *in situ* tetramer staining. Four rhesus macaques (rh2515, rh2516, rh2520 and rh2588) in early chronic phase of SIVmac239 infection (59 days post-infection) received 50 mg/kg anti-CD8 monoclonal antibody (mAb) MT-87R1 (Nonhuman Primate Reagent Resource, Boston, MA.) to deplete $CD8^+$ cells.

In situ tetramer staining combined with Immunohistochemistry

In situ tetramer staining combined with immunohistochemistry was performed on fresh lymph tissue specimens shipped overnight, sectioned with a compresstome (208) and stained essentially as previously described (73, 74, 209). Biotinylated MHC-class I monomers were loaded with peptides (National Institute of Health Tetramer Core Facility, Emory University, Atlanta GA) and converted to MHC-class I tetramers. Mamu-A1*001 molecules loaded with SIV

Gag CM9 (CTPYDINQM) peptides (210) or irrelevant negative control peptides FV10 (FLPSDYFPSV) from the hepatitis B virus core protein; Mamu-B*008 molecules loaded with Nef RL10 (RRHRILDIYL) peptides (211) and Env KL9 (KRQQELLRL) peptides (211); and Mamu-A1*002 monomers loaded with Nef YY9 (YTSGPGIRY) peptides (212). Fresh lymph node and spleen tissues sections were incubated with MHC-class I tetramers (0.5 μg/ml) alone or along with goat-anti-human PD-1 Abs (1 μg/mL, polyclonal, R&D Systems). For secondary incubations, sections were incubated with rabbit-anti-FITC Abs (0.5 μg/mL, BioDesign, Saco, ME) and mouse-anti-human Ki67 Abs (1:500 dilution, clone MM1, Vector), or mouse-anti-human perforin Abs (0.1 μg/mL, clone 5B10, Novacastra), or mouse-anti-human Foxp3 Abs (2.5 μg/mL, clone 206D, BioLegend) or mouse-anti-human CD20 Abs (0.19 μg/mL, clone L26, Novocastra). For the tertiary incubations, the sections stained with goat-anti-human PD-1 Abs were incubated with Cy3-conjugated donkey-anti-rabbit Abs (0.3 μg/mL, Jackson ImmunoResearch Laboratories, West Grove, PA), Alexa 488-conjugated donkey-anti-goat Abs (0.75 μg/mL, Jackson ImmunoResearch Laboratories), and Cy5-conjugated donkey-anti-mouse Abs (0.3 μg/mL, Jackson ImmunoResearch Laboratories). All other sections were incubated with Cy3-conjugated goat-anti-rabbit Abs (0.3 μg/mL, Jackson ImmunoResearch Laboratories), Alexa 488-conjugated goat-anti-mouse Abs (0.75 μg/mL, Molecular probes), and Dylight 649-conjugated goat anti-human IgM (0.3 μg/mL, Jackson ImmunoResearch Laboratories). Sections were imaged using an Olympus FluoView 1000 microscope. Confocal z-series were collected from ~6 μm from the surface of the section to 35-45 μm into the tissue. Montage images of multiple 800 × 800 pixels were created and used for analysis.

Quantitative image analysis

For the determination of levels of SIV-specific $CD8^+$ T cells and percentages of SIV-specific $CD8^+$ T cells that co-expressed specific molecules, follicular areas were identified

morphologically as clusters of brightly stained closely aggregated $CD20^+$ or IgM^+ cells. Follicular and extrafollicular areas were delineated using Olympus FluoView 1000 software. Areas that showed loosely aggregated B cells that were ambiguous as to whether the area was a follicle were not included. Quantification of MHC-tetramer stained cells within GC and non-GC areas was performed on sections stained with IgM and Ki67 antibodies, where IgM antibody staining was used to delineate follicles and Ki67 antibody staining to delineate GC areas of follicles. GC delineation was only done in the studies that included Ki67 antibody staining. For PD-1 expression analysis, an average of 174 $tetramer^+$ cells (range, 46-313) was analyzed in follicular regions and 445 (range, 120-883) in extrafollicular regions. For quantification of $tetramer^+$ cells that were in contact with $Foxp3^+$ cells and express $Foxp3^+$, an average of 271 $tetramer^+$ cells (range, 100-486) was analyzed in follicular regions and 294 (range, 119-498) in extrafollicular regions. For perforin expression level analysis, an average of 107 $tetramer^+$ cells (range, 22-300) was analyzed in follicular region and 201 (range, 82-389) in extrafollicular region. To determine levels of perforin expression, $tetramer^+$ cells were scored using the following objective criteria as follows. $Tetramer^+$ cells with no detectable perforin staining above background levels were scored as perforin negative. $Tetramer^+$ cells with perforin staining 2-3X greater than background were scored as perforin low, with perforin staining 4-9X higher than background as perforin medium, and those with 10X or greater than background levels and with perforin staining detectable throughout much of the cytoplasm were scored as perforin high. Cell counts were done on single z-scans. While doing the cells counts, we stepped up and down through the z-scans to distinguish tops and bottoms of cells from non-specific background staining and demarcated cells using a software tool to avoid counting the same cell twice. Quantitative image analysis of PD-1, Foxp3 and perforin staining were done with lymph node tissues, while quantitative image analysis of Ki67 staining was done with both lymph node and splenic tissues. An average of 1.97 mm^2 (range, 1.19-2.64 mm^2) was analyzed for each lymph node and 2.82 mm^2 (range, 1.67-3.98 mm^2) for each spleen.

In situ hybridization combined with immunohistochemistry

In situ hybridization for SIV RNA was performed as previously described (73, 75). This technique identifies cells that are actively transcribing SIV, but not extracellular virions encapsulated in envelope glycoprotein and bound to FDC. Briefly, 6 µm frozen sections were fixed in 3% paraformaldehyde (Sigma-Aldrich, St. Lousis, MO), hybridized overnight with digoxygenin labeled SIVmac239 antisense probes (Lofstrand Labs, Gaithersburg, MD) and visualized using NBT/5-bromo-4-chloro-3-indolyl phosphate (Roche, Nutley, NJ). Immunohistochemistry staining for B cells was performed in the same tissues using mouse-anti-human CD20 (clone 7D1; AbD Serotec, Raleigh, NC) and detected using HRP-labeled polymer anti-mouse IgG (ImmPressKit; Vector Laboratories, Burlingame, CA) and Vector NovaRed substrate (Vector Laboratories). SIV RNA^+ cells were counted by visual inspection and classified as either inside or outside of B cell follicles which were identified morphologically as a cluster of $CD20^+$ cells as previously described (73, 75). Total tissue area and area of follicles was determined by quantitative image analysis (Qwin Pro version 3.4.0; Leica, Cambridge, U.K.) and used to calculate the frequency of SIV^+ cells per mm^2. An average of 49.3 mm^2 (4.7 mm^2 – 95.2 mm^2) was analyzed.

Statistical analysis

All statistical analysis assumed two-sided tests with a significant level of 0.05. Count data were analyzed using generalized linear mixed models that would accommodate within subject correlation, over-dispersion (negative binomial with log link) and an offset for total cells or area, as appropriate. Simple linear regression was used for modeling $\log_{10}$ viral load. Perforin expression on $tetramer^+$ cells was analyzed using repeated measures ordinal (proportional odds)

logistic regression (213-215) with standard errors estimated via bootstrap. SAS version 9.3 (Cary, NC), R (216) and GraphPad Prism (6.0) software was used. Because of there were only four animals in the CD8 depletion study, statistics were not performed.

Results

SIV-specific CD8$^+$ T cells can migrate into germinal centers (GCs)

Although HIV- and SIV-specific CD8$^+$ T cells are typically most concentrated in T cell zones of lymphoid tissues, some localize inside of lymphoid B cell follicles (73, 74). Within B cell follicles, HIV- and SIV-specific CD8$^+$ T cells are typically distributed near the border of follicles adjacent to the T cell zone (73, 74). In addition, greater than 50% of the follicular area is devoid of HIV- and SIV-specific CD8$^+$ T cells in most follicles (73, 74). These findings led us to wonder whether virus-specific CD8$^+$ T cells are restricted to certain areas of the follicle or are able to migrate throughout the entire follicular area including the GC where FDCs hold virus in immune complexes and are presumably actively infecting passing CD4$^+$ T cells.

To address this question, we used MHC-class I tetramers to stain SIV-specific CD8$^+$ T cells *in situ* in lymph node tissue sections from SIV-infected rhesus macaques (Table 1). We counter-stained tissue sections with antibodies directed against IgM to label B cell follicles, and antibodies directed against Ki67 to label proliferating cells and allow us to delineate GCs. We found that within follicles, tetramer$^+$ SIV-specific CD8$^+$ T cells were located both inside and outside of GCs (Fig. 1A). They were present in similar levels inside and outside of the GC area of B cell follicles ($p = 0.85$), and as anticipated, were present at significantly lower levels in follicles compared to extrafollicular regions (Fig. 1B) ($p < 0.0001$). Thus, although SIV-specific CD8$^+$ T cells are typically relatively low in numbers within B cell follicles, they can migrate throughout the entire follicle, including the GC.

In addition, we previously found a positive correlation between levels of follicular and extrafollicular tetramer$^+$ virus-specific CD8$^+$ T cells (73). We performed a similar comparison in our current study including 7 animals from our previously published work (73), plus 8 additional animals. The results from the increased animal cohort strongly support our previous finding and showed a highly significant positive correlation between levels of follicular and extrafollicular

tetramer$^+$ virus-specific CD8$^+$ T cells in lymph node and spleen tissues (Fig. 1C) ($p = 0.0001$). Furthermore, we also observed significant correlation between levels of GC and extrafollicular tetramer$^+$ virus-specific CD8$^+$ T cells (Fig. 1D) ($p = 0.014$), and between levels of non-GC follicular and extrafollicular tetramer$^+$ virus-specific CD8$^+$ T cells (Fig. 1E) ($p = 0.0030$). Thus, as total numbers of virus-specific CD8$^+$ T cells increase, there is a corresponding increase in extrafollicular as well as follicular virus-specific CD8$^+$ T cells, including cells in the GC as well areas outside of the GC in follicles.

Many follicular SIV-specific CD8$^+$ T cells express PD-1 during chronic SIV infection

As mentioned above, we previously showed that many SIV-specific CD8$^+$ T cells appear to down-modulate surface expression of CD8 upon entering B cell follicles (205). Here we investigated additional factors that might inhibit follicular SIV-specific CD8$^+$ T cells function. We investigated the inhibitory receptor PD-1, which is a marker of functional exhaustion of CD8$^+$ T cells (178, 179) as well as a marker of CD8$^+$ T cells that have recently been exposed to antigenic stimulation (178). PD-1 is markedly upregulated on the surface of dysfunctional virus-specific CD8$^+$ T cells during chronic HIV and SIV infections (217, 218), and blockade of PD-1 *in vivo* enhanced SIV-specific CD8$^+$ T cells responses (219). The degree to which follicular SIV-specific CD8$^+$ T cells express PD-1 has not yet been investigated. To investigate this, we stained tissue sections from chronically SIV infected rhesus macaques with MHC-class I tetramers, antibodies directed against PD-1, and antibodies directed against CD20 to label B cell follicles (Table 1 and Fig. 2A) We found a broad range of 10-86% (average 54%) of follicular tetramer$^+$ SIV-specific CD8$^+$ T cells expressing PD-1. Levels were lowest in the animals Rh2515 and Rh2520 sacrificed at the earliest time point evaluated at 42 days post-infection. Comparison of viral loads and percentages of follicular tetramer$^+$ SIV-specific CD8$^+$ T cells expressing PD-1 showed no significant correlation ($p = 0.10$). PD-1 expression was slightly higher (11.2%, p =

0.047) in follicular compared to extrafollicular tetramer$^+$ SIV-specific CD8$^+$ T cells (Fig. 2B). These results indicate that PD-1 expression is quite variable in animals during chronic SIV infection, and suggest that many follicular as well as extrafollicular SIV-specific CD8$^+$ T cells are continually being exposed to antigen and likely exhausted. Importantly, these results also indicate that subsets follicular SIV-specific CD8$^+$ T cells in each animal do not express PD-1, and are not exhausted.

Foxp3$^+$ cells likely inhibit follicular and extrafollicular SIV-specific CD8$^+$ T cell function

We also investigated whether Foxp3$^+$ cells were in contact with, and potentially inhibiting, follicular SIV-specific CD8$^+$ T cells. Tregs play a pivotal role in maintaining immunological self-tolerance, controlling autoimmune disease (64, 65) and suppressing immune activation (66, 67). A large subset of Tregs is characterized by the expression of the transcription factor Foxp3 (101, 220, 221). Although most Tregs are CD4$^+$, a subpopulation of CD8$^+$ T cells also functions as Tregs (222-224). During chronic infection, Tregs can suppress CD8$^+$ T cell activity in a contact dependent manner (225). In this study, we investigated whether Foxp3$^+$ Tregs were in contact with and potentially inhibiting follicular SIV-specific CD8$^+$ T cells function. We stained lymph node tissue sections from chronically SIV infected rhesus macaques with MHC-class I tetramers to label SIV-specific CD8$^+$ T cells, anti-Foxp3 antibodies to label Foxp3$^+$ Tregs, and anti-IgM antibodies to label B cell follicles, and quantified numbers of tetramer$^+$ cells in contact with Foxp3$^+$ cells and levels of tetramer$^+$ Foxp3$^+$ cells (Table 2). We found that on average 7% (range 5-9%) of follicular tetramer$^+$ SIV-specific CD8$^+$ T cells were in direct contact with Foxp3$^+$ cells (Fig. 3A and 3B). In extrafollicular regions, significantly higher levels of tetramer$^+$ SIV-specific CD8$^+$ T cells were in contact with Foxp3$^+$ cells (average 16%, range 9-29%, Fig. 3B). In addition, a small subset of tetramer$^+$ SIV-specific CD8$^+$ T cells expressed Foxp3 (Fig. 3C). An average of 0.35% (range 0-1%) of follicular and 0.7% of extrafollicular

(range 0-3%) SIV-specific $CD8^+$ T cells were $Foxp3^+$ (Fig. 3D). No significant difference between follicular and extrafollicular $Foxp3^+$ SIV-specific $CD8^+$ T cells was observed. Thus, $Foxp3^+$ cells are contacting and likely inhibiting subsets of follicular as well as extrafollicular SIV-specific $CD8^+$ T cells.

In addition, consistent with previous studies (5, 6, 30, 31), we found that $tetramer^+$ SIV-specific $CD8^+$ T cells levels were significantly lower in follicular compared extrafollicular areas ($p = 0.017$; Fig. 3E). Similarly, $Foxp3^+$ cells levels were significantly lower in follicular compared extrafollicular areas ($p = 0.0004$; Fig. 3F). Combining these data, we found that the ratio of $tetramer^+$ SIV-specific $CD8^+$ T cells: $Foxp3^+$ cells tended to be higher in follicular areas than extrafollicular areas ($p = 0.052$; Fig. 3G). Moreover, the absolute number of $tetramer^+$ cells in contact with $Foxp3^+$ cells was higher in extrafollicular region (272 of 2057 $tetramer^+$ SIV-specific $CD8^+$ T cells) than follicular region (115 of 1900 $tetramer^+$ SIV-specific $CD8^+$ T cells) as well (Table 2). These findings suggest that contact-mediated suppression of SIV-specific $CD8^+$ T cells by $Foxp3^+$ cells may be lower in follicular compared to extrafollicular compartments.

Interestingly, when the percentages of follicular and extrafollicular $tetramer^+$ SIV-specific $CD8^+$ T cells in contact with $Foxp3^+$ cells were evaluated together vis-a-vis viral load (SIV RNA Copies/ml) in each animal, the percentage of follicular but not extrafollicular $tetramer^+$ SIV-specific T cells in contact with $Foxp3^+$ cells was a borderline significant predictor of viral load ($p = 0.08$, and $p = 0.26$ respectively; Fig. 3H).

Levels of lymphoid SIV-specific $CD8^+$ T cells tend to predict viral load

We compared levels of follicular and extrafollicular $tetramer^+$ SIV-specific $CD8^+$ T cells and viral loads. We found that follicular tended to negatively predict ($p = 0.060$), and extrafollicular significantly negatively predicted plasma viral load ($p = 0.036$; Fig. 4). These

findings suggest that SIV-specific $CD8^+$ T cells located in lymph nodes are important in controlling plasma viral loads.

Activated proliferating SIV-specific $CD8^+$ T cells are found in follicles

We next assessed whether follicular SIV-specific $CD8^+$ T cells express Ki67 in lymph nodes and spleen (Table 1). In T cells, Ki67 is a marker of activation and proliferation (226, 227). We found $Ki67^+$ $tetramer^+$ SIV-specific $CD8^+$ T cells in follicular (Fig. 5A) as well as in extrafollicular regions (Fig. 5B), at similar levels (Fig. 5C). On average 11% (range 5-22%) of $tetramer^+$ SIV-specific $CD8^+$ T cells were $Ki67^+$ in the GCs, 12% (range 0-33%) in non-GC follicular areas, and 13% (range 5-24%) in extrafollicular areas. These data indicate that a subset of both follicular and extrafollicular SIV-specific $CD8^+$ T cells are activated and proliferating.

Many follicular SIV-specific $CD8^+$ T cells express low levels of perforin

Perforin is an important cytolytic effector molecule which CTL use to lyse virus-infected cells. We previously showed that approximately 35% of follicular SIV-specific $CD8^+$ T cells express perforin and that most expressed another effector molecule, granzyme B, which typically works in concert with perforin to lyse infected cells (73).

Central memory (T_{CM}) and effector memory (T_{EM}) T cells are two distinct populations of memory T lymphocytes. T_{CM} subsets of CTL can be identified by their absence of perforin expression (103, 228). T_{CM} are known for proliferating and secreting cytokines upon contact with antigen, which serves to propagate antigen-specific CTL and send inflammatory signals to propagate inflammatory responses, but they are not able to kill infected cells immediately upon contact (103, 228). In contrast, T_{EM} express perforin and importantly, can kill infected cells immediately upon contact (103, 228).

Based on the importance of perforin expression on the ability of CTL to immediately kill infected cells, we set out to characterize perforin expression levels within follicular and extrafollicular SIV-specific $CD8^+$ T cells in lymph nodes during chronic SIV infection (Table 1). We determined levels of perforin (negative, low, medium, and high) in $tetramer^+$ follicular and extrafollicular SIV-specific $CD8^+$ T cells (Fig. 6A). We found that a wide range $tetramer^+$ follicular SIV-specific $CD8^+$ T cells did not express perforin (mean: 56%; range: 20-81%), consistent with being T_{CM}. The percentage of $perforin^-$ $tetramer^+$ cells was slightly higher in follicular compared with extrafollicular regions ($p = 0.026$, Fig. 6B). Among $perforin^+$ $tetramer^+$ cells, most expressed low to medium levels of perforin (mean 94%, range 91-97%), consistent with being T_{EM}. A small population of $perforin^+$ $tetramer^+$ cells (mean 6%, range 3-9%) expressed high levels of perforin consistent with being effector T cells or terminally differentiated memory populations. Percentages of both follicular ($p = 0.90$) and extrafollicular ($p = 0.91$) $tetramer^+$ SIV-specific $CD8^+$ T cells expressing perforin showed no significant correlation with plasma viral loads. The percentages of follicular and extrafollicular $tetramer^+$ cells that expressed each level of perforin were not significantly different (Fig. 6C). Thus, there were similar levels of $tetramer^+$ SIV-specific $CD8^+$ T cells expressing low, medium, and high levels of perforin in follicular and extrafollicular compartments.

Increase in follicular SIV-producing cells post-CD8 depletion

To evaluate the ability of SIV-specific $CD8^+$ T cells to kill SIV-producing cells in follicular and extrafollicular compartments *in vivo*, we temporarily depleted $CD8^+$ T cells in four chronically SIV infected rhesus macaques using anti-CD8 antibodies. As anticipated from previous CD8 depletion studies (168-170), after administration of anti-CD8 antibodies, $CD8^+$ T cells including tetramer-binding SIV-specific $CD8^+$ T cells were temporarily depleted from blood and plasma viral loads increased (Fig. 7A). Ten days post-depletion, animals were sacrificed and

tissues collected for *in situ* analyses. Using *in situ* tetramer staining combined with immunohistochemistry, we visualized and quantified tetramer$^+$ SIV-specific CD8$^+$ T cells inside and outside of B cell follicles before (Fig. 7B) and after (Fig. 7C) CD8 depletion. Tetramer$^+$ SIV-specific CD8$^+$ T cells were largely depleted in both follicular and extrafollicular areas (Fig. 7D and 7E). Using *in situ* hybridization combined with immunohistochemistry, we visualized and quantified SIV RNA$^+$ cells inside and outside of B cell follicles before (Fig. 7F) and after (Fig. 7G) CD8 depletion. We found higher levels of both follicular and extrafollicular SIV-producing cells after CD8$^+$ T cells depletion in all four animals examined, albeit the increase was much more substantial in extrafollicular areas, with the average change in follicles being 3.8 cell/mm^2 (range 0.6 to 10.3 cells/mm^2; Fig. 7H) and in extrafollicular areas 8.9 cells/mm^2 (range 0.6 to 22.4 cells/mm^2; Fig 7I). These results suggest that both follicular as well as extrafollicular CD8$^+$ T cells suppress viral replication *in vivo*.

Discussion

B cell follicles are a major reservoir of HIV and SIV replication and remain a critical obstacle to the elimination of HIV and SIV infection. During chronic infection, HIV and SIV producing cells are highly concentrated within B cell follicles (73-75, 78), while virus-specific $CD8^+$ T cells fail to accumulate in large number in these areas (73, 74, 79, 80), indicating that B cell follicles are somewhat of an immune privileged site where low concentrations of anti-viral $CD8^+$ T cells permit ongoing viral replication. Furthermore during HIV and SIV infections, FDC within the GC of mature B cell follicles have large quantities of virions attached to their cell surface via complement and antibody complexes (2, 143, 202), and are thought to be continually infecting follicular $CD4^+$ T cells.

Prior to our study presented here, it was not clear whether SIV-specific $CD8^+$ T cells are able to migrate throughout the entire follicular area including the GC, or are limited to only certain regions of the follicle. We found that $tetramer^+$ SIV-specific $CD8^+$ T cells were located throughout the entire follicle, including the GC as well as non-GC regions of follicles, at similar levels, in both lymph node and spleen tissues. These findings indicate that virus-specific $CD8^+$ T cells are able to migrate throughout the entire follicular area, including GC areas laden with virus.

In addition to there being relatively low levels of SIV-specific $CD8^+$ T cells in follicles, other factors or cellular processes likely contribute to the inability of CTL to fully control follicular viral replication. We previously found that virus-specific $CD8^+$ T cells often down-modulate cell surface expression of the important CD8 co-receptor upon entering follicles (205), which likely impairs cytolytic function. We show here that some follicular $tetramer^+$ SIV-specific $CD8^+$ T cells are PD-1 positive and likely struggling with exhaustion. We also found that small subsets were in contact with $Foxp3^+$ cells, or were $Foxp3^+$, indicating a potential source of T cell inhibition. Thus, there exists several mechanisms that likely contribute to the failure of virus-specific $CD8^+$ T cells to fully control follicular HIV and SIV replication.

Although several mechanisms contribute to the failure of follicular virus-specific CTL to fully control HIV and SIV replication, evidence exists that at least a subset of follicular virus-specific $CD8^+$ T cells are able to suppress viral replication. In support of this hypothesis, we recently reported that virus-specific $CD8^+$ T cells in lymphoid compartments predict levels of SIV-producing cells in those compartments (73), and as we show here, also tended to predict plasma viral load. We also show here that many follicular $tetramer^+$ virus-specific $CD8^+$ T cells express the effector molecule perforin at low to medium levels and small subset at high levels, and thus have machinery needed for cytolytic function. In addition, we show here that a subset of follicular $tetramer^+$ SIV-specific $CD8^+$ T cells express Ki67 indicating they are activated and proliferating. Importantly, we also show here a rise in follicular virus-producing cells after CD8 depletion in all four animals examined, suggesting that follicular $CD8^+$ T cells actively suppress follicular viral replication *in vivo*.

Additional evidence that follicular $CD8^+$ T cells suppress viral replication is presented in two recently published studies (174, 229). Follicular, $CXCR5^+$ LCMV-specific $CD8^+$ T cells were shown to control LCMV infection of T_{FH} cells and reduce viral loads significantly better than $CXCR5^-$ $CD8^+$ T cells adoptively transferred into LCMV infected mice (174, 229). In addition, and importantly, HIV-specific $CXCR5^+$ $CD8^+$ T cell levels in blood inversely correlated with viral loads in chronically infected untreated HIV infected patients (174). Thus, substantial evidence is accumulating supporting an important role for follicular anti-viral $CD8^+$ T cells in controlling infection.

While the focus of this study is follicular $CD8^+$ T cells, it is important to also discuss extrafollicular cells. We found a greater increase of extrafollicular SIV-producing cells after CD8 depletion, compared to the increase seen to follicular areas. This finding supports the hypothesis that we have been promoting for a decade that HIV- and SIV-specific $CD8^+$ T are able to effectively clear virus producing cells in the extrafollicular region, but are not as effective in

targeting virus-producing cells in B cell follicles (73, 74, 163). These findings support the recent findings by Fukazawa Y. et al, who found that *in vivo* $CD8^+$ lymphocyte depletion of chronically SIV-infected rhesus macaques led to a redistribution of SIV infection from T_{FH} cells within the follicles to extrafollicular $CD4^+$ T cells (78), which similarly suggest a superior ability of extrafollicular relative to follicular $CD8^+$ T cells in controlling viral replication.

There is clearly a need to develop strategies to eliminate of HIV and SIV virions and infected cells from B cell follicles. Studies presented here by us and others, support HIV cure strategies that augment functional follicular virus-specific $CD8^+$ T cells to enhance viral control. This might be achieved in a number of ways. First, we found a highly significant positive correlation between levels of extrafollicular and follicular $tetramer^+$ virus-specific $CD8^+$ T cells, suggesting that increasing total numbers of lymphoid virus-specific $CD8^+$ T cells may increase total numbers of both extrafollicular as well as follicular virus-specific $CD8^+$ T cells. Second, given that CXCR5 directs $CD8^+$ T cells to B cell follicles (174, 229), adoptive transfer of autologous HIV-specific $CXCR5^+$ $CD8^+$ T cells could increase levels of HIV-specific $CXCR5^+$ $CD8^+$ T cells in follicles and reduce follicular viral replication. Third, combining a therapy that increases levels of follicular HIV-specific $CD8^+$ T cells with other therapies may be synergistic. For example, adding blockade of PD-1/PD-L1 to a therapy that augments levels of follicular HIV-specific $CD8^+$ T cells may enhance reductions in viral replication, as it has been shown that blockade of PD-1/PD-L1 pathway *in vivo* restored the function of exhausted $CD8^+$ T cells during chronic viral infection (178) and enhanced SIV-specific $CD8^+$ T cells responses (219). In addition, anti-PD-L1 treatment synergistically strengthened the control of LCMV replication mediated by the adoptive transfer of $CXCR5^+$ $CD8^+$ T cells in mice (174). Inhibition of Tregs might also synergize with a therapy that augments levels of follicular HIV-specific $CD8^+$ T cells. IL-15 is a cytokine that facilitates $CD8^+$ and $CD4^+$ effector T cell expansion, while having no preferential effect on the proliferation of Tregs (230). In addition, IL-27 delivery inhibits $CD4^+CD25^+Foxp3^+$

Tregs expansion and potentiates tumor-specific CTL reactivity (231). Based on these properties, it is reasonable to speculate that therapeutic IL-15 and IL-27 administration might lead to relatively lower levels of Tregs and increased effector T cells in lymphoid tissues, and lead to improved viral control. A therapy that increases levels of follicular HIV-specific $CD8^+$ T cells might also be combined with HIV latency reversal agents to improve killing of reactivated cells. It might also be combined with approaches that create $CD4^+$ T cells that are resistant to infection to improve overall viral suppression. Fourth, therapies that eliminate infectious HIV from the FDC network may be developed, and these may be combined with the strategies suggested above. Thus, there is a critical need to reduce HIV replication in B cell follicles, and there exist many new approaches that may tackle this problem.

In summary, studies presented here suggest that during chronic SIV infection, despite likely inhibition of SIV-specific $CD8^+$ T cells by $Foxp3^+$ cells, and a subset of follicular SIV-specific $CD8^+$ T cells likely being exhausted, some follicular SIV-specific $CD8^+$ T cells express the functional markers perforin and Ki67, and appear to suppress viral replication *in vivo*. These findings support HIV cure strategies that augment functional follicular virus-specific $CD8^+$ T cells to enhance viral control.

TABLE 1 Rhesus macaques included in studies

ID number	DPI	MHC-genotype[a] Peptide	Virus	Plasma SIV RNA (log_{10} Copies/ml)	Route[b]	Ki67	Perf	Foxp3	PD-1
Rh2515	42	A01 Gag-CM9	SIVmac239	4.22	IV	ND	-	+	+
Rh2516	42	A01 Gag-CM9	SIVmac239	5.55	IV	+	ND	+	+
Rh2520	42	A01 Gag-CM9	SIVmac239	7.58	IV	+	ND	+	+
Rh2306	84	A01 Gag-CM9	SIVmac239	6.15	R	-	+	-	-
R03111	105	A02 Nef-YY9	SIVmac239	6.45	R	-	+	-	-
R03094[c]	154	A01 Gag-CM9	SIVmac239	6.20	R	-	+	-	-
R03116	161	A01 Gag-CM9	SIVmac239	3.78	R	-	+	-	-
11-57	258	A01 Gag-CM9	SIVmac251	4.51	V	+[d]	+	-	+
11-45	272	A01 Gag-CM9	SIVmac251	6.26	V	ND	+	+	+
JD85	272	A01 Gag-CM9	SIVmac251	4.56	V	+[d]	ND	+	+
11-89	349	A01 Gag-CM9	SIVmac251	5.15	V	+[d]	+	+	+
11-061	545	A01 Gag-CM9	SIVmac251	3.72	V	+[d]	ND	+	+
Rhax18[c]	546	B08 Nef-RL10	SIVmac239	4.97	R	+[d]	+	-	+
R01106	567	B08 Env-KL9	SIVmac239	4.73	R	+[d]	+	-	-
Rhau10	1687	B08 Nef-RL10	SIVmac239	4.11	IV	-	+	-	-

[a] Full MHC allele names are as follows. A01 is *Mamu-A1*001:01;* A02 is *Mamu-A1*002:01;* and B08 is *Mamu-B*008:01*

[b] Route of SIV infection.

[c] Animals had < 200 $CD4^+$ T cells/mm^3.

[d] Quantitative image analysis was done with both lymph node and splenic tissue.

- There were not enough follicles in stained tissue sections for quantitative image analysis.

DPI: Days Post-Infection.

ND: Not Done.

TABLE 2 Numbers of tetramer$^+$ cells counted in contact with Foxp3$^+$ cells

ID number	Total # tet$^+$ cells counted in follicular areas	Total # tet$^+$ cells that contact Foxp3$^+$ cells in follicular areas	Total # tet$^+$ cells counted in extrafollicular areas	Total # tet$^+$ cells that contact Foxp3$^+$ cells in extrafollicular areas
Rh2515	100	6	176	40
Rh2516	261	17	352	47
Rh2520	212	20	119	35
11-45	406	23	486	43
JD85	486	23	498	63
11-89	129	11	129	18
11-061	306	15	297	26
Total	**1900**	**115**	**2057**	**272**

Figure 1

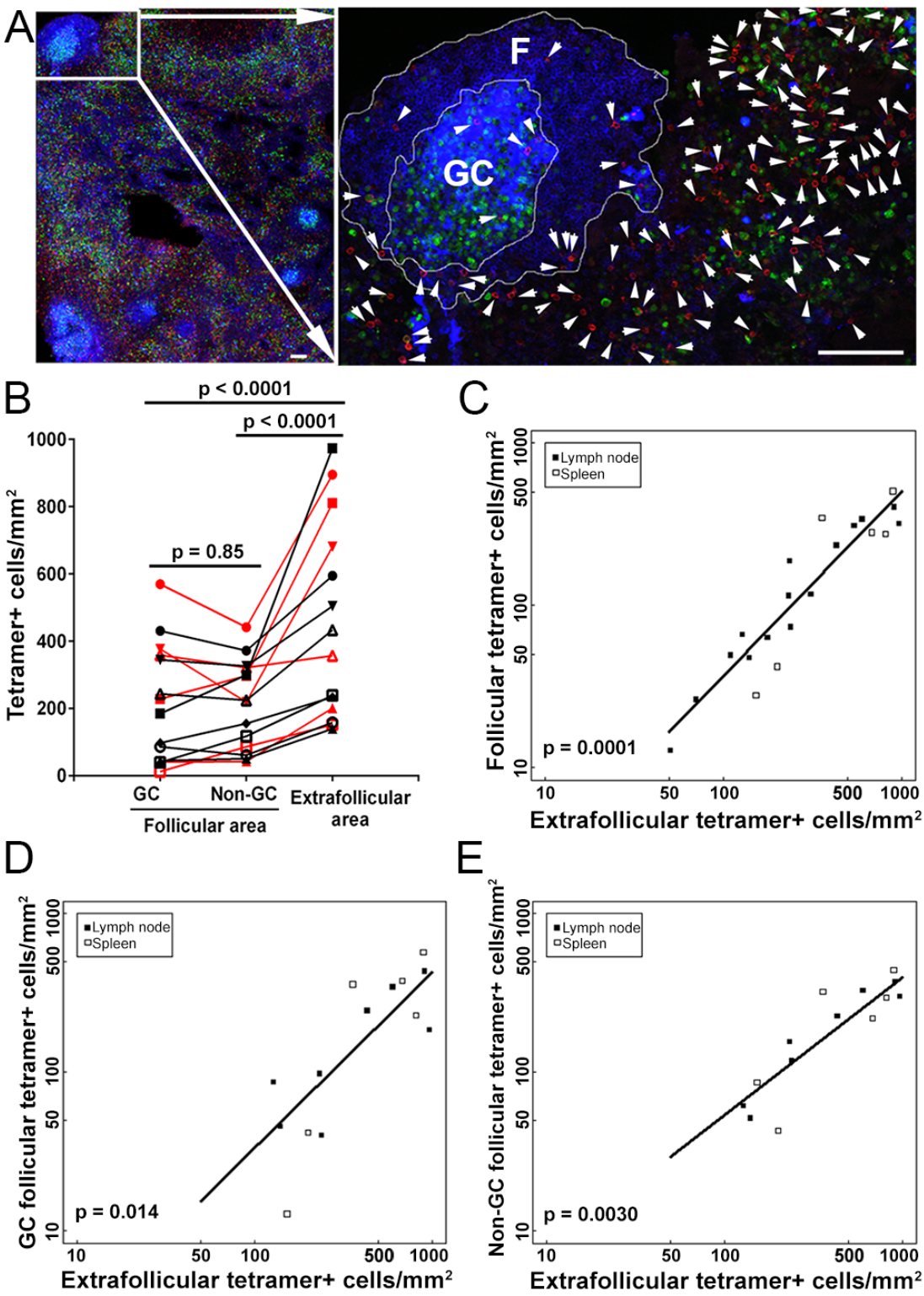
A
F
GC
B
p < 0.0001
p < 0.0001
p = 0.85
Tetramer+ cells/mm²
1000
800
600
400
200
0
GC
Non-GC
Extrafollicular area
Follicular area
C
Lymph node
Spleen
Follicular tetramer+ cells/mm²
p = 0.0001
Extrafollicular tetramer+ cells/mm²
10
50
100
500
1000
D
Lymph node
Spleen
GC follicular tetramer+ cells/mm²
p = 0.014
Extrafollicular tetramer+ cells/mm²
E
Lymph node
Spleen
Non-GC follicular tetramer+ cells/mm²
p = 0.0030
Extrafollicular tetramer+ cells/mm²

FIG 1 SIV-specific $CD8^+$ T cells can migrate throughout B cell follicles including germinal centers (GC). (A) Representative lymph node section stained with Mamu-A1*001/Gag CM9 tetramers to label SIV-specific $CD8^+$ T cells (red, and indicted with arrows in the enlargement), IgM antibodies (blue) to define follicles (F), and Ki67 antibodies (green) to label GC. Confocal images were collected with a 20X objective and each scale bar indicates 100 μm. (B) Frequencies of $tetramer^+$ SIV-specific $CD8^+$ T cells in different compartments of lymph nodes and spleen during chronic SIV infection. Samples from spleen are indicated with red, whereas all others are from lymph node. There were no significant differences between frequencies of $tetramer^+$ SIV-specific $CD8^+$ T cells in GC and non-GC follicular areas ($p = 0.85$). Frequencies of extrafollicular $tetramer^+$ SIV-specific $CD8^+$ T cells were 109% (95% CI, 60, 172%) higher than GC ($p < 0.0001$) and 104% (95% CI, 56, 166%) higher than non-GC follicular areas ($p < 0.0001$). (C) Relationship between frequencies of follicular and extrafollicular $tetramer^+$ SIV-specific $CD8^+$ T cells. The frequency of extrafollicular $tetramer^+$ SIV-specific $CD8^+$ T cells predicted the frequency of follicular $tetramer^+$ SIV-specific $CD8^+$ T cells. For every 1 $\log_{10}$ increase in extrafollicular $tetramer^+$ SIV-specific $CD8^+$ T cells, there was an estimated 1.14 $\log_{10}$ (95% CI 0.86, 1.42) increase in the frequency of follicular $tetramer^+$ SIV-specific $CD8^+$ T cells ($p = 0.0001$). After adjusting for extrafollicular $tetramer^+$ SIV-specific $CD8^+$ T cells, tissue type (LN or Spleen) was not a significant predictor of follicular $tetramer^+$ SIV-specific $CD8^+$ T cells ($p = 0.39$). (D) The relationship between frequencies of GC and extrafollicular $tetramer^+$ SIV-specific $CD8^+$ T cells. The frequency of extrafollicular $tetramer^+$ SIV-specific $CD8^+$ T cells predicted the frequency of GC $tetramer^+$ SIV-specific $CD8^+$ T cells. For every 1 $\log_{10}$ increase in extrafollicular $tetramer^+$ SIV-specific $CD8^+$ T cells, there was an estimated 1.11 $\log_{10}$ (95% CI 0.34, 1.88) increase in the frequency of GC $tetramer^+$ SIV-specific $CD8^+$ T cells ($p = 0.014$). After adjusting for extrafollicular $tetramer^+$ SIV-specific $CD8^+$ T cells, tissue type (LN or spleen) was not a significant predictor of GC $tetramer^+$ SIV-specific $CD8^+$ T cells ($p = 0.96$). (E) Relationship between frequencies of non-GC follicular and extrafollicular $tetramer^+$ SIV-specific $CD8^+$ T cells.

The frequency of extrafollicular tetramer$^+$ SIV-specific CD8$^+$ T cells predicted the frequency of non-GC follicular tetramer$^+$ SIV-specific CD8$^+$ T cells. For every 1 $\log_{10}$ increase in extrafollicular tetramer$^+$ SIV-specific CD8$^+$ T cells, there was an estimated 0.87 $\log_{10}$ (95% CI 0.45, 1.28) increase in the frequency of non-GC follicular tetramer$^+$ SIV-specific CD8$^+$ T cells ($p = 0.0030$). After adjusting for extrafollicular tetramer$^+$ SIV-specific CD8$^+$ T cells, tissue type (LN or spleen) was not a significant predictor of non-GC follicular tetramer$^+$ SIV-specific CD8$^+$ T cells ($p = 0.86$).

Figure 2

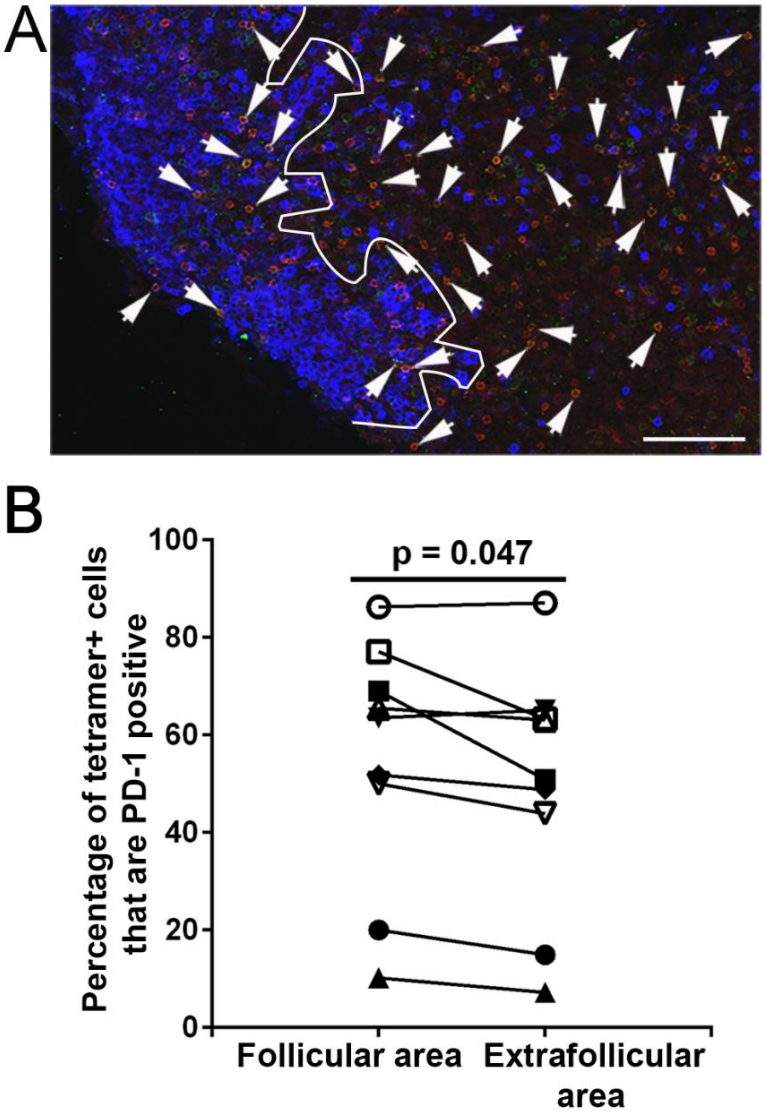
A
B
p = 0.047
Percentage of tetramer+ cells that are PD-1 positive
100
80
60
40
20
0
Follicular area
Extrafollicular area

FIG 2 Many follicular tetramer$^+$ SIV-specific CD8$^+$ T cells express PD-1. (A) Representative lymph node section stained with Mamu-A*001:01/Gag CM9 tetramers to label SIV-specific CD8$^+$ T cells (red), PD-1 antibodies (green) to label PD-1 expressing cells and CD20 antibodies (blue) to define follicles. Confocal images were collected with a 20X objective and the scale bar is 100 µm. (B) The percentage of PD-1$^+$ cells within the tetramer-binding population was 11.2% ($p = 0.047$, 95% CI, 0.2, 23%) higher in follicles compared with extrafollicular regions.

Figure 3

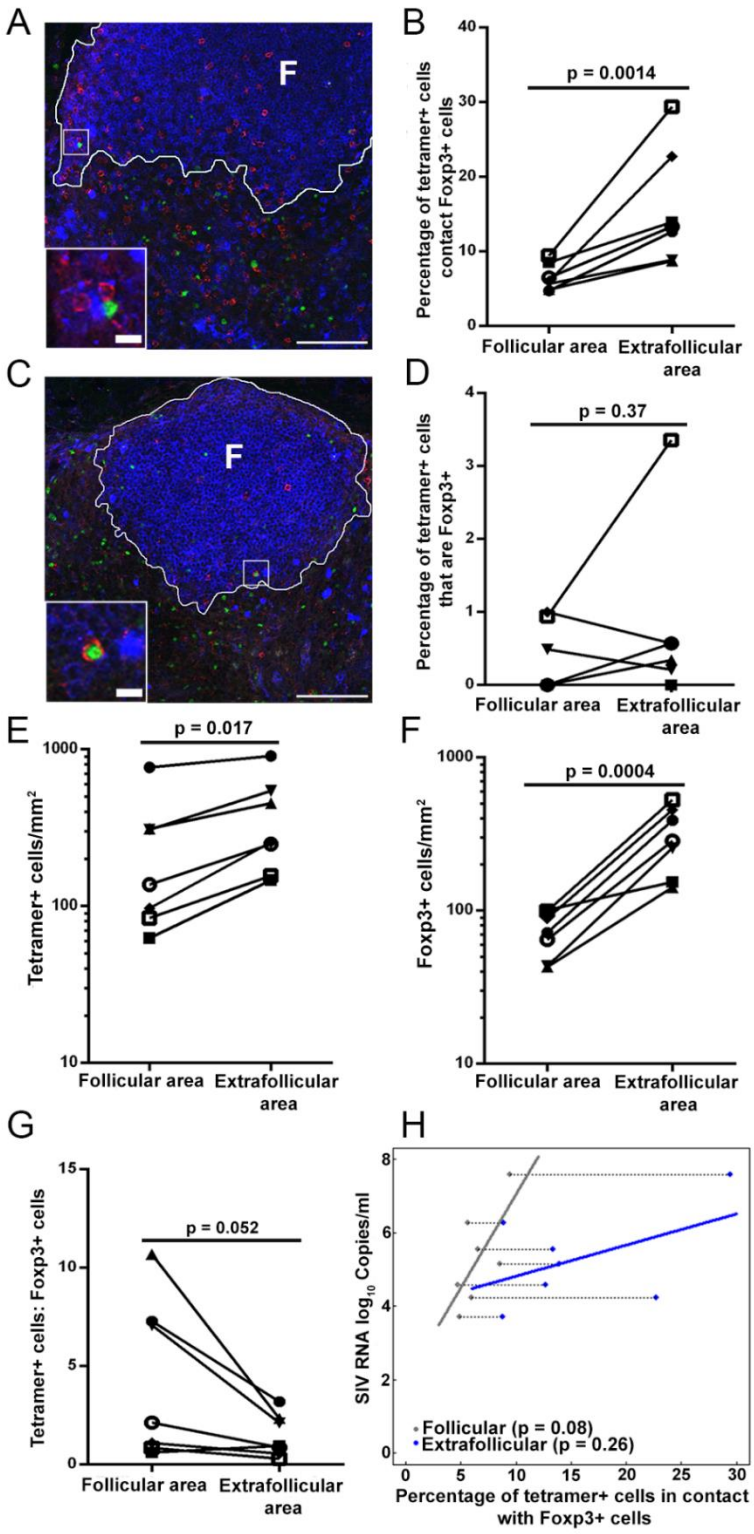
A
F
B
p = 0.0014
Percentage of tetramer+ cells contact Foxp3+ cells
Follicular area
Extrafollicular area
C
F
D
p = 0.37
Percentage of tetramer+ cells that are Foxp3+
Follicular area
Extrafollicular area
E
p = 0.017
Tetramer+ cells/mm²
Follicular area
Extrafollicular area
F
p = 0.0004
Foxp3+ cells/mm²
Follicular area
Extrafollicular area
G
p = 0.052
Tetramer+ cells: Foxp3+ cells
Follicular area
Extrafollicular area
H
SIV RNA log10 Copies/ml
Follicular (p = 0.08)
Extrafollicular (p = 0.26)
Percentage of tetramer+ cells in contact with Foxp3+ cells

FIG 3 A subset of follicular tetramer$^+$ SIV-specific CD8$^+$ T cells are likely inhibited by Foxp3$^+$ cells. (A) Representative lymph node section stained with Mamu-A*001:01/Gag CM9 tetramers (red), IgM (blue), and Foxp3 (green) showing tetramer$^+$ cells in contact with Foxp3$^+$ cells. (B) Percentages of follicular tetramer$^+$ SIV-specific CD8$^+$ T cells that were in direct contact with Foxp3$^+$ cells were significantly lower than extrafollicular tetramer$^+$ SIV-specific CD8$^+$ T cells ($p = 0.0014$). (C) Representative image showing tetramer$^+$ cells are Foxp3$^+$. (D) There was no significant difference between percentages of tetramer$^+$ SIV-specific CD8$^+$ T cells inside and outside follicle that were Foxp3$^+$ ($p = 0.37$). (E) Frequencies of tetramer$^+$ SIV-specific CD8$^+$ T cells inside follicles were significantly lower than outside of follicle ($p = 0.017$). (F) Frequencies of Foxp3$^+$ cells inside follicles were significantly lower than outside of follicle ($p = 0.0004$) as well. (G) Tetramer$^+$ SIV-specific CD8$^+$ T cells: Foxp3$^+$ cells ratios inside follicle tend to be higher than outside of follicle ($p = 0.052$). (H) Separate linear regression lines were fit for follicular and extrafollicular percentage of tetramer$^+$ cells in contact with Foxp3$^+$ cells as predictors of plasma viral load. In the follicle, for every 1% increase in percentage of tetramer$^+$ cell in contact with Foxp3$^+$ cells, there was an estimated 0.51 (-0.09, 1.1) $\log_{10}$ increase in SIV RNA $\log_{10}$ copies/ml ($p = 0.08$, $R^2 = 38.8\%$). In the extrafollicular region, for every 1% increase in tetramer$^+$ cell in contact with Foxp3$^+$ cells, there was an estimated 0.09 (-0.09, 0.26) $\log_{10}$ increase in SIV RNA $\log_{10}$ copies/ml ($p = 0.26$, $R^2 = 9.5\%$).

Figure 4

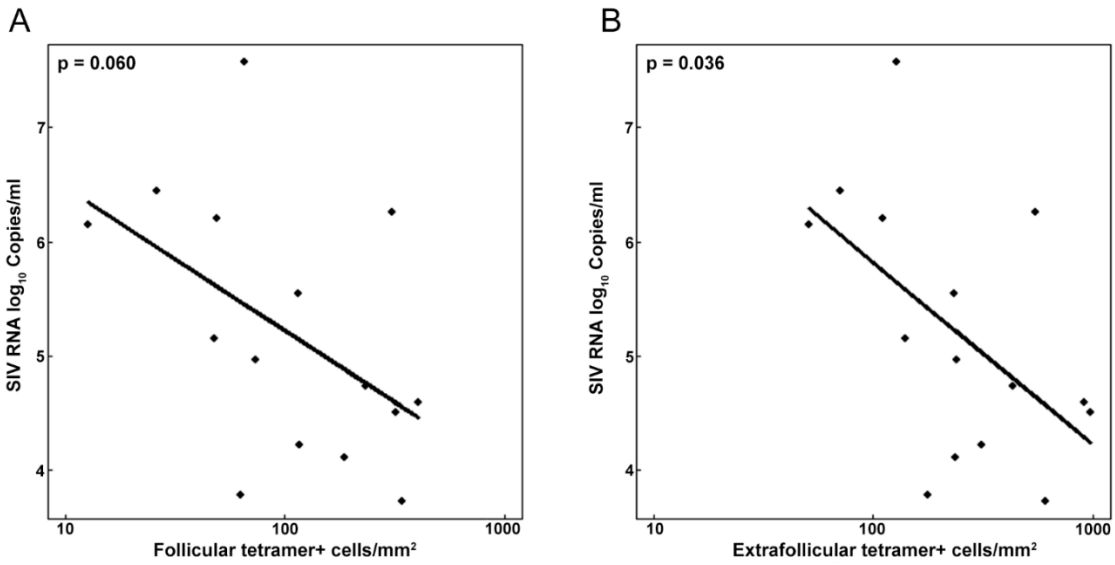

FIG 4 Relationship between plasma viral load and frequencies of tetramer$^+$ SIV-specific CD8$^+$ T cells in follicular and extrafollicular areas. (A) Log_{10} follicular tetramer$^+$ SIV-specific CD8$^+$ T cells tended to predict plasma viral load ($p = 0.060$). For every 1 log_{10} increase in follicular tetramer$^+$ SIV-specific CD8$^+$ T cells, there was an estimated 1.25 (95% CI: -0.064, 2.57) log_{10} decrease in SIV RNA log_{10} copies/ml; (B) Log_{10} extrafollicular tetramer$^+$ SIV-specific CD8$^+$ T cells significantly predicted plasma viral load ($p = 0.036$). For every 1 log_{10} increase in follicular tetramer$^+$ SIV-specific CD8$^+$ T cells, there was an estimated 1.60 (95% CI: 0.12, 3.08) log_{10} decrease in SIV RNA log_{10} copies/ml.

Figure 5

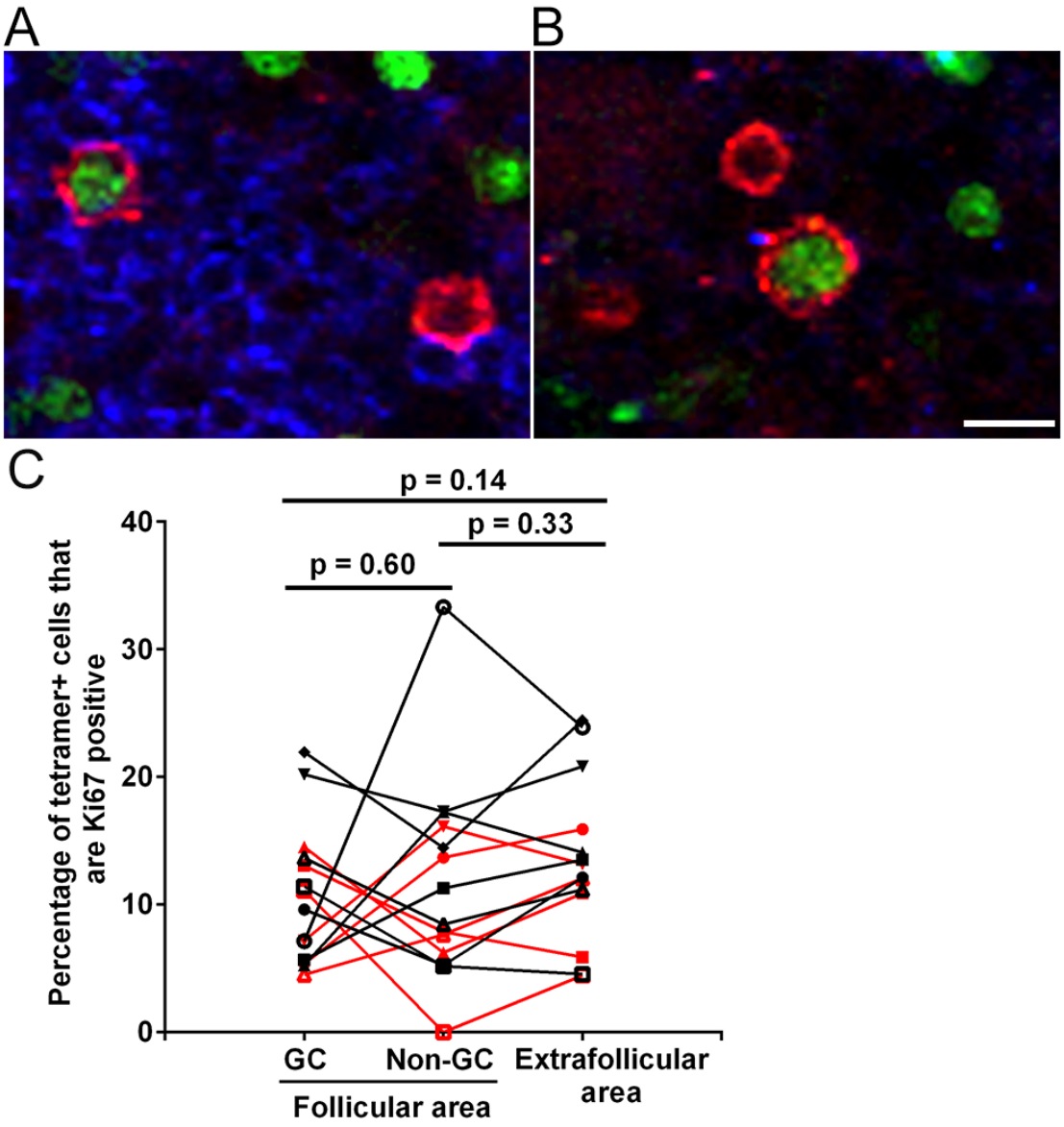
A
B
C
p = 0.14
p = 0.33
p = 0.60
Percentage of tetramer+ cells that are Ki67 positive
40
30
20
10
0
GC
Non-GC
Extrafollicular area
Follicular area

FIG 5 Ki67 expression levels in follicular and extrafollicular tetramer$^+$ SIV-specific CD8$^+$ T cells. Representative lymph node section stained with Mamu-A*001:01/Gag CM9 tetramers (red), IgM (blue), and Ki67 (green) showing tetramer$^+$ Ki67$^+$ cells in follicle (A) and in extrafollicular region (B). Scale bar indicates 10 μm. (C) Percentages of tetramer$^+$ SIV-specific CD8$^+$ T cells that expressed Ki67 in GC, non-GC areas of follicles and extrafollicular areas in lymph nodes and spleen. Samples from spleen are indicated with red, and from lymph node black. There was no significant difference between compartments in lymph nodes or spleen ($p = 0.13$).

Figure 6

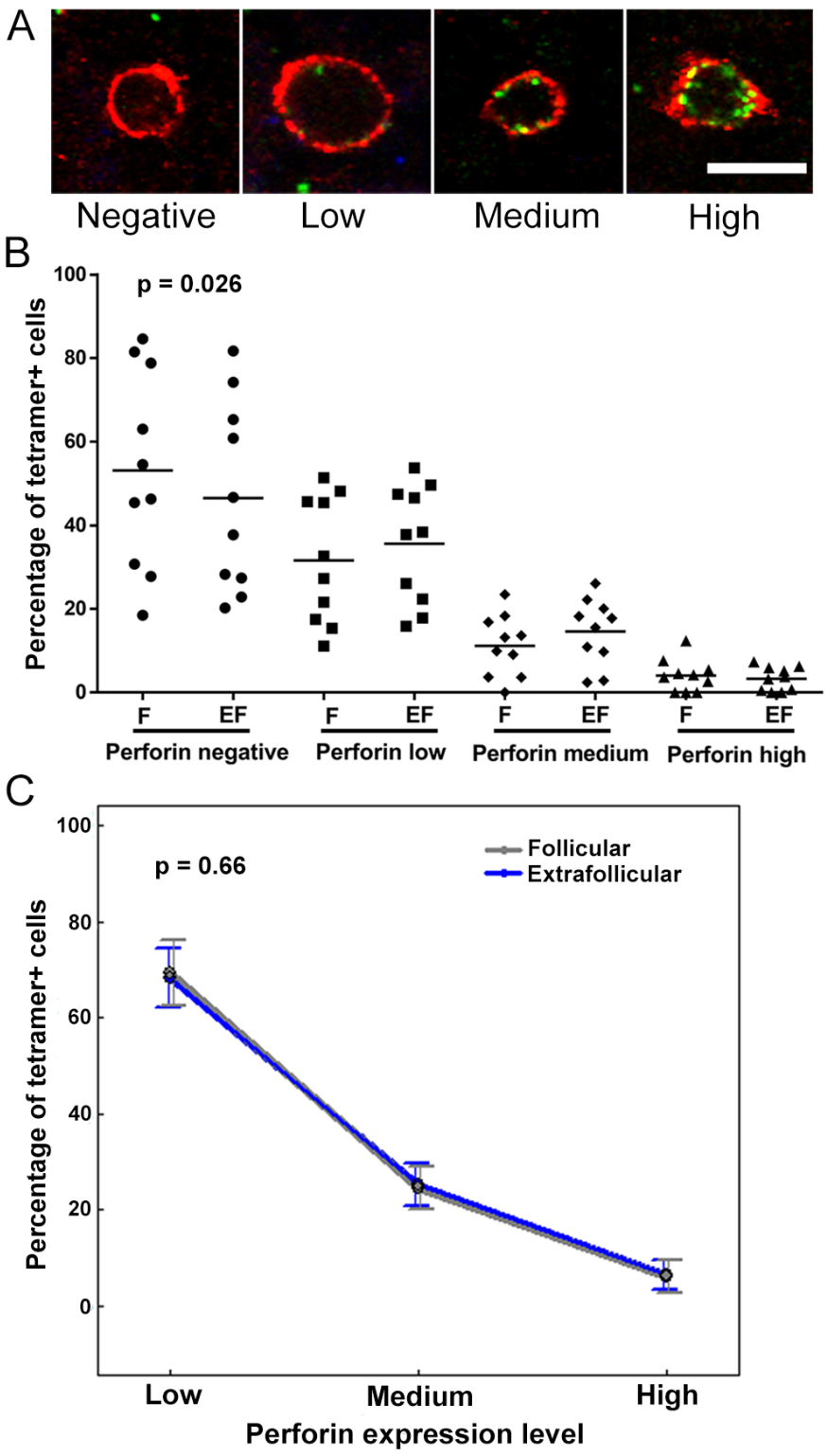
A
Negative
Low
Medium
High
B
p = 0.026
Percentage of tetramer+ cells
100
80
60
40
20
0
F
EF
Perforin negative
Perforin low
Perforin medium
Perforin high
C
p = 0.66
Follicular
Extrafollicular
Percentage of tetramer+ cells
Low
Medium
High
Perforin expression level

FIG 6 Perforin expression levels in follicular and extrafollicular tetramer$^+$ SIV-specific CD8$^+$ T cells. (A) Representative lymph node section stained with Mamu-A*001:01/Gag CM9 tetramers (red) and perforin (green) showing perforin negative, perforin low, perforin medium and perforin high MHC-class I tetramer$^+$ SIV-specific CD8$^+$ T cells. Scale bar indicates 10 µm. (B) Percentages of tetramer$^+$ SIV-specific CD8$^+$ T cells that expressed perforin in follicular and extrafollicular regions. The percentage of tetramer$^+$ SIV-specific CD8$^+$ T cells didn't express perforin was ~5.4% (95% CI, 0.7, 10.2%) higher in follicles compared with extrafollicular regions ($p = 0.026$). (C) Among tetramer$^+$ cells that express perforin, the distribution of cells across low, medium and high perforin expression is not significantly different between follicular and extrafollicular regions ($p = 0.66$).

Figure 7

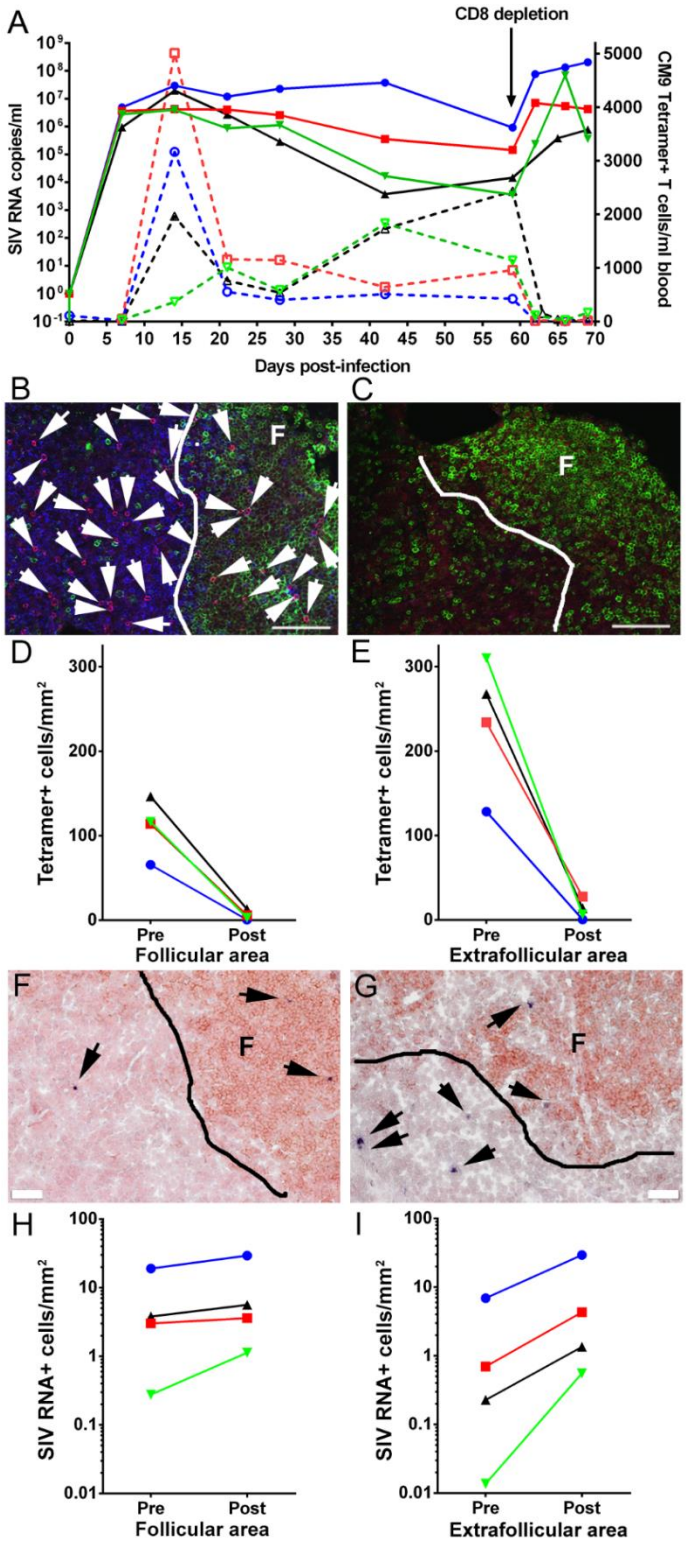
A
CD8 depletion
SIV RNA copies/ml
CM9 Tetramer+ T cells/ml blood
Days post-infection
B
C
F
D
E
Tetramer+ cells/mm²
Pre
Post
Follicular area
Extrafollicular area
F
G
H
I
SIV RNA+ cells/mm²

FIG 7 Increase in follicular and extrafollicular SIV-producing cells post-CD8 depletion. (A) Dynamics of plasma viral loads and Mamu-A*001:01/Gag CM9 tetramer$^+$ cells concentration in blood post SIV infection. Representative lymph node tissue sections stained with MHC-class I tetramers (red) to label SIV-specific CD8$^+$ T cells, CD8 antibodies (blue) to label T cells and CD20 antibodies (green) to label B cells and define B cell follicles. (B) A lymph node section from animal Rh2516 stained with Mamu-A*001:01/Gag CM9 tetramers demonstrating an example of tetramer$^+$ SIV-specific CD8$^+$ T cells level and location before CD8 depletion. (C) A lymph node section from animal Rh2516 stained with Mamu-A*001:01/Gag CM9 tetramers demonstrating an example after CD8 depletion. Scale bars indicate 100 μm. (D) Tetramer$^+$ SIV-specific CD8$^+$ T cells in B cell follicles were almost completely depleted after CD8 depletion in all four animals. (E) Tetramer$^+$ SIV-specific CD8$^+$ T cells in extrafollicular regions dramatically decreased after CD8 depletion. Representative images of *in situ* hybridization for SIV RNA to identify virus-producing cells (blue/black cells indicated by arrows) and CD20 staining (brown) to morphologically identify B cell follicles in lymph node before (F) and after CD8 depletion (G). Scale bars indicate 50 μm. (H) Frequencies of SIV RNA$^+$ cells in B cell follicles increased after CD8 depletion in all four animals. (I) Frequencies of SIV RNA$^+$ cells in extrafollicular regions dramatically increased after CD8 depletion.

Chapter 3

Low levels of SIV-specific CD8 T cells in germinal centers during early infection may set the stage for persistent chronic infection

Synopsis

HIV-specific CD8 T cells contribute to the control of HIV infection. These cells are typically excluded from B cell follicles during chronic infection. It is not known whether this phenomenon also occurs during early infection. Here, we determined the distribution and phenotype of simian immunodeficiency virus (SIV)-specific CD8 T cells in lymph nodes from SIV-infected rhesus macaques during early infection. We found that levels of SIV-specific CD8 T cells in B cell follicles were also significantly lower than in extrafollicular regions. Furthermore, follicular SIV-specific CD8 T cells were largely excluded from germinal centers (GCs). Despite high level of PD-1 expression and potential inhibition from Foxp3+ cells, subsets of follicular SIV-specific CD8 T cells express the proliferation molecule Ki67 and high levels of cytolytic molecule perforin. We found a small population of follicular SIV-specific CD8 T cells are PARP+ in early chronic infection, suggesting that cell death is not a critical factor for low levels of follicular SIV-specific CD8 T cells. Taken together, these data suggest that despite high levels of exhaustion and likely inhibition by Foxp3+ cells, a subset of follicular SIV-specific CD8 T cells likely possess cytolytic function and suppress viral replication. Furthermore, these data suggest that the low levels of follicular SIV-specific CD8 T cells in GCs may be a major factor for the establishment of persistent chronic infection. These findings here provide important insights into SIV immunopathogenesis and may help inform future cure strategies.

Introduction

Most human immunodeficiency virus (HIV)-infected individuals fail to adequately control persistent high-level viral replication that results in gradual loss of CD4 T cells and AIDS ultimately in the absence of combination antiretroviral therapy (cART). B cell follicles in secondary lymphoid tissues have been identified as important sanctuaries that contain large amounts of virus-producing cells during chronic HIV and simian immunodeficiency virus (SIV) infection (73-77). CD4+ T follicular helper (T_{FH}) cells, a specialized CD4 T cell population that mainly resides in B cell follicles and provides pivotal help to B cell activation and maturation, serve as a major site of productive HIV and SIV infection in B cell follicles (2, 74-76, 137, 232, 233). In SIV-infected rhesus macaques with full control of viral replication, either via natural highly effective immune response or receiving long-term, fully suppressive cART, residual productive SIV infection is strikingly restricted to T_{FH} cells (78). In HIV infected aviremic individuals treated with long-term ART, T_{FH} also serves as a major reservoir for active and persistent virus transcription (136). Therefore, understanding the immune activity needed to kill virus-infected T_{FH} cells in B cell follicle is necessary for developing novel therapies to fully eradicate HIV or SIV infection.

Development of HIV-specific CD8 T cells during acute infection is associated with a decline in plasma viremia, suggesting that the positive effect of these cells in initial viral control (147, 148, 234). Moreover, CD8 depletion experiments in acute SIV and SHIV infection induces continuously high levels of plasma viremia which don't reduce until reconstitution of CD8+ lymphocytes (169, 170). Certain individual HIV-specific CD8 T cell responses elicited in acute HIV infection have been shown to modulate the subsequent immune control of viremia (235). Similarly, multiple studies to date have determined the indispensable role of anti-viral CD8 T cell responses in suppressing viral replication during chronic SIV infection (168, 170). Furthermore, strong HIV-specific CD8 T cell activity is directly associated with long-term elite control of

infection (150, 151, 203). We previously showed a significant inverse relationship between SIV-specific CD8 T cell frequency and SIV-producing cell levels in different compartments of the lymph node during chronic SIV infection (73). However, in spite of the notable anti-viral effect in both acute and chronic infection, HIV/SIV-specific CD8 T cells fail to fully eliminate viral replication and the vast majority of HIV/SIV-infected individuals eventually develop disease in the absence of cART.

We and others previously showed that HIV- and SIV-specific CD8 T cells are largely excluded from B cell follicles in lymph node and spleen tissues during chronic infection (73, 74, 79, 80, 236). The apparent paucity of virus-specific CD8 T cells inside B cell follicles, where HIV- and SIV-producing cells are highly concentrated, has been identified as an important mechanism of immune evasion by HIV and SIV. This mechanism may, at least partially, account for the failure to eradicate HIV/SIV infection. However, the exclusion of anti-viral CD8 T cells from B cell follicles is not absolute. A couple of recent studies reported a population of functional CD8 T cells expressing CXCR5 in B cell follicles in chronic LCMV, HIV and SIV infections (174, 229, 237, 238). Moreover, modest increases in SIV-producing cells in B cell follicles were observed following CD8 depletion experiments during chronic SIV infection (236), suggesting follicular CD8 T cells mediate control of SIV replication to some extent in B cell follicles. Together, these studies strongly suggest that at least a fraction of follicular CD8 T cells are functional and able to suppress HIV/SIV replication during chronic infection. However, whether virus-specific CD8 T cells migrate into B cell follicles during early HIV/SIV infection remains unaddressed and if so, whether these cells are functional in controlling virus replication has not been tested.

In this study, we sought to determine and characterize the distribution of SIV-specific CD8 T cells in lymph nodes, the functional phenotype of these cells and the local relationship of these cells to Foxp3+ cells during early SIV infection. We observed a small population of SIV-

specific CD8 T cells migrate into B cell follicles. Furthermore, follicular SIV-specific CD8 T cells were largely excluded from germinal centers (GCs). Although suffering from potential suppression of regulatory T cells (Tregs) and possible exhaustion, a subset of follicular SIV-specific CD8 T cells are activated and proliferating, and exhibit effector phenotype and possibly contribute to control of viral replication in early SIV infection. We next compared the functional phenotype of follicular SIV-specific CD8 T cells in early and chronic SIV infection. We found that more follicular SIV-specific CD8 T cells in early infection are activated and display effector phenotype. These findings suggest that, similar to chronic infection, SIV-specific CD8 T cells in early infection are also largely excluded from B cell follicles, and these cells are likely possess cytolytic potential. Importantly, low levels of follicular SIV-specific CD8 T cells from GCs during early infection may pave the road for persistent chronic infection.

Materials and Methods

Tissues from animals in early SIV infection

Lymph nodes were obtained from captive-bred rhesus macaques of Indian origin infected with SIVmac239 intravenously (IV) (Table 1). All animals were housed and cared for according to American Association for Accreditation of Laboratory Animal Care standards in accredited facilities. All animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committees of the Wisconsin National Primate Research Center. Portions of fresh lymphoid tissues were immediately snap frozen in OCT and/or formalin fixed and embedded in paraffin. Simultaneously, portions of fresh lymphoid tissues were also collected in RPMI 1640 medium with sodium heparin (18.7 U/ml) and shipped overnight to the University of Minnesota for *in situ* tetramer staining.

In situ tetramer staining combined with Immunohistochemistry

In situ tetramer staining combined with immunohistochemistry was performed on fresh lymph tissue specimens shipped overnight, sectioned with a compresstome (208) and stained essentially as previously described (73, 74, 209). Biotinylated MHC-class I monomers were loaded with peptides (National Institute of Health Tetramer Core Facility, Emory University, Atlanta GA) and converted to MHC-class I tetramers. Mamu-A1*001 molecules loaded with SIV Gag CM9 (CTPYDINQM) peptides (210) or irrelevant negative control peptides FV10 (FLPSDYFPSV) from the hepatitis B virus core protein. Fresh lymph node sections were incubated with MHC-class I tetramers (0.5 µg/ml) alone or along with goat-anti-human PD-1 Abs (1 µg/mL, polyclonal, R&D Systems). For secondary incubations, sections were incubated with rabbit-anti-FITC Abs (0.5 µg/mL, BioDesign, Saco, ME) and mouse-anti-human Ki67 Abs (1:500 dilution, clone MM1, Vector), or mouse-anti-human perforin Abs (0.1 µg/mL, clone

5B10, Novacastra), or mouse-anti-human Foxp3 Abs (2.5 μg/mL, clone 206D, BioLegend) or mouse-anti-human CD20 Abs (0.19 μg/mL, clone L26, Novocastra), or mouse-anti-human PARP Abs (3.5 μg/mL, clone Asp214, Cell signaling). For the tertiary incubations, the sections stained with goat-anti-human PD-1 Abs were incubated with Cy3-conjugated donkey-anti-rabbit Abs (0.3 μg/mL, Jackson ImmunoResearch Laboratories, West Grove, PA), Alexa 488-conjugated donkey-anti-goat Abs (0.75 μg/mL, Jackson ImmunoResearch Laboratories), and Cy5-conjugated donkey-anti-mouse Abs (0.3 μg/mL, Jackson ImmunoResearch Laboratories). All other sections were incubated with Cy3-conjugated goat-anti-rabbit Abs (0.3 μg/mL, Jackson ImmunoResearch Laboratories), Alexa 488-conjugated goat-anti-mouse Abs (0.75 μg/mL, Molecular probes), and Dylight 649-conjugated goat anti-human IgM (0.3 μg/mL, Jackson ImmunoResearch Laboratories). Sections were imaged using a Zeiss LSM 800 confocal microscope. Montage images of multiple 512 × 512 pixels were created and used for analysis.

Quantitative image analysis

For the determination of levels of SIV-specific CD8+ T cells and percentages of SIV-specific CD8+ T cells that co-expressed specific molecules, follicular areas were identified morphologically as clusters of brightly stained closely aggregated IgM+ or CD20+ cells. Follicular and extrafollicular areas were delineated using ImageJ software. Areas that showed loosely aggregated B cells that were ambiguous as to whether the area was a follicle were not included. For PD-1 expression analysis, an average of 112 tetramer+ cells (range, 67-190) was analyzed in follicular regions and 213 (range, 117-272) in extrafollicular regions. For quantification of tetramer+ cells that were in contact with Foxp3+ cells and express Foxp3+, an average of 102 tetramer+ cells (range, 57-193) was analyzed in follicular regions and 298 (range, 168-560) in extrafollicular regions. For Ki67 expression analysis, an average of 133 tetramer+ cells (range, 30-246) was analyzed in follicular regions and 307 (range, 130-464) in

extrafollicular regions. For perforin expression level analysis, an average of 97 tetramer+ cells (range, 22-193) was analyzed in follicular region and 276 (range, 98-530) in extrafollicular region. To determine levels of perforin expression, tetramer+ cells were scored using the following objective criteria as follows. Tetramer+ cells with no detectable perforin staining above background levels were scored as perforin negative. Tetramer+ cells with perforin staining 2-3X greater than background were scored as perforin low, with perforin staining 4-9X higher than background as perforin medium, and those with 10X or greater than background levels and with perforin staining detectable throughout much of the cytoplasm were scored as perforin high. Cell counts were done on single z-scans. While doing the cells counts, we demarcated cells using a software tool to avoid counting the same cell twice. All quantitative image analyse were done with lymph node tissues. An average of 7.42 mm2 (range, 5.63-10.08 mm2) was analyzed for each lymph node.

In situ hybridization combined with immunohistochemistry

In situ hybridization for SIV RNA was performed as previously described (73, 75). This technique identifies cells that are actively transcribing SIV, but not extracellular virions encapsulated in envelope glycoprotein and bound to FDC. Briefly, 6 μm frozen sections were fixed in 3% paraformaldehyde (Sigma-Aldrich, St. Lousis, MO), hybridized overnight with digoxygenin labeled SIVmac239 antisense probes (Lofstrand Labs, Gaithersburg, MD) and visualized using NBT/5-bromo-4-chloro-3-indolyl phosphate (Roche, Nutley, NJ). Immunohistochemistry staining for B cells was performed in the same tissues using mouse-anti-human CD20 (clone 7D1; AbD Serotec, Raleigh, NC) and detected using HRP-labeled polymer anti-mouse IgG (ImmPressKit; Vector Laboratories, Burlingame, CA) and Vector NovaRed substrate (Vector Laboratories). SIV RNA+ cells were counted by visual inspection and classified as either inside or outside of B cell follicles which were identified morphologically as a cluster of

CD20+ cells as previously described (73, 75). Total tissue area and area of follicles was determined by quantitative image analysis (Qwin Pro version 3.4.0; Leica, Cambridge, U.K.) and used to calculate the frequency of SIV+ cells per mm^2. An average of 12.5 mm^2 (7.1 mm^2 – 87.2 mm^2) was analyzed.

Statistical analysis

All statistical analysis assumed two-sided tests with a significant level of 0.05. GraphPad Prism version 6.0 (GraphPad Software, La Jolla, California, USA) was used to conduct statistical analyses.

Results

SIV-specific CD8 T cells are largely excluded from B cell follicles in early infection

We and others previously showed that CD8 T cells in HIV-infected individuals and SIV-specific CD8 T cells in rhesus macaques are able to immigrate into entire follicular area including the GCs during chronic infection (236-238). Here, to understand whether SIV-specific CD8 T cells are able to enter B cell follicles in early infection, we determined the distribution and magnitude of Gag CM9 tetramer+ CD8 T cells (Tet+) in the lymph nodes on 21 days post infection in a group of SIV-infected Mamu A01+ rhesus macaques.

In addition to Gag CM9 tetramer staining *in situ* in lymph node sections to label SIV-specific CD8 T cells, antibodies against IgM were used to label B cell follicles. We found that, similar to chronic infection, Tet+ cells also migrate into B cell follicles in early infection (Fig. 1A). Moreover, level of follicular Tet+ cells was significantly lower than their extrafollicular counterparts (Fig. 1B) ($p = 0.0023$). We previously showed a positive correlation between levels of follicular and extrafollicular Tet+ cells (73). We next investigated the relationship between Tet+ cells inside and outside B cell follicles in the current study. A highly significant positive correlation between levels of follicular and extrafollicular Tet+ cells in lymph node was also observed in early SIV infection (Fig. 1C) ($p < 0.0001$). These data demonstrated that SIV-specific CD8 T cells are largely excluded from B cell follicles in early SIV infection and the level of follicular SIV-specific CD8 T cells is positively correlated to the level of SIV-specific CD8 T cells in extrafollicular regions.

We next analyzed whether Tet+ cells accumulate within GCs inside follicular areas where follicular dendritic cells (FDCs) hold large amount of virus in immune complexes and are potently infectious to CD4+ T cells around. To address this question, we used Gag CM9 tetramer to stain SIV-specific CD8 T cells, antibodies against IgM to label B cell follicles and antibodies

against Ki67 to identify GCs. Among all GCs that were counted here, 60.3% (44/73) were completely devoid of Tet+ cells (Fig. 1D). Furthermore, throughout the rest of the B cell follicles (39.7%, (29/73)), very few Tet+ cells in GCs were found (Fig. 1E). These data indicate that follicular SIV-specific CD8 T cells are largely excluded from GCs in early infection.

Many follicular SIV-specific CD8 T cells express PD-1 in early SIV infection

PD-1 is a marker of functional exhaustion of CD8 T cells (178, 179) as well as a marker of CD8 T cells that have recently been exposed to antigenic stimulation (178). PD-1 is markedly upregulated on the surface of dysfunctional virus-specific CD8 T cells during chronic HIV and SIV infections (217, 218), and blockade of PD-1 *in vivo* enhanced SIV-specific CD8 T cells responses (219). Moreover, recent studies found that high percentages of follicular CD8 T cells in chronic HIV and SIV infection express inhibitory molecule PD-1 (236, 237). However, the degree to which follicular SIV-specific CD8 T cells in early infection express PD-1 has not yet been investigated.

To understand this, we stained lymph node tissue sections from SIV infected rhesus macaques with MHC-class I tetramers, antibodies directed against PD-1, and antibodies directed against CD20 to label B cell follicles. We found PD-1+ Tet+ cells in both follicular and exteafollicular areas in early SIV infection (Fig. 2A). To our surprise, quantitative analysis showed that more than 50% of both follicular and extrafollicular Tet+ cells express PD-1 in all animals except one (Rh2588). No significant difference was observed between the percentage of PD-1+ Tet+ cells inside and outside B cell follicles (Fig. 2B). We further compared the percentage of Tet+ cells that express PD-1 between early and chronic SIV infection in follicular and extrafollicular regions respectively. Again, no significant differences were observed (Fig. 2C and 2D). These data indicate that, even though around half of follicular SIV-specific CD8 T cells

are PD-1+, a subset of follicular SIV-specific CD8 T cells evade the inhibitory effect of PD-1 in early SIV infection.

Foxp3+ cells likely inhibit follicular SIV-specific CD8 T cell function in early infection

Tregs play a crucial role in maintaining immunological self-tolerance and controlling autoimmune diseases (64, 65). However, they also get involved in suppressing immune activation in viral infection (66, 67). A large proportion of Tregs is characterized by the expression of the transcription factor Foxp3 (220, 221, 239). While most Tregs are CD4+, there exist a small population of CD8+ Tregs (222-224). Directed contact is an important mechanism mediates suppression of Tregs on CD8 T cells (225).

We next investigated whether Foxp3+ cells were in contact with and potentially inhibiting function of follicular SIV-specific CD8 T cells in early infection. We stained lymph node tissue sections from rhesus macaques in early stage of SIV infection with MHC-class I tetramers to label SIV-specific CD8 T cells, anti-Foxp3 antibodies to label Foxp3+ Tregs, and anti-IgM antibodies to label B cell follicles. We found follicular Tet+ cells that directly contact Foxp3+ cells (Fig. 3A) and follicular Tet+ cells that are Foxp3+ (Fig. 3B). An average of 12.4% (range 7-20%) follicular Tet+ cells were in direct contact with Foxp3+ cells (Fig. 3C) and the corresponding number in extrafollicular was 18.6% (9-26%). No significant difference was shown between the percentage of Tet+ cells that contact Foxp3+ cells inside and outside B cell follicles in early SIV infection (Fig. 3C). Simultaneously, the percentages of Foxp3+ Tet+ cells inside and outside B cell follicles showed no significant difference either (Fig. 3D). Therefore, these data suggest that function of a small population of follicular as well as extrafollicular SIV-specific CD8 T cells are likely inhibited by Foxp3+ cells in early SIV infection.

Similar to SIV-specific CD8 T cells, Foxp3+ cells levels were also significantly lower in follicular than extrafollicular regions (Fig. 3E and 3F) ($p = 0.008$). In addition, there was no significant difference between the ratios of Tet+ cells: Foxp3+ cells in follicular and extrafollicular regions (data not shown). These findings suggest that contact mediated suppression of Foxp3+ Tregs on SIV-specific CD8 T cells are similar in follicular and extrafollicular regions in early SIV infection.

We then evaluated whether the effects of Foxp3+ Tregs on SIV-specific CD8 T cells in early and chronic infection are different. We first found significantly higher level of Foxp3+ cells in early infection than chronic infection in follicular area (Fig. 4A) ($p = 0.0382$), but not in extrafollicular (Fig. 4B) ($p = 0.1007$). Second, the percentage of follicular Tet+ cells that contact Foxp3+ cells in early infection was significantly higher than chronic infection (Fig. 4C) ($p = 0.0194$), but the this difference was not observed in extrafollicular SIV-specific CD8 T cells (Fig. 4D) ($p = 0.4999$). Third, the percentage of Tet+ cells in follicular area that express Foxp3 tended to be higher in early infection (Fig. 4E) ($p = 0.0508$) and the same percentage in extrafollicular area was significantly higher in early infection (Fig. 4F) ($p = 0.0468$) than chronic infection. Last, the ratios of Tet+ cells: Foxp3+ cells in both follicular and extrafollicular regions were dramatically lower in early infection compared to chronic infection (Fig. 4G and 4H) ($p = 0.0419$ and $p = 0.0197$). Taken together, these data suggest that the suppressive effect of Foxp3+ Tregs on follicular SIV-specific CD8 T cells is stronger in acute infection compared to chronic infection. Similar but milder difference in effect of Foxp3+ Tregs on extrafollicular SIV-specific CD8 T cells in different phase of infection was observed as well.

Activated proliferating follicular SIV-specific CD8 T cells are found in early infection

Ki67 is an activation and proliferation marker of T cells (226, 227). We next assessed the level of follicular SIV-specific CD8 T cells express Ki67 in lymph nodes in early SIV infection. Ki67+ Tet+ cells were found in follicular as well as in extrafollicular regions (Fig. 5A). Quantitative analysis showed that the percentage of follicular Ki67+ Tet+ cells was significantly lower than their extrafollicular counterparts (Fig. 5B) ($p = 0.0007$). An average of 39.7% (range 7-61%) of Tet+ cells were Ki67+ in follicular area and this percentage was 54.4% (range 19-76%) in extrafollicular areas. By comparing the level of Ki67+ Tet+ cells among all Tet+ cells in early and chronic infection, we found that significantly more Tet+ cells in both follicular and extrafollicular regions from early SIV infection express Ki67 (Fig. 5C and 5D) ($p = 0.002$ and $p = 0.0004$). These data demonstrate that significantly lower level of follicular SIV-specific CD8 T cells are activated and proliferating than their extrafollicular counterparts in early SIV infection. Besides, SIV-specific CD8 T cells in early infection generally proliferate more vigorously than SIV-specific CD8 T cells during chronic infection.

A large proportion of follicular SIV-specific CD8 T cells express perforin in early infection

Perforin is a crucial factor for cytolytic function in virus-specific CD8 T cells. We previously showed that approximately 35% of follicular SIV-specific CD8+ T cells express perforin and that most expressed another cytolytic effector molecule, granzyme B, which typically works in concert with perforin to lyse infected cells (73).

We next characterized the expression of perforin in SIV-specific CD8 T cells in early infection. SIV-specific CD8 T cells were divided into four categories (negative, low, medium, and high) according to perforin expression levels (Fig. 6A). We found that a wide range of follicular Tet+ cells were perforin negative (mean 26.7%; rang 6-65%) (Fig. 6B). There was no significant difference between the percentage of perfroin- Tet+ cells in follicular and

extrafollicular regions (Fig. 6B). Among follicular perforin+ Tet+ cells, a large fraction expressed low to medium levels of perforin. At the same time, a population of perforin+ Tet+ cells express high levels of perforin (mean 21.5%; range 0-44%), consistent with being effector T cells. No significant differences were observed between the percentages of follicular and extrafollicular Tet+ cells that expressed each level of perforin (Fig. 6B). By comparison of perforin expression between Tet+ cells in early and chronic infection, we found that proportion of follicular perforin+ Tet+ cells in early SIV infection was significantly higher than chronic infection (Fig. 6C) ($p = 0.0206$). While the percentage of extrafollicular perforin+ Tet+ cells in early SIV infection tended to be higher than chronic infection (Fig. 6D) ($p = 0.0502$). Furthermore, there were significantly more follicular and extrafollicular perforin^{high} Tet+ cells in early SIV infection than chronic infection (Fig. 6E and 6F) ($p = 0.0242$ and $p = 0.0135$). Taken together, these data showed that similar levels of follicular and extrafollicular SIV-specific CD8 T cells express perforin in early infection. The subset of SIV-specific CD8 T cells in early infection express high level of perforin may be effector CD8 T cells can immediately kill virus-infected cells.

Cell death is not a major factor accounts for the low levels of follicular SIV-specific CD8 T cells

Poly (ADP-ribose) polymerase (PARP) is involved in cells death by promoting release of apoptosis-inducing factor (AIF) (240). Here we assessed whether SIV-specific CD8 T cells express PARP in early chronic SIV infection (50-60 dpi) to test whether the cell death is responsible for the low frequency of follicular SIV-specific CD8 T cells. We found a small population of Tet+ cells were themselves PARP+ (Fig. 7A). Quantitative analysis showed no significant difference between the levels of PARP+ Tet+ cells inside and outside B cell follicles (Fig. 7B). Interestingly, we found some Tet+ cells that directly contact PARP+ cells (Fig. 7A). Quantitative analysis showed that the percentage of Tet+ cells that contact PARP+ cells in

follicular area was significantly higher than extrafollicular area (Fig. 7C) ($p = 0.0043$). These results suggest that even though a small number of follicular SIV-specific CD8 T cells are likely undergoing apoptosis, cell death is not a major factor accounts for the low levels of follicular SIV-specific CD8 T cells.

Discussion

Eradication of HIV-infected cells *in vivo* remains a critical obstacle to cure HIV infection. B cell follicles are major anatomical reservoirs that permit active HIV and SIV replication during chronic infection (73, 74, 78). However, virus-specific CD8 T cells fail to accumulate in B cell follicles in high frequency (73, 74, 236, 237). The ongoing viral replication in B cell follicles during chronic infection is due, at least partially, to the paucity of follicular anti-viral CD8 T cell responses (73-75, 78, 163). It is not known whether this phenomenon also occurs during early stages of infection. Here, we determined the location, abundance, and phenotype of SIV-specific CD8 T cells in follicular and extrafollicular regions during early SIV infection. We found that a small population of SIV-specific CD8 T cells accumulate in B cell follicles during early SIV infection, similar to what we reported in chronic disease (73). Subsets of these cells are activated and proliferating, and likely have cytolytic potential. Moreover, our data suggest that a population of follicular SIV-specific CD8 T cells may be actively killing virus infected cells exist in B cell follicles in early chronic SIV infection.

Previous studies demonstrated that high magnitude of anti-viral CD8 T cell responses in early HIV and SIV infection contribute to initial viral control (147, 148, 169, 170, 234). But these cells fail to completely suppress viral replication in early infection. Here we found that frequency of SIV-specific CD8 T cells located inside B cell follicles was significantly lower than outside B cell follicles in early SIV infection. Furthermore, follicular SIV-specific CD8 T cells are largely excluded from GCs. The relatively low levels of follicular SIV-specific CD8 T cells and its uneven distribution in follicles may, at least in part, account for the inability to fully control viral replication despite strong virus-specific CD8 T cells responses elicited in primary infection and set the stage for persistent chronic infection.

In addition to relatively low levels of follicular SIV-specific CD8 T cells during early infection, other negative regulators likely influence the functionality of these cells. We found

some follicular SIV-specific CD8 T cells are PD-1 positive and likely struggling with exhaustion. However, multiple studies showed that follicular CD8 T cells demonstrate strong cytotoxic capacity despite the expression of inhibitory receptors such as PD-1 (174, 237). Besides, we found a small subset of these cells directly contacted with Foxp3+ cells, or were themselves Foxp3+, suggesting a potential inhibitory effect from Tergs. We also found a small fraction of follicular SIV-specific CD8 T cells in early chronic infection were PARP+, suggesting they were undergoing apoptosis.

The presence of follicular virus-specific CD8 T cells with strong ability to control viral replication were identified during chronic LCMV, HIV and SIV infections (73, 174, 229, 236-238). In particular, a perforin- and granzyme B-dependent mechanism was suggested as the dominant killing mechanism of follicular CD8 T cells in chronic HIV and SIV infection (237, 238). In line with this data, we found a large fraction of follicular SIV-specific CD8 T cells during early infection exhibit cytolytic potential characterized by perforin expression. In particular, many of follicular SIV-specific CD8 T cells expressed high levels of perforin, consistent with being effector T cells that are able to kill virus-infected cells immediately after recognition. Perforin expression level during early SIV infection was significantly higher than chronic infection. Moreover, large proportions of follicular SIV-specific CD8 T cells expressed Ki67 in early infection which was also significantly higher than chronic SIV infection, suggesting they were activated and proliferating. A recent study demonstrated that frequency of CD8 T cells express high levels of Ki67 during acute HIV infection was inversely correlated with plasma viral load set point, suggesting CD8 T cells proliferation elicited in acute HIV infection is associated with ensuing viral control (234). During early chronic infection, we found a fraction of SIV-specific CD8 T cells that contact PARP+ cells, suggesting these cells are actively killing virus-infected cells. Taken together, our data suggested that follicular SIV-specific CD8 T cells have

vigorous cytolytic potential to eliminate SIV-infected cells in B cell follicles during early infection.

Simultaneously, we found a significantly higher fraction of extrafollicular SIV-specific CD8 T cells express Ki67 than their follicular counterparts during early infection. In addition to lack of CXCR5 expression which is necessary for homing to B cell follicles, this may be another factor contributes to the low level of follicular SIV-specific CD8 T cells. Moreover, we found similar level of extrafollicular SIV-specific CD8 T cells express perforin as their counterparts in B cell follicles. In consideration of higher frequency, extrafollicular SIV-specific CD8 T cells in early infection likely have strong potential in controlling viral replication as well.

Rapid and high magnitude of anti-viral CD8 T cell responses are critical for immune control of acute HIV infection (234). The strategy to induce augmented frequency of functional follicular virus-specific CD8 T cells may further enhance viral control. We found a significant positive correlation between follicular and extrafollicular Tet+ cells during early SIV infection, suggesting that induction of increased level of total SIV-specific CD8 T cells via vaccination may also generate more follicular SIV-specific CD8 T cells during early infection. IL-15 is a cytokine that facilitates CD8 T cells expansion, but has no preferential effect on the proliferation of Tregs (190, 230). Blockade of PD-1/PD-L1 *in vitro* and in vivo recovered the function of exhausted CD8 T cells (178, 219, 237) and synergistically strengthened the viral control mediated by adoptive transfer of CXCR5+CD8+ T cells in LCMV infected mice (174). Given the high level of PD-1 expression in follicular SIV-specific CD8 T cells during early infection, blocking PD-1/PD-L1 interaction may further assist vaccines or therapies in reducing viral replication. Moreover, IL-27 impedes CD4+CD25+Foxp3+ Tregs expansion and promotes activity of tumor-specific CD8 T cells (231). Therefore, vaccination accompanied by therapeutic administration of IL-15 and IL-27 might result in relatively lower suppressive effect of Tregs and enhanced anti-viral CD8 T cell responses. A recent study demonstrated that the presence of a bi-specific antibody promotes the

cytolytic activity of follicular CD8 T cells to kill HIV infected cells (237). This justifies the potential of a bi-specific antibody in facilitating the elimination of HIV-infected cells. Another study showed that a significant fraction of newly differentiated effector CD8 T cells undergo apoptosis in spite of ongoing viral replication in acute HIV infection (234). Therefore, induction of more follicular CD8 T cells with long-term memory phenotype is necessary to generate long-lasting viral suppression.

In summary, our data suggest that despite high levels of exhaustion and likely inhibition by Foxp3+ cells, the scarce follicular SIV-specific CD8 T cells during early infection are likely possess cytolytic potential and contribute to suppression of viral replication. Importantly, we found follicular SIV-specific CD8 T cells are largely excluded from GCs during early infection. This low levels of follicular SIV-specific CD8 T cells in GCs may set the stage for subsequent persistent chronic infection. These findings support the strategy that induction of a large magnitude of follicular virus-specific CD8 T cells in early HIV and SIV infection via vaccination can contribute to the elimination of virus infected cells, and thus have implications for future strategies of HIV vaccines.

TABLE 1 Rhesus macaques included in studies

ID number	DPI	MHC-genotype[a] Peptide	Virus	Plasma SIV RNA (log_{10} Copies/ml)	Route[b]	Ki67	Perf	Foxp3	PD-1
Rh2515	21	A01 Gag-CM9	SIVmac239	5.94	IV[c]	+	+	+	-[d]
Rh2516	21	A01 Gag-CM9	SIVmac239	6.61	IV	+	+	+	+
Rh2520	21	A01 Gag-CM9	SIVmac239	7.08	IV	+	+	+	+
Rh2578	21	A01 Gag-CM9	SIVmac239	6.87	IV	+	+	-	-
Rh2579	21	A01 Gag-CM9	SIVmac239	7.13	IV	+	+	+	-
Rh2583	21	A01 Gag-CM9	SIVmac239	7.00	IV	+	+	+	-
Rh2584	21	A01 Gag-CM9	SIVmac239	6.78	IV	+	+	+	+
Rh2587	21	A01 Gag-CM9	SIVmac239	6.75	IV	+	+	-	+
Rh2588	21	A01 Gag-CM9	SIVmac239	6.43	IV	-	-	-	+

[a] Full MHC allele name is A01 is *Mamu-A1*001:01*.

[b] Route of SIV infection.

[c] Rhesus macaques were infected with SIVmac 239 intravenously.

[d] There were not enough follicles in stained tissue sections for quantitative image analysis.

DPI: Days Post-Infection.

Figure 1

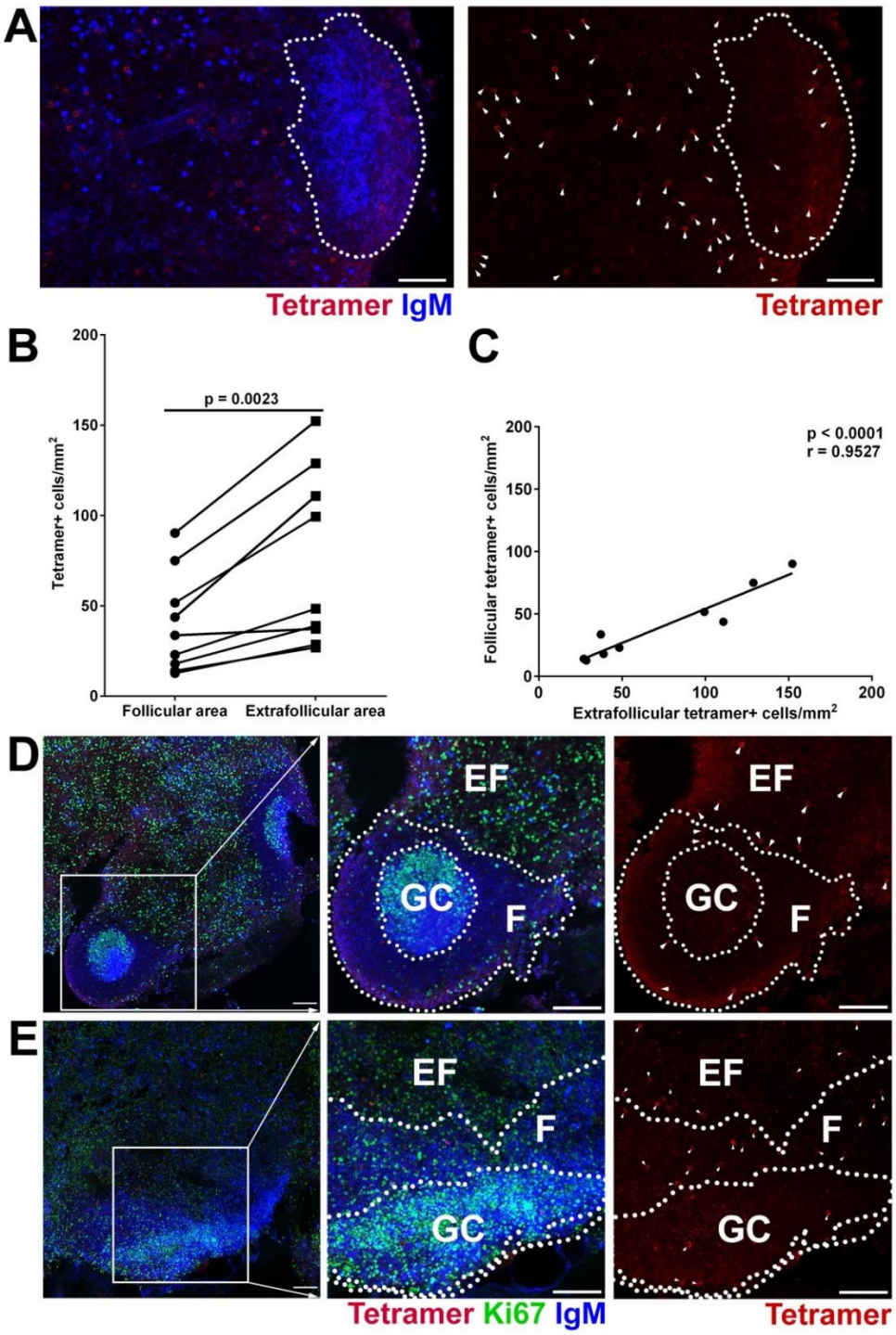
A
Tetramer IgM
Tetramer
B
p = 0.0023
Tetramer+ cells/mm²
200
150
100
50
0
Follicular area
Extrafollicular area
C
p < 0.0001
r = 0.9527
Follicular tetramer+ cells/mm²
Extrafollicular tetramer+ cells/mm²
0
50
100
150
200
D
EF
GC
F
E
Tetramer Ki67 IgM
Tetramer

FIG 1 SIV-specific CD8 T cells are largely excluded from B cell follicles in early SIV infection. (**A**) Representative lymph node section shows the distribution of SIV-specific CD8 T cells in different compartments of lymph node. This section was stained with Mamu-A1*001/Gag CM9 tetramers to label SIV-specific CD8 T cells (red, and indicted with arrows in the image on the right which shows tetramer staining alone), IgM antibodies (blue) to define follicles (F). Confocal images were collected with a 20X objective and each scale bar indicates 100 μm. (**B**) Frequencies of tetramer+ SIV-specific CD8 T cells in different compartments of lymph nodes during early SIV infection. Frequencies of tetramer+ SIV-specific CD8 T cells in B cell follicle were significantly lower ($p = 0.0023$) than those in extrafollicular region. (**C**) Relationship between frequencies of follicular and extrafollicular tetramer+ SIV-specific CD8 T cells. The frequency of follicular tetramer+ SIV-specific CD8 T cells is significantly correlated with those located in extrafollciular area ($p < 0.0001$). Representative images demonstrate the distribution of SIV-specific CD8 T cells within B cell follicle during early SIV infection (**D** and **E**). Sections were stained with Mamu-A1*001/Gag CM9 tetramers to label SIV-specific CD8 T cells (red, and indicted with arrows in the image on the right which shows tetramer staining alone), IgM antibodies (blue) to define follicles (F), and Ki67 antibodies (green) to label GC. Confocal images were collected with a 20X objective and each scale bar indicates 100 μm. We observed that 60.3% (44/73) of GCs were totally devoid of tetramer+ SIV-specific CD8 T cells (**D**). Very few of tetramer+ SIV-specific CD8 T cells were found in the rest of GCs (**E**).

Figure 2

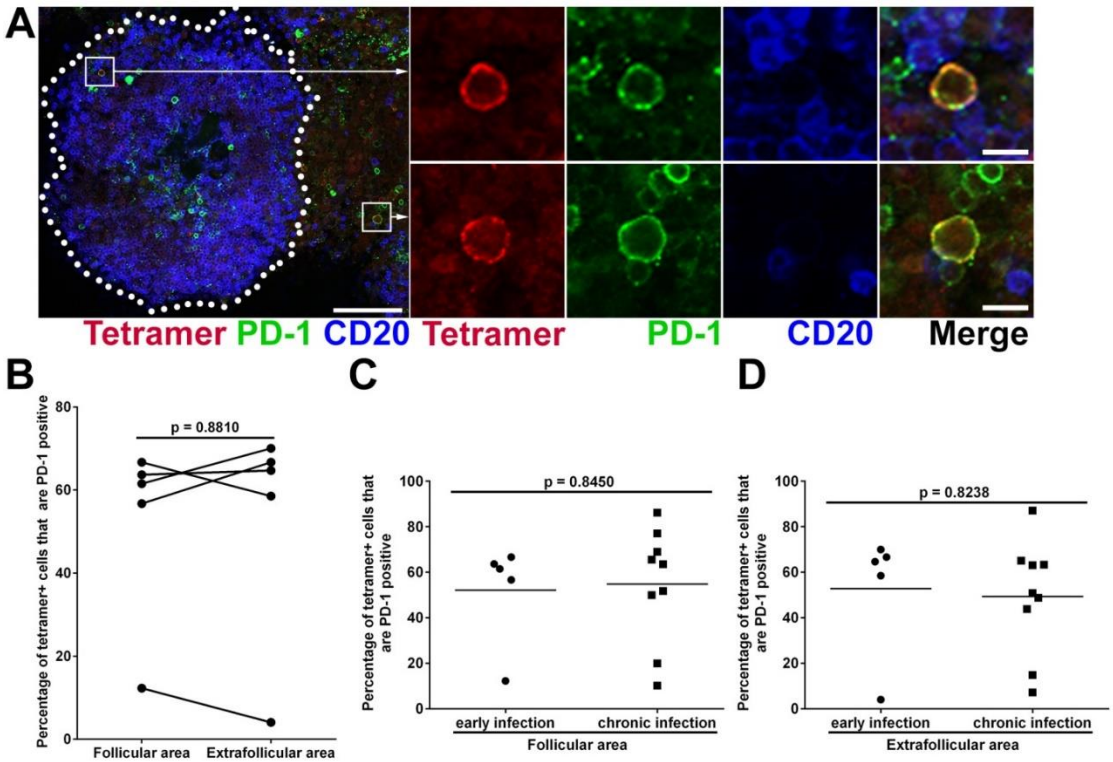

FIG 2 Many follicular tetramer+ SIV-specific CD8 T cells express PD-1 during early SIV infection. (**A**) Representative lymph node section shows tetramer+ SIV-specific CD8 T cells express PD-1 inside and outside B cell follicle. This section was stained with Mamu-A*001:01/Gag CM9 tetramers to label SIV-specific CD8+ T cells (red), PD-1 antibodies (green) to label PD-1 expressing cells and CD20 antibodies (blue) to define follicles. Confocal images were collected with a 20X objective and the scale bar is 100 µm in the image on the left and 10 µm in the enlargement. (**B**) There is no significant difference between the percentages of PD-1+ cells within the tetramer-binding population locate in follicular and extrafollicular regions. There is no significant difference between percentages of tetramer+ SIV-specific CD8 T cells that are PD-1+ during early and chronic SIV infection in both follicular ($p = 0.8450$) (**C**) and extrafollicular area ($p = 0.8238$) (**D**).

Figure 3

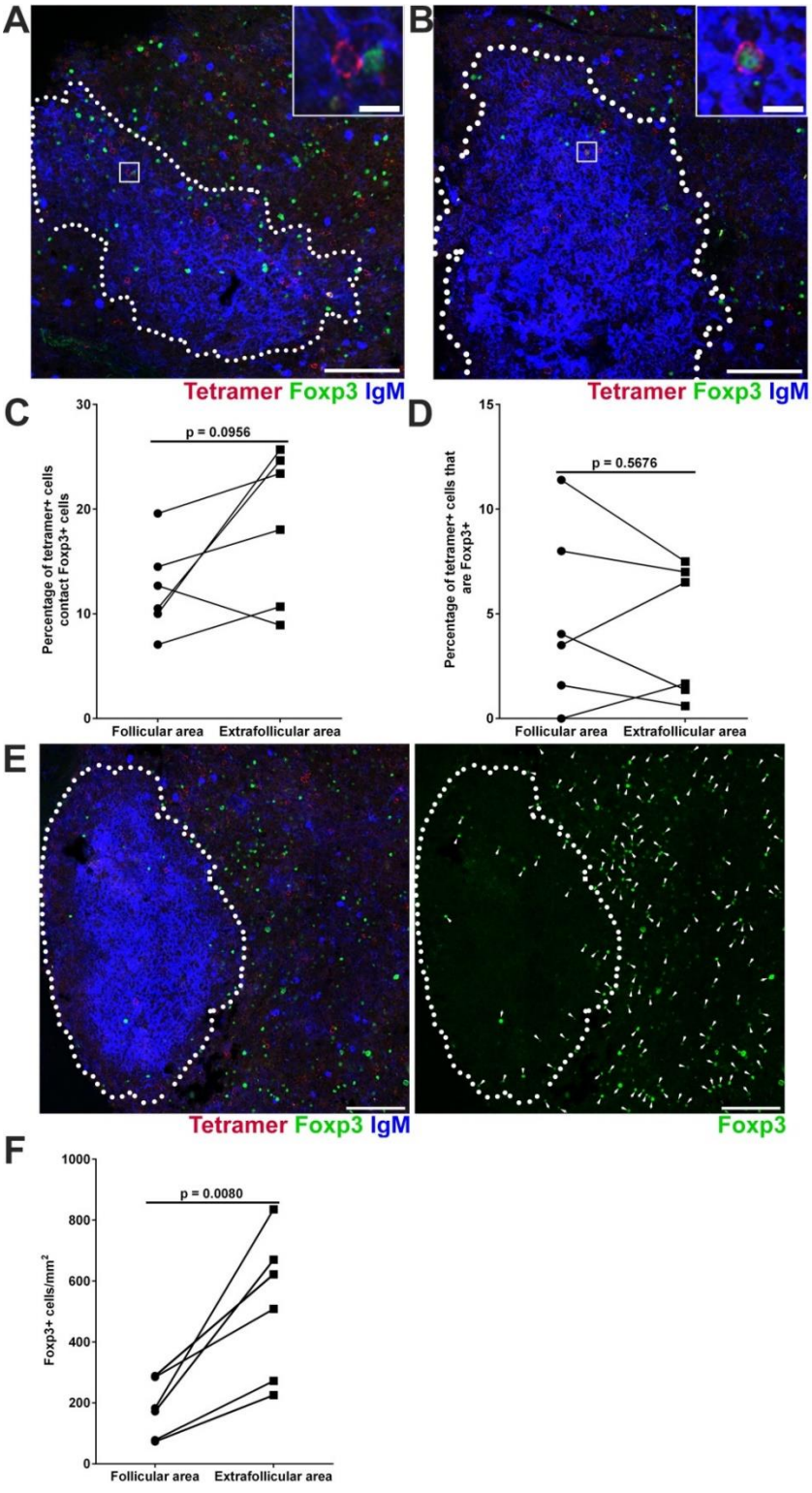
A
B
Tetramer Foxp3 IgM
Tetramer Foxp3 IgM
C
p = 0.0956
Percentage of tetramer+ cells contact Foxp3+ cells
30
20
10
0
Follicular area
Extrafollicular area
D
p = 0.5676
Percentage of tetramer+ cells that are Foxp3+
15
10
5
0
Follicular area
Extrafollicular area
E
Tetramer Foxp3 IgM
Foxp3
F
p = 0.0080
Foxp3+ cells/mm²
1000
800
600
400
200
0
Follicular area
Extrafollicular area

FIG 3 A subset of follicular tetramer+ SIV-specific CD8 T cells are likely inhibited by Foxp3+ cells during early SIV infection. (**A**) Representative lymph node section stained with Mamu-A*001:01/Gag CM9 tetramers (red), IgM (blue), and Foxp3 (green) showing tetramer+ cells in contact with Foxp3+ cells. Confocal images were collected with a 20X objective and the scale bar is 100 µm and 10 µm in low- and high-magnification images respectively. (**B**) Representative image showing tetramer+ cell is Foxp3+. (**C**) Percentages of follicular tetramer+ SIV-specific CD8 T cells that were in direct contact with Foxp3+ cells tend to be lower than extrafollicular tetramer+ SIV-specific CD8 T cells ($p = 0.0956$). **(D**) There was no significant difference between percentages of tetramer+ SIV-specific CD8+ T cells inside and outside follicle that were Foxp3+ ($p = 0.5676$). (**E**) Representative image showing distribution of Foxp3+ cells in lymph node. Scale bar is 100 µm. (**F**) Frequencies of Foxp3+ cells inside follicles were significantly lower than outside of follicle ($p = 0.0080$)

Figure 4

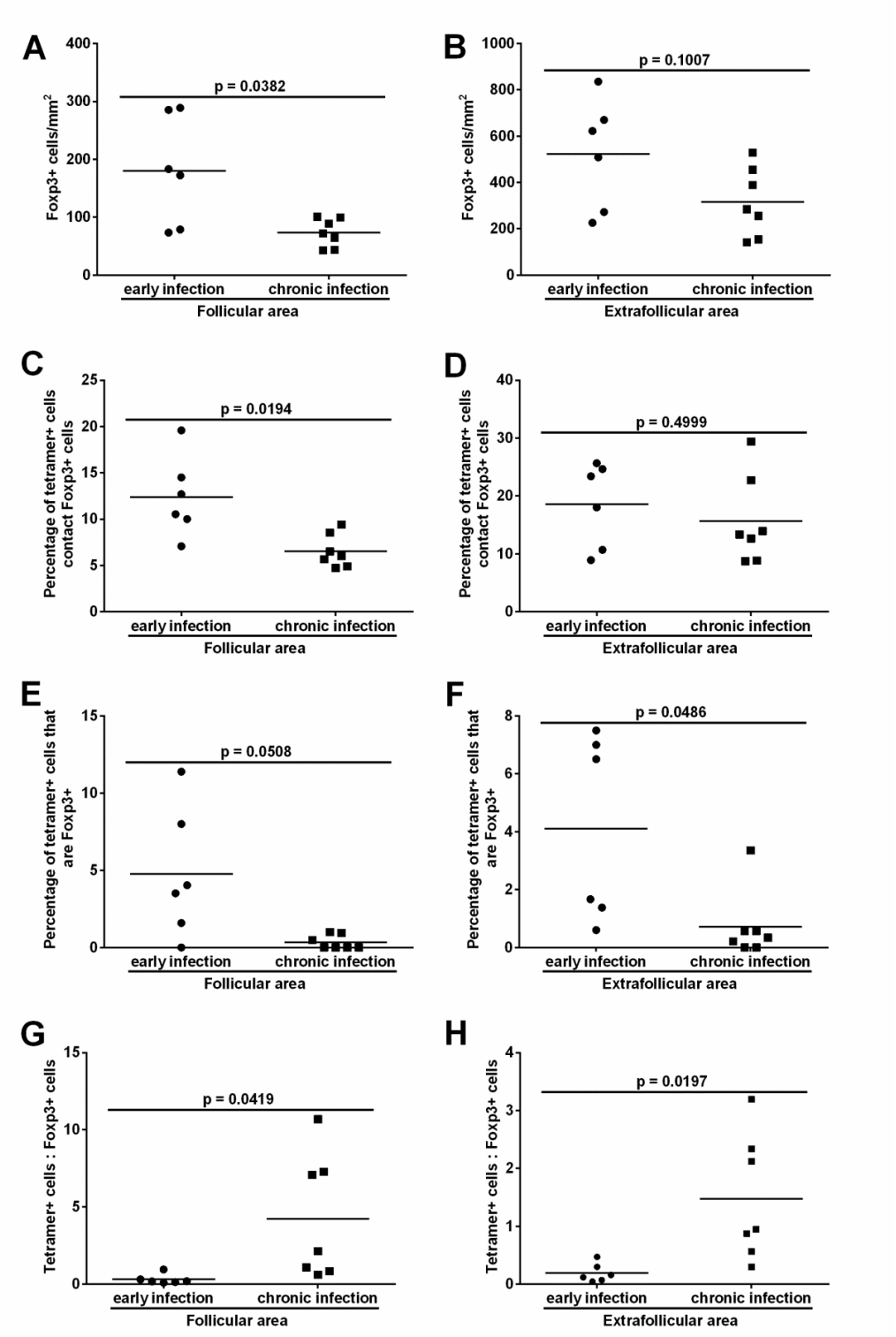
A
Foxp3+ cells/mm2
p = 0.0382
early infection
chronic infection
Follicular area
B
Foxp3+ cells/mm2
p = 0.1007
early infection
chronic infection
Extrafollicular area
C
Percentage of tetramer+ cells contact Foxp3+ cells
p = 0.0194
early infection
chronic infection
Follicular area
D
Percentage of tetramer+ cells contact Foxp3+ cells
p = 0.4999
early infection
chronic infection
Extrafollicular area
E
Percentage of tetramer+ cells that are Foxp3+
p = 0.0508
early infection
chronic infection
Follicular area
F
Percentage of tetramer+ cells that are Foxp3+
p = 0.0486
early infection
chronic infection
Extrafollicular area
G
Tetramer+ cells : Foxp3+ cells
p = 0.0419
early infection
chronic infection
Follicular area
H
Tetramer+ cells : Foxp3+ cells
p = 0.0197
early infection
chronic infection
Extrafollicular area

FIG 4 Foxp3+ Tregs may have a stronger suppressive effect on follicular SIV-specific CD8 T cells during early SIV infection. There was significantly higher frequency of Foxp3+ cells during early SIV infection than chronic SIV infection in follicular area ($p = 0.0382$) (**A**), but not in extrafollicular area ($p = 0.1007$) (**B**). Significantly higher percentage of tetramer+ SIV-specific CD8 T cells during early SIV infection contact Foxp3+ cells than those during chronic SIV infection in follicular area ($p = 0.0194$) (**C**), but not in extrafollicualr area ($p = 0.4999$) (**D**). (**E**) Percentage of follicular tetramer+ SIV-specific CD8 T cells contact Foxp3+ cells during early SIV infection tend to be higher than chronic infection ($p = 0.0508$). (**F**) At the same time, the percentage of extrafollicular tetramer+ SIV-specific CD8 T cells contact Foxp3+ cells during early SIV infection is significnatly higher than chronic infection ($p = 0.0486$). Ratios of tetramer+ SIV-specific CD8 T cells to Foxp3+ cells during early SIV infection were significantly lower than chronic SIV infection in both follicular ($p = 0.0419$) (**G**) and extrafollicular area ($p = 0.0197$) (**H**).

Figure 5

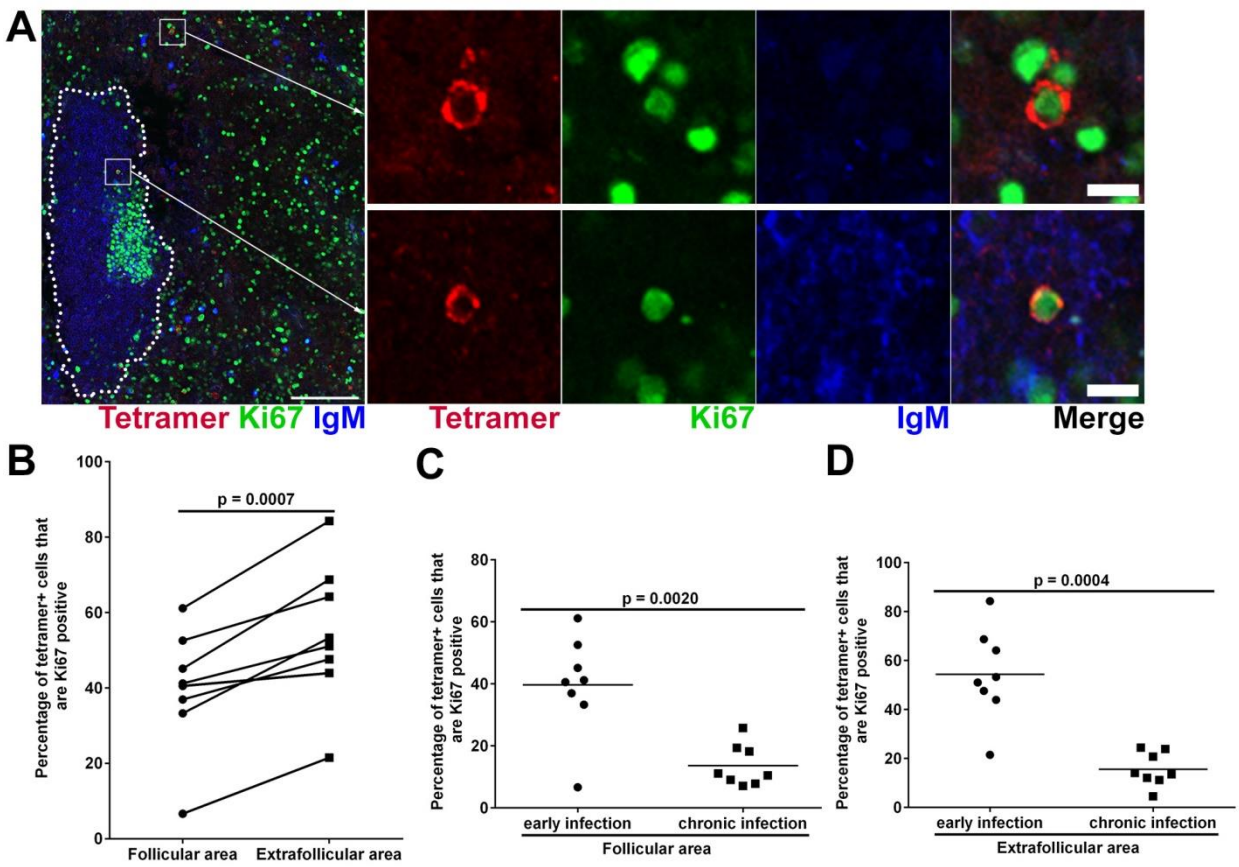

FIG 5 Ki67 expression levels in follicular and extrafollicular tetramer+ SIV-specific CD8 T cells during early SIV infection. Representative lymph node section stained with Mamu-A*001:01/Gag CM9 tetramers (red), IgM (blue), and Ki67 (green) showing tetramer+ Ki67+ cells in follicular and extrafollicular region (**A**). Scale bars indicate 100 µm and 10 µm in low- and high-magnification images respectively. (**B**) Percentages of tetramer+ SIV-specific CD8 T cells that expressed Ki67 in follicular areas is significantly lower than extrafollicular areas in lymph nodes during early SIV infection ($p = 0.0007$). Percentages of tetramer+ SIV-specific CD8 T cells that are Ki67+ during early SIV infection were significantly higher than chronic SIV infection in both follicular ($p = 0.0020$) (**C**) and extrafollicular area ($p = 0.0004$) (**D**).

Figure 6

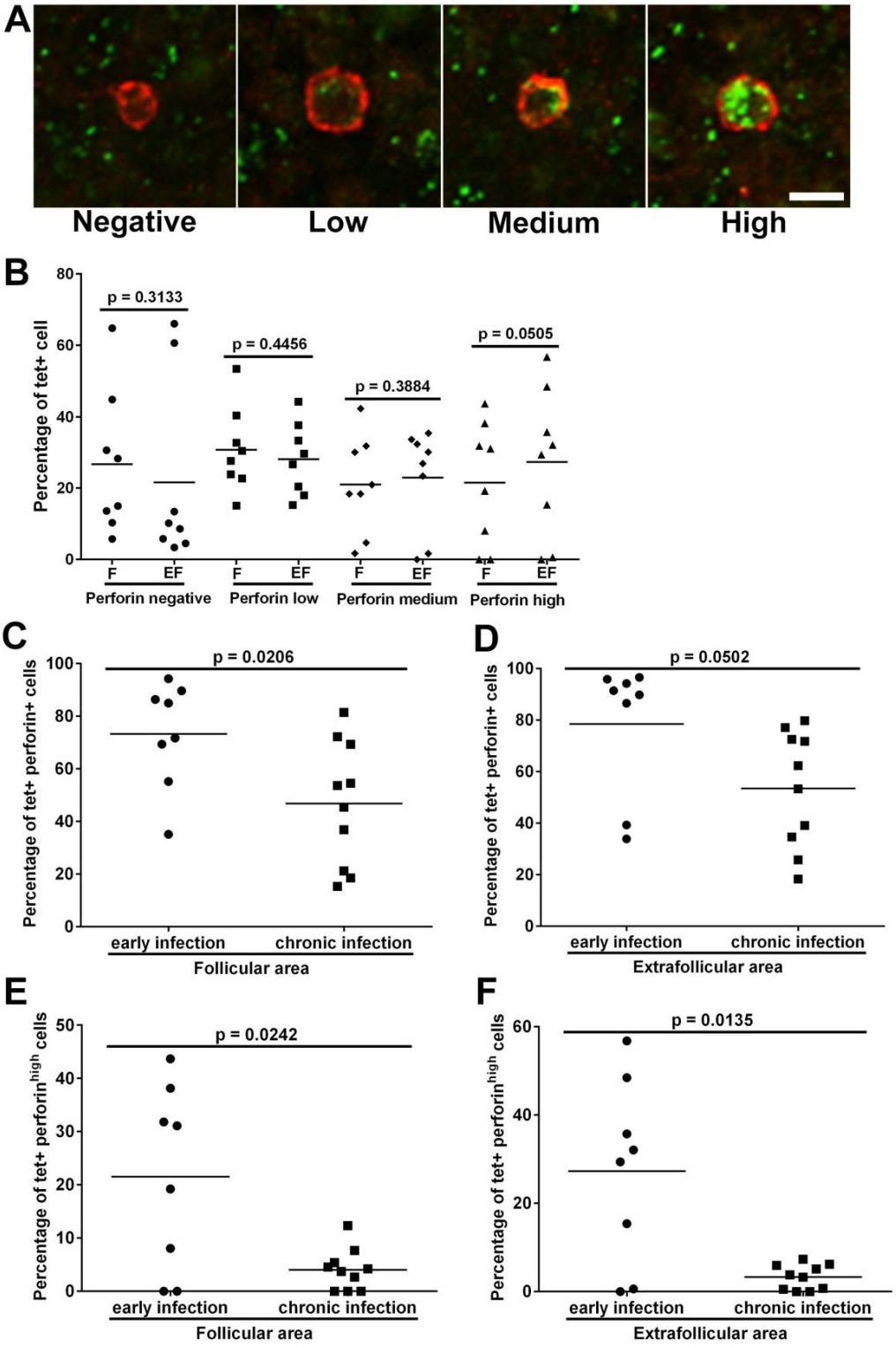
A
Negative
Low
Medium
High
B
Percentage of tet+ cell
p = 0.3133
p = 0.4456
p = 0.3884
p = 0.0505
F
EF
Perforin negative
Perforin low
Perforin medium
Perforin high
C
Percentage of tet+ perforin+ cells
p = 0.0206
early infection
chronic infection
Follicular area
D
Percentage of tet+ perforin+ cells
p = 0.0502
early infection
chronic infection
Extrafollicular area
E
Percentage of tet+ perforin^high cells
p = 0.0242
early infection
chronic infection
Follicular area
F
Percentage of tet+ perforin^high cells
p = 0.0135
early infection
chronic infection
Extrafollicular area

FIG 6 There are significantly more tetramer+ SIV-specific CD8 T cells express perforin during early SIV infection than during chronic SIV infection in follicular area. (**A**) Representative lymph node section stained with Mamu-A*001:01/Gag CM9 tetramers (red) and perforin (green) showing perforin negative, perforin low, perforin medium and perforin high MHC-class I tetramer+ SIV-specific CD8 T cells. Scale bar indicates 10 μm. (**B**) Percentages of tetramer+ SIV-specific CD8 T cells that expressed perforin in follicular and extrafollicular regions. Among tetramer+ SIV-specific CD8 T cells, the distribution of cells across perfroin negative, low, medium and high is not significantly different between follicular and extrafollicular regions. Significantly higher percentage of tetramer+ SIV-specific CD8 T cells during early SIV infection express perforin than those during chronic SIV infection in follicular area ($p = 0.0206$) (**C**), but not in extrafollicualr area ($p = 0.0502$) (**D**). Simultaneously, significantly higher percentages of tetramer+ SIV-specific CD8 T cells during early SIV infection express high level of perforin than those during chronic SIV infection in both follicular ($p = 0.0242$) (**E**), and extrafollicular regions ($p = 0.0135$) (**F**).

Figure 7

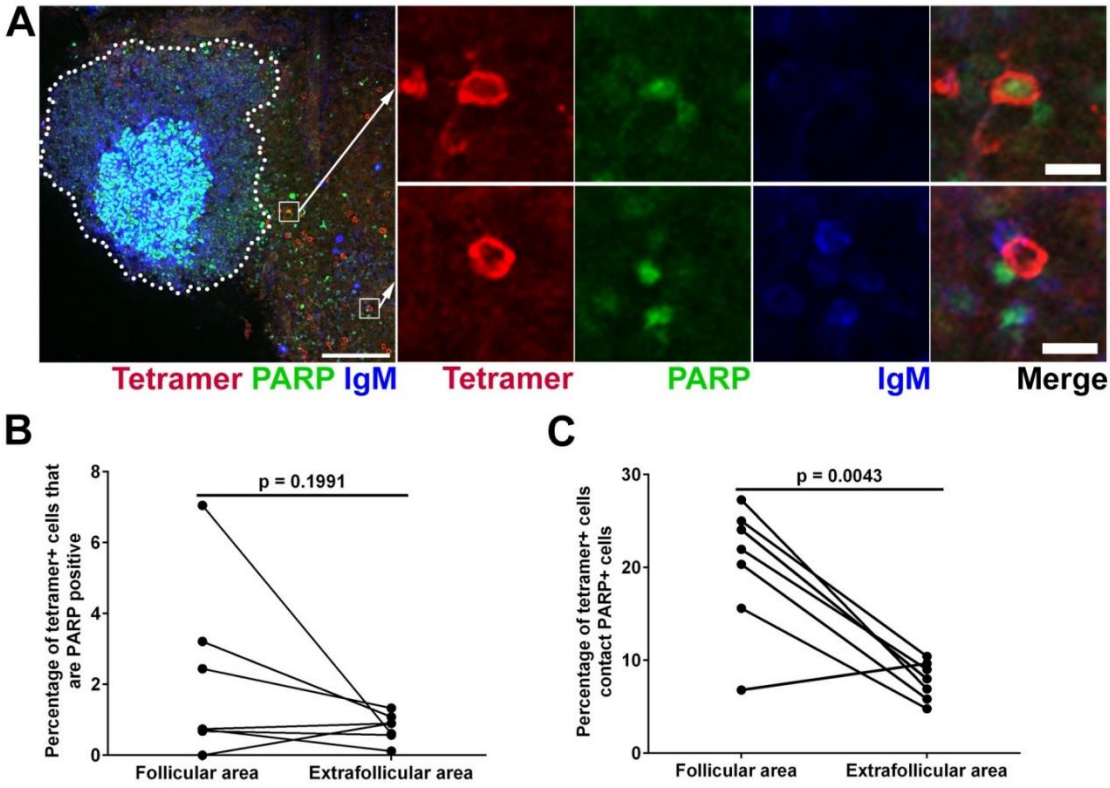

FIG 7 Cell death is not a major factor accounts for the low levels of follicular SIV-specific CD8 T cells. (**A**) Representative lymph node section stained with Mamu-A*001:01/Gag CM9 tetramers (red), IgM (blue), and PARP (green) showing tetramer+ PARP+ cells and tetramer+ cells in contact with PARP+ cells. Confocal images were collected with a 20X objective and the scale bar is 100 µm and 10 µm in low- and high-magnification images respectively. (**B**) The percentage of tetramer+ SIV-specific CD8 T cells that are PARP+ is not significantly different between follicular and extrafollicular regions (p = 0.1991). (**C**) However, percentages of follicular tetramer+ SIV-specific CD8 T cells that were in direct contact with PARP+ cells was significantly higher than extrafollicular tetramer+ SIV-specific CD8 T cells (p = 0.0043).

Chapter 4

The human IL-15 superagonist ALT-803 directs SIV-specific CD8 T cells into B cell follicles

Synopsis

During chronic asymptotic HIV and SIV infection, virus-producing cells are highly concentrated in B cell follicles in secondary lymphoid tissues. However, virus-specific CD8 T cells are largely excluded from this area. Therefore, limited access of virus-specific CD8 T cells to follicular virus-producing cells is an important obstacle to eradicate HIV. Here, by using SIV-infected rhesus macaques, we show that ALT-803, a novel human IL-15 superagonist and potent immunostimulatory molecule, drives dramatic expansion of SIV-specific CD8 T cells in blood and lymphoid tissues. Importantly, ALT-803 drives significant accumulation of SIV-specific CD8 T cells in B cell follicles, reducing the number of SIV-producing cells within B cell follicles. These data justify the further evaluation of ALT-803 for eradication of HIV-infected cells.

Introduction

While many studies demonstrated that virus-specific CD8+ T cells are crucial for viral control in HIV and SIV infection (73, 147, 148, 150, 151, 168-170, 203), these cells fail to fully control persistent viral replication. B cell follicles have been identified as major sites of productive HIV and SIV infection during asymptomatic chronic infection (73, 74, 78, 136). CD4+ follicular helper T cells (T_{FH}) within B cell follicles are significant reservoirs responsible for the persistent HIV/SIV infection (78, 136). However, virus-specific CD8 T cell typically fail to accumulate in large numbers in B cell follicles (73, 74, 79, 80, 236). The paucity of functional follicular virus-specific CD8 T cells is an important cause for the failure in controlling persistent HIV/SIV infection. Therefore, induction of high levels of follicular virus-specific CD8 T cells is a promising immunotherapeutic approach to reduce or eliminate virus-infected T_{FH} hide in B cell follicles.

Interleukin-15 (IL-15), a common γ-chain cytokine, is critical for mediating T cell and NK cell activation and proliferation. It has been previously explored as a candidate to decrease HIV reservoir size in patients on combined antiretroviral therapy (cART). Previous studies showed that IL-15 triggers NK cell expansion and preferentially stimulates proliferation of CD4+ and CD8+ effector memory T cells in non-human primate models for HIV infection (192, 193, 241). Besides, IL-15 has been demonstrated to effectively reactivate HIV replication in latently infected cells (242). Therefore, IL-15 is a good candidate for immunotherapy against HIV infection.

However, the short half-life and limited bioavailability of the soluble IL-15 molecule restrict its clinical application. Moreover, in contrast to other γ-chain cytokines such as IL-2 and IL-7 that circulate as soluble protein until they bind to their receptor directly on target immune cells, IL-15 needs to bind the IL-15 receptor alpha chain (IL-15Rα) first to be ready for subsequent presentation to target cells (194, 195). These limitations impede the use of free IL-15

in clinical immunotherapy. Development of the IL-15 superagonist, ALT-803, circumvents these limitations, allowing for a more comprehensive evaluation of IL-15 administration during HIV infection.

ALT-803 consists of a human IgG1 Fc fused onto two IL-15Rα units, each of which bounds to a novel IL-15 monomer mutant, IL-15$_{N72D}$, which possesses 5-fold higher activity than wild type IL-15 (198). Through these modifications, ALT-803 exhibits 25-fold higher biological activity and a 35-fold longer half-life in serum compared to free IL-15 (199) . Therefore, it results in potent stimulation of NK and memory T cells (199). ALT-803 is well tolerated in both mice and cynomolgus macaques at a dose of 100 μg/kg and no systemic cytokine storm was induced after ALT-803 administration (198). In consideration of the safety and promising results in cancer immunotherapy research, ALT-803 is being explored as a method to enhance anti-viral immune response during chronic HIV infection. For example, ALT-803 has been demonstrated to drive NK cells activation and suppress HIV replication during acute infection in a humanized mouse model of HIV infection (200). Moreover, ALT-803 was shown to markedly reverse HIV latency and strengthen the ability of CD8 T cells to kill HIV-producing cells in an *in vitro* cell culture model (201).

Here, we explored the *in vivo* effect of ALT-803 in the setting of established chronic viral infections using SIV-infected rhesus macaques. We showed that ALT-803 drove dramatic expansion of SIV-specific CD8 T cells within peripheral blood and lymph nodes. Moreover, in the presence of ALT-803, CD8+ T cells displayed a 50-fold increase in CXCR5 mRNA and a corresponding increase in surface expression of CXCR5. Importantly, we observed significant accumulation of SIV-specific CD8 T cells inside B cell follicle and more even distribution of these cells in the follicular and extrafollicular regions. These findings suggest that ALT-803 is a good candidate to promote progress in HIV eradication.

Materials and Methods

Reagents, animals, and veterinary procedures.

All rhesus macaques (*Macaca mulatta*) and Mauritian cynomolgus macaques (*Macaca fascicularis*) used in this study were housed at the Oregon National Primate Research Center (ONPRC) and utilized for studies under the approval of the Oregon Health and Science University (OHSU) Institutional Animal Care and Use Committee (IACUC). All macaques in this study were managed according to the ONPRC animal care program, which is fully accredited by AAALAC International and is based on the laws, regulations, and guidelines set forth by the United States Department of Agriculture (e.g., the Animal Welfare Act and its regulations, and the Animal Care Policy Manual), Institute for Laboratory Animal Research (e.g., Guide for the Care and Use of Laboratory Animals, 8th edition), and the Public Health Service Policy on Humane Care and Use of Laboratory Animals. The IL-15 superagonist ALT-803 was generated by Altor Biosciences as previously described[6]. All ALT-803 injections were given as intravenous bolus doses of either 6 μg/kg or 100 μg/kg. For BrdU injections, BrdU was suspended at 10 mg/ml in HBSS (HyClone Laboratories, Logan, UT, USA), and then injected intravenously in the saphenous vein at a rate of 2–3 ml/min for a total dose of 60 mg/kg of BrdU.

Blood and tissue processing.

Whole blood was collected into EDTA-treated tubes (BD Biosciences, San Jose, Ca, USA). Blood was assessed for complete blood counts using an ABX Pentra 60 C+ (Horiba, Irvine, CA, USA). Lymph node and spleen were diced with scalpels and then forced through a 70-μm cell strainer. The strainer was rinsed repeatedly with R10 to obtain a single-cell suspension. Immune cell phenotyping was conducted on whole blood samples that were washed twice in 1X PBS and then surface-stained for 30 minutes at room temperature. Samples were then

incubated in 1 ml FACSLyse for 10 minutes, spun at 830 *g* for 4 minutes, and washed three times in 1X PBS, supplemented with 10% FCS (FACS buffer). For Ki67 assessment, fixed cells were washed twice in 1 mg/ml saponin (saponin buffer) and stained overnight at 4°C. For BrdU assessment, fixed cells were washed twice in 1:1 mixture of saponin buffer and 2X BD FACSPerm, then washed once in saponin buffer, and stained for 1 hour at room temperature in the presence of 0.5 mg/ml DNase I. Following staining, samples were washed twice in saponin buffer and then run on an LSR II (Becton Dickinson, Franklin Lakes, NJ, USA). Flow cytometric data were analyzed using FlowJo, version 10 (TreeStar Ashland, OR, USA).

Quantitative real-time reverse transcriptase PCR.

PBMC were sorted for CD8β+ T cells via magnetic sorting using a PE-conjugated anti-CD8β antibody (clone 2ST8.5H7, Beckman Coulter) and anti-PE MicroBeads (Miltenyi Biotec). Purified cells were resuspended in R10 and incubated with or without 10nM ALT803. Cells were subsequently collected at days 1, 3, and 5 and RNA was extracted using AllPrep DNA/RNA mini kit (Qiagen). Relative quantitative real-time reverse transcriptase PCR was performed using AgPath-ID One-Step RT-PCR reagents (ThermoFisher Scientific). Primer pairs for detection of rhesus CXCR5 and internal beta-actin control are as follows: *CXCR5* (forward, 5'-TTCACCTCCCGATTCCTCTA-3'; reverse, 5'-CAACCTGTGCACTACCCC-3'), *beta-actin* (forward, 5'-ATGCTTCTAGGCGGACTGTG-3'; reverse, 5'-AAAGCCATGCCAATCTCATC-3'). The TaqMan probe sequence for CXCR5 was 5'-GGATTCCTGCTGCCCATGCT-3' and for beta-actin, the probe sequence was 5'-TGCGTTACACCCTTTCTTGACAAAACC-3'. Both probes were labeled at the 5' end with a 5' 6-carboxyfluorescein (6-FAM) and a 3' black hole quencher-1 (BHQ-1). The Applied Biosystems StepOnePlus Real-Time PCR System (ThermoFisher Scientific) was used for real-time PCR analysis. Thermal cycling conditions were designed as follows: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C

for 15 s and 60°C for 45s. RNA levels in all samples are relative to housekeeping gene, beta-actin.

***In situ* tetramer staining combing with immunohistochemistry.**

In situ tetramer staining combined with immunohistochemistry was performed on fresh lymph tissue specimens shipped overnight, sectioned with a compresstome and stained essentially as previously described[2]. Biotinylated peptide-loaded MHC-class I monomers for Mamu-A1*001:01 $Gag_{181-189}$CM9, Mamu-A1*002:01 $Nef_{159-167}$YY9, and Mamu-B*08:01 $Nef_{137-146}$RL10 (National Institute of Health Tetramer Core Facility, Emory University, Atlanta GA) were converted to FITC-labeled MHC-class I tetramers. Fresh lymph node sections were incubated with MHC-class I tetramers (0.5 µg/ml) and rat-anti-human CD8 antibody (2 µg/mL, clone YTC182.20, Acris). For secondary incubations, sections were incubated with 1) rabbit-anti-FITC Abs (0.5 µg/mL, BioDesign, Saco, ME) and mouse-anti-human CD20 Abs (0.19 µg/mL, clone L26, Novocastra), or 2) mouse-anti-human CD20 Abs (0.19 µg/mL, clone L26, Novocastra) and rat-anti-human CD3 Abs (2 µg/mL, clone CD3-12, BioRad). For the tertiary incubations, all sections were incubated with Cy3-conjugated goat-anti-rabbit Abs (0.3 µg/mL, Jackson ImmunoResearch Laboratories), Alexa 488-conjugated goat-anti-mouse Abs (0.75 µg/mL, Molecular probes), and Cy5-conjugated goat anti-rat Abs (0.3 µg/mL, Jackson ImmunoResearch Laboratories). Sections were imaged using a Zeiss LSM 800 confocal microscope. Montage images of multiple 512 × 512 pixels were created and used for analysis. Confocal z-series were collected in a step size of 1.23 µm.

Detection and quantification of SIVmac239 RNA producing cells in lymphoid tissue.

SIVmac239 RNA producing cells were detected on 5 µm thick formalin fixed paraffin embedded sections of lymph node (LN) using RNAscope® technology (Advanced Cell Diagnostics, ACD, Newark, CA). Briefly, fresh cut sections were dewaxed in Xylene, subjected to epitope unmasking by heating in citrate buffer (ACD, treatment 2), protease treated (ACD) and incubated with probe to SIVmac239 for 2 hours at 40˚C. RNAscope® 2.5 detection kit with FastRed was used to amplify and detect the probe. Sections were counterstained by blocking with 1% normal donkey serum (Jackson Labs, Bar Harbor, ME) and 0.5% Casein (Vector laboratories, Burlingame, CA) in Tris Buffered Saline (TBS), staining with Rabbit anti CD20 (abcam, Cambridge, MA) to identify follicles, followed by secondary staining with AF647 labeled donkey anti Rabbit IgG (Invitrogen). Sections were counterstained with DAPI, mounted in Slowfade™ Diamond antifade (Invitrogen) and scanned at 40X on an Aperio Versa 8 (Leica Biosystems). Aperio Image Scope (vs12.3.2.5030, Leica Biosystems) was used to quantify virus producing cells and measure total and follicular areas. Four sections at least 30 µm apart were evaluated for each LN.

Statistical analysis.

The repeated-measures ANOVA test was used for all longitudinal studies. Statistical analyses were conducted using GraphPad Prism version 6.0 (GraphPad Software, La Jolla, California, USA). Statistical significance of the findings was set at a *p*-value of less than 0.05.

Results

ALT-803 induces expansion of T cells and NK cells

Due to its effects on T cell and NK cell proliferation within the blood, administration of IL-15 has been previously explored as a potential candidate for immune restoration during chronic SIV infection in macaques (192, 193). Since ALT-803 has a longer serum half-life, greater tissue distribution, and is more biologically active than wild-type IL-15, we hypothesized that ALT-803 would also positively impact anti-SIV immune response, particularly in elite controller macaques with low plasma viremia. To explore this hypothesis, three SIV-infected controller rhesus macaques were administered 100 μg/kg of ALT-803 intravenously.

Similar to IL-15, ALT-803 induced expansion of both CD4 and CD8 T cells in peripheral blood (Fig. 1A). The change in T cell populations were associated with a significant increase in expression of the cell proliferation marker Ki67 after ALT-803 injection (Fig. 1B). Simultaneously, CD16+ NK cells exhibited a marked expansion on 5 days post ALT-803 treatment (Fig. 1A). Consistent with increased NK cell populations, we observed significantly increased expression of Ki67 in CD16+ NK cells on day 5 post treatment too (Fig. 1C). These data indicate that ALT-803 stimulates dramatic proliferation of CD4, CD8 T cells and NK cells *in vivo*.

ALT-803 drives significant increase of SIV-specific CD8 T cells within lymph nodes

After evaluating the effect of ALT-803 on total CD8 T cells, we next assess its effect on SIV-specific CD8 T cells. Six SIV-infected rhesus macaques (3 progressors and 3 controllers) received 100 μg/kg ALT-803. Lymph nodes were sampled before ALT-803 treatment and 5 days post-treatment. Percentage of SIV-specific CD8 T cells was measured by MHC class I tetramer

staining in lymph nodes and PBMC. There was a trend toward increased numbers of SIV-specific CD8+ T cell in peripheral blood, but it did not reach statistical significance. In contrast, there was a marked and statistically significant increase of SIV-specific CD8 T cells within lymph nodes post ALT-803 (Fig. 2).

CXCR5 expression in CD8 T cells increases after ALT-803 treatment *in vitro*

Chemokine receptor, CXCR5, is typically upregulated in activated T cells and promotes T cell migration into B cell follicles within secondary lymphoid tissues (174, 207, 229). We next measured whether ALT-803 treatment affect expression of CXCR5 on CD8+ T cells by treating PBMC with 15 nM ALT-803 for 5 days *in vitro*. Quantitative real-time reverse transcriptase PCR detected that CD8+ T cells exposed to ALT-803 exhibited a 50-fold increase in CXCR5 mRNA (Fig. 3A). Accordingly, flow cytometry showed that ALT-803 treated CD8+ T cells displayed a significantly increased surface expression of CXCR5 (Fig. 3C).

ALT-803 stimulates significant accumulation of SIV-specific CD8 T cells within B cell follicles

A major hurdle to eliminate HIV infection is that limited ability of virus-specific CD8+ T to enter B cell follicles to kill HIV-infected intrafollicular CD4+ T cells within lymph nodes of HIV-infected patients (73, 74, 78). This exclusion of virus-specific CD8+ T cells from B cell follicles allows for the persistence of HIV infection. The significant increase of SIV-specific CD8 T cell in lymph nodes and elevated expression of B cell follicle homing molecule CXCR5 in CD8 T cells led us to test whether virus-specific CD8 T cells were gaining access to the B cell follicles.

In order to address this, we attained lymph node samples from six SIV-infected rhesus macaques (progressor n=3 and controller n=3) before and five days after 100 μg/kg ALT-803 treatment and subsequently performed *in situ* staining of lymph node sections with MHC class I tetramers to stain for SIV-specific CD8 T cells. We also counterstained with antibodies against CD8 to specify CD8+ T cells as well as CD20, allowing us to identify B cells and thus define B cell follicles. As previously described (73, 74), SIV-specific CD8 T cells localized predominantly in the extrafollicular space in lymph nodes of SIV-infected rhesus macaques before treatment (Fig. 4A). However, following ALT-803 treatment, the total number of SIV-specific CD8 T cells within the lymph node increased dramatically, with significant accumulation within B cell follicles (Fig. 4B and C). This infiltration of SIV-specific CD8 T cells into B cell follicles normalized the distribution of anti-viral CD8+ T cells between the follicular and extrafollicular space (Fig. 4D). In accordance with the presence of increased anti-viral effector CD8+ T cells in follicles, lower numbers of SIV-producing cells were found within the B cell follicles of the controller rhesus macaques following ALT-803 treatment (Fig. 5).

Moderate increase of NK cells in B cell follicles is observed after ALT-803 treatment

In contrast to significantly increased percent of CD8+ T cells in lymph nodes, we observed similar percent of NK cells in lymph node before and after ALT-803 treatment (Fig. 1D). Given the dramatic accumulation of SIV-specific CD8 T cell in B cell follicle after treatment, we tested if ALT-803 affected the distribution of NK cells in lymph nodes.

To answer this question, we performed immunohistochemistry on lymph node sections from three SIV-infected rhesus macaques before and five days after 100 μg/kg ALT-803 treatment. We used anti-NKG2A antibody to stain NK cells. Simultaneously, we counted-stained sections with antibodies directly against CD20 to label B cell follicles, and antibodies against

CD3 to label T cells. We found that NK cells located both inside and outside B cell follicles before and after treatment (Fig. 6A and B). Quantitative analysis showed no significant increase in total and extrafollicular NK cells. However, we observed a modest increase of NK cells in B cell follicle after ALT-803 treatment (Fig. 6C). Similar to SIV-specific CD8 T cells, there was lower level of NK cells located in B cell follicles compared to extrafollicular regions (Fig. 6D). These results suggest that ALT-803 can induce elevated level of NK cells within B cell follicle in SIV-infected rhesus macaques.

Discussion

IL-15 is considered a powerful candidate for immunotherapy in the context of cancer and HIV infection because of its ability to promote T cell and NK cell proliferation. Moreover, therapeutic administration of IL-15 induces restoration of the primary target of HIV, the CD4+ effector memory T cell population, in the context of ART (193). Additionally, IL-15 causes HIV reactivation in latently infected cells *in vitro* (201). Although IL-15 treatment is a viable strategy as an immunotherapy, the special trans-presentation mechanism, restricted bioavailability and short half-life in serum of IL-15 limit its application in clinic.

ALT-803, a novel IL-15 superagonist, exhibits potent immunostimulatory effect on CD8 T cells and NK cells, and prolonged half-life in serum than IL-15. Moreover, it demonstrated superior antitumor activity and has been proved as an ideal substitute for IL-15 in cancer immunotherapy (198, 199, 243, 244). Recent studies also showed that ALT-803 can not only activate NK cells that can suppress *in vivo* acute HIV infection in humanized mice (200), but also can reverse viral latency (201). In this study, we found that a single administration of ALT-803 substantially increased proliferation of CD4 and CD8 T cells, and CD16+ NK cells in SIV-infected rhesus macaques. Many studies demonstrated that CD8 T cells have a critical role in controlling both HIV and SIV infection (73, 147, 148, 150, 151, 168-170, 203). Expanded populations of CD8 T cells in peripheral blood and lymphoid tissues induced by ALT-803 may further strengthen the viral control. A previous study showed that IL-15 may promote restoration of CD4 T cell compartments in the setting of ART (193). ALT-803 may also contribute to the restoration of CD4 T cells through triggering significant proliferation of this cell population. However, consistent with previous observations (198), we showed that the stimulation of ALT-803 on lymphocytes proliferation was transient, which is inadequate for long-term viral control. ALT-803 is capable of improving NK cell activity *in vitro* through inducing increased expression of cytolytic molecules perforin and granzyme B, as well as increased CD107a, a marker of

degranulation (200). Thus the impaired NK cell responses induced by HIV infection may be ameliorated by ALT-803 treatment which is able to enhance the proliferation and cytotoxicity of NK cells (245).

It is well known that HIV- and SIV-CD8 T cells are largely excluded from B cell follicles (73, 74, 79, 80). Moreover, it has been demonstrated that these B cell follicles become major sites for viral replication and can avoid the threat of antiviral CD8 T cells during asymptomatic HIV and SIV infection (73, 78, 136, 238). In the apparent lack of virus-specific CD8 T cell surveillance, B cell follicles are established as long-lasting anatomical viral reservoirs of the HIV and SIV infection. Here we demonstrated that, in SIV-infected rhesus macaques, ALT-803 triggers significant upregulation of CXCR5 which is the key homing molecule for CD8 T cells to lymphoid follicles. Importantly, more SIV-specific CD8 T cells accumulate in B cell follicles *in vivo*. Given our observations, ALT-803 promotes the trafficking of virus-specific CD8 T cells into B cells follicles in lymph nodes, thus providing a powerful tool to more effectively bring potent antiviral CD8+ T cells to the site of the latent viral reservoir. Furthermore, we also found a significantly lower level of NK cells in B cell follicles. Importantly, ALT-803 drove moderate increase of NK cells in B cell follicles too. These findings suggest that, in addition to enhancing NK cells activity, ALT-803 also induces increased frequency of NK cells in the major sites of viral replication during SIV infection.

In conclusion, by using SIV-infected rhesus macaques, we demonstrated that ALT-803 is a good candidate as immunotherapeutic agent for HIV eradication strategies. First, given its potent immunostimulatory effect on memory T cells and cytotoxic NK cells, ALT-803 may improve immune cell effector functions to contribute to reduction and clearance of the viral reservoir. Secondly, we found that ALT-803 stimulates accumulation of SIV-specific CD8 T cells and NK cells in follicular sanctuaries of virus-producing cells. Taken together, these data

highlight the great potential of the application of ALT-803 in seeking strategies to eradicate HIV infection.

Figure 1

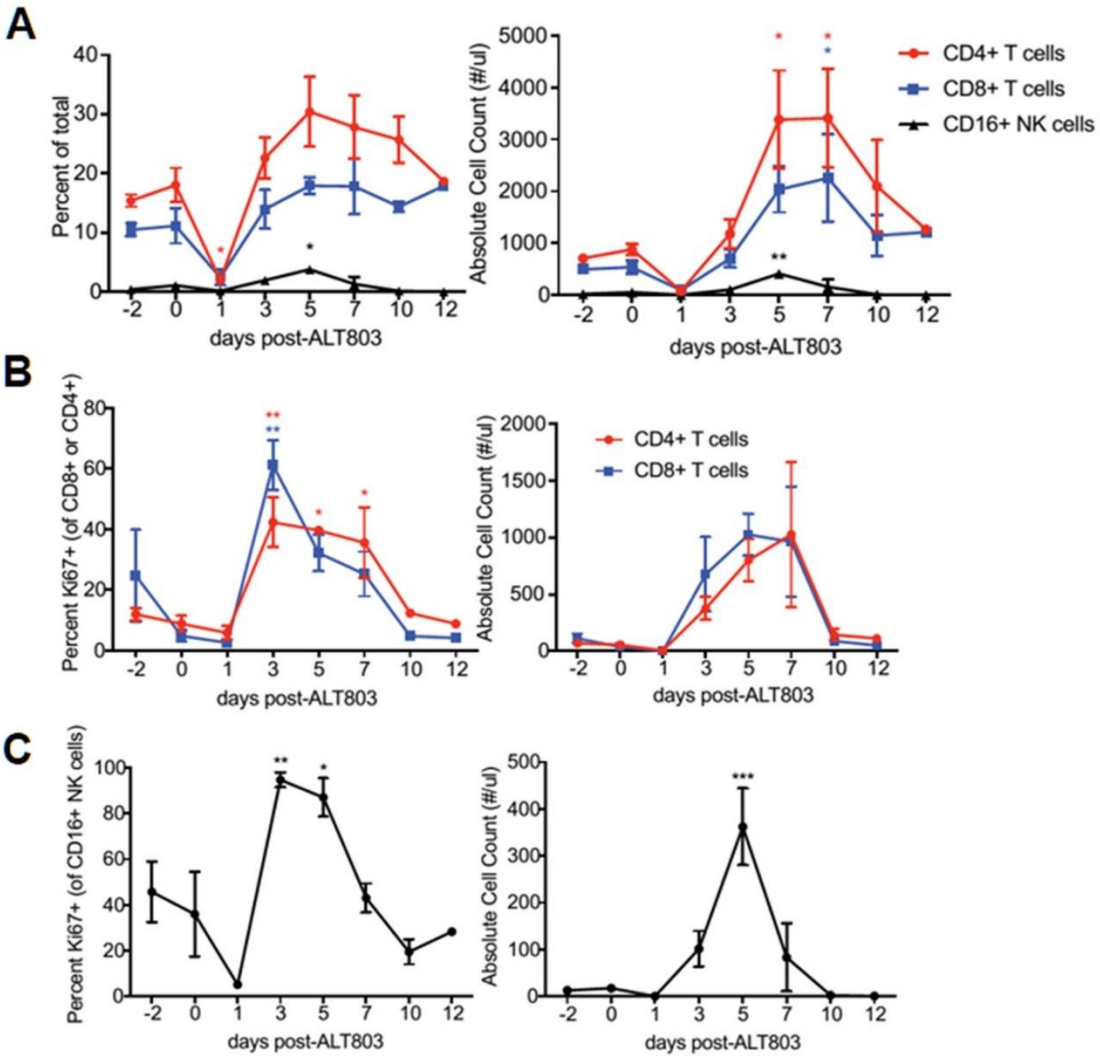
A
Percent of total
Absolute Cell Count (#/ul)
CD4+ T cells
CD8+ T cells
CD16+ NK cells
days post-ALT803
B
Percent Ki67+ (of CD8+ or CD4+)
Absolute Cell Count (#/ul)
CD4+ T cells
CD8+ T cells
days post-ALT803
C
Percent Ki67+ (of CD16+ NK cells)
Absolute Cell Count (#/ul)
days post-ALT803

FIG 1 *In vivo* administration of ALT-803 induces expansion of CD4, CD8 and NK cells in peripheral blood. SIV-infected controller rhesus macaques (n=3) were administered 100 μg/kg of ALT-803 intravenously. (**A**) Dynamics of CD4, CD8 and NK cells in whole blood before and after ALT-804 administration. Proliferation of CD4, CD8 T cells (**B**) and NK cells (**C**) were determined as a percentage of Ki67+ cells of that particular lymphocyte population. Absolute counts were calculated based on the percentage of the particular cell subset and the WBC count. Data shown are means (± SEM) of combined data from all three animals. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ comparing time points to time point zero.

Figure 2

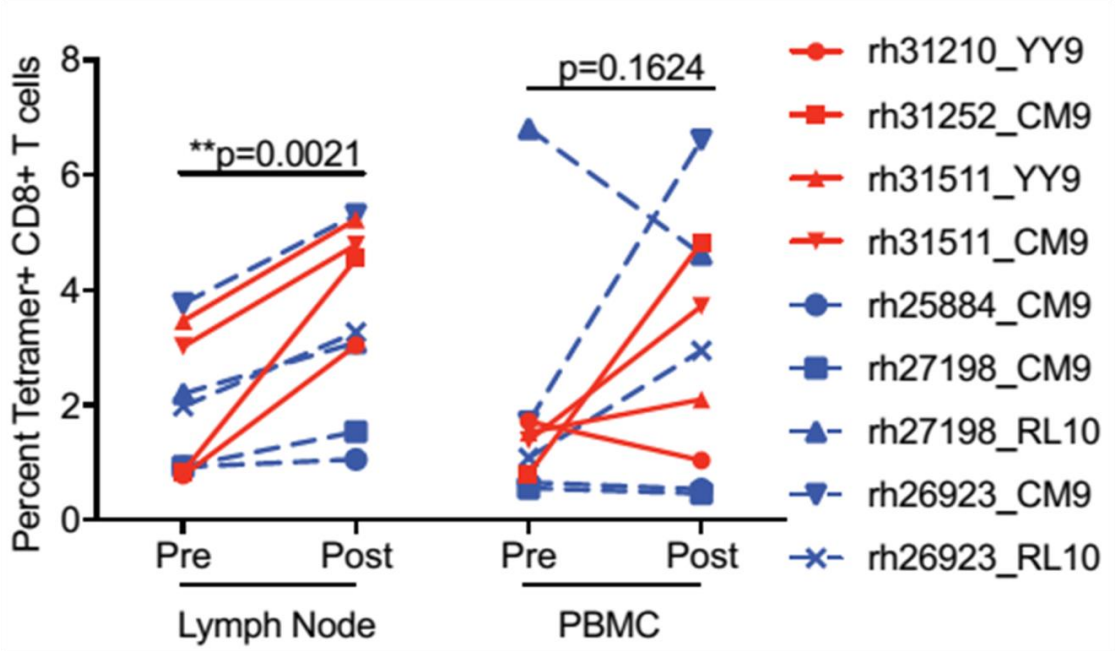

FIG 2 ALT-803 drives significant increase of SIV-specific CD8 T cells within lymph nodes. Percentage of SIV-specific CD8 T cells, measured by MHC class I tetramer staining, increased significantly after ALT-803 administration in lymph nodes but not in PBMC.

Figure 3

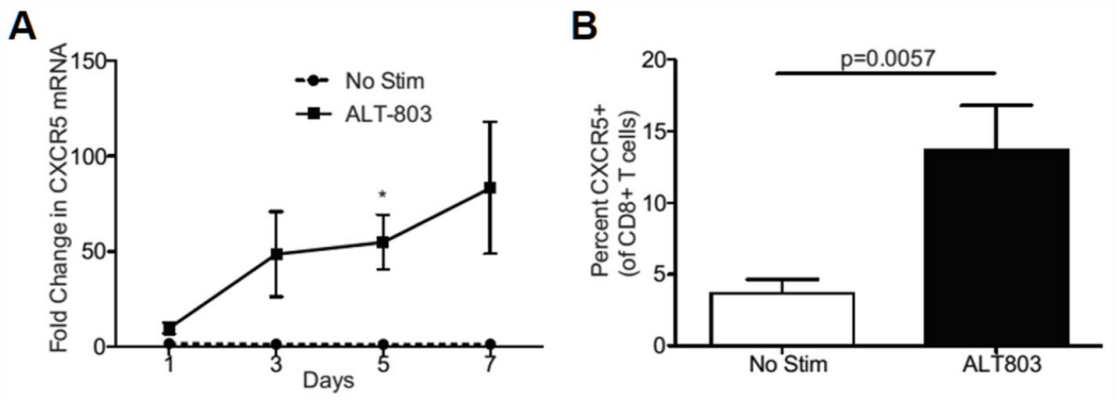

FIG 3 ALT-803 stimulates increased CXCR5 expression of CD8 T cell *in vitro*. (**A**) CD8β-sorted T cells from 6 rhesus macaques were cultured in vitro for 7 days with or without 15 nM ALT-803 and CXCR5 mRNA levels were determined via quantitative RT-PCR at days 1, 3, 5 and 7 post-treatment. (**B**) PBMC from 9 rhesus macaques and 3 cynomolgus macaques were cultured *in vitro* for 5 days with or without 15 nM ALT-803. CD8+ T cells were then assessed for surface expression of CXCR5.

Figure 4

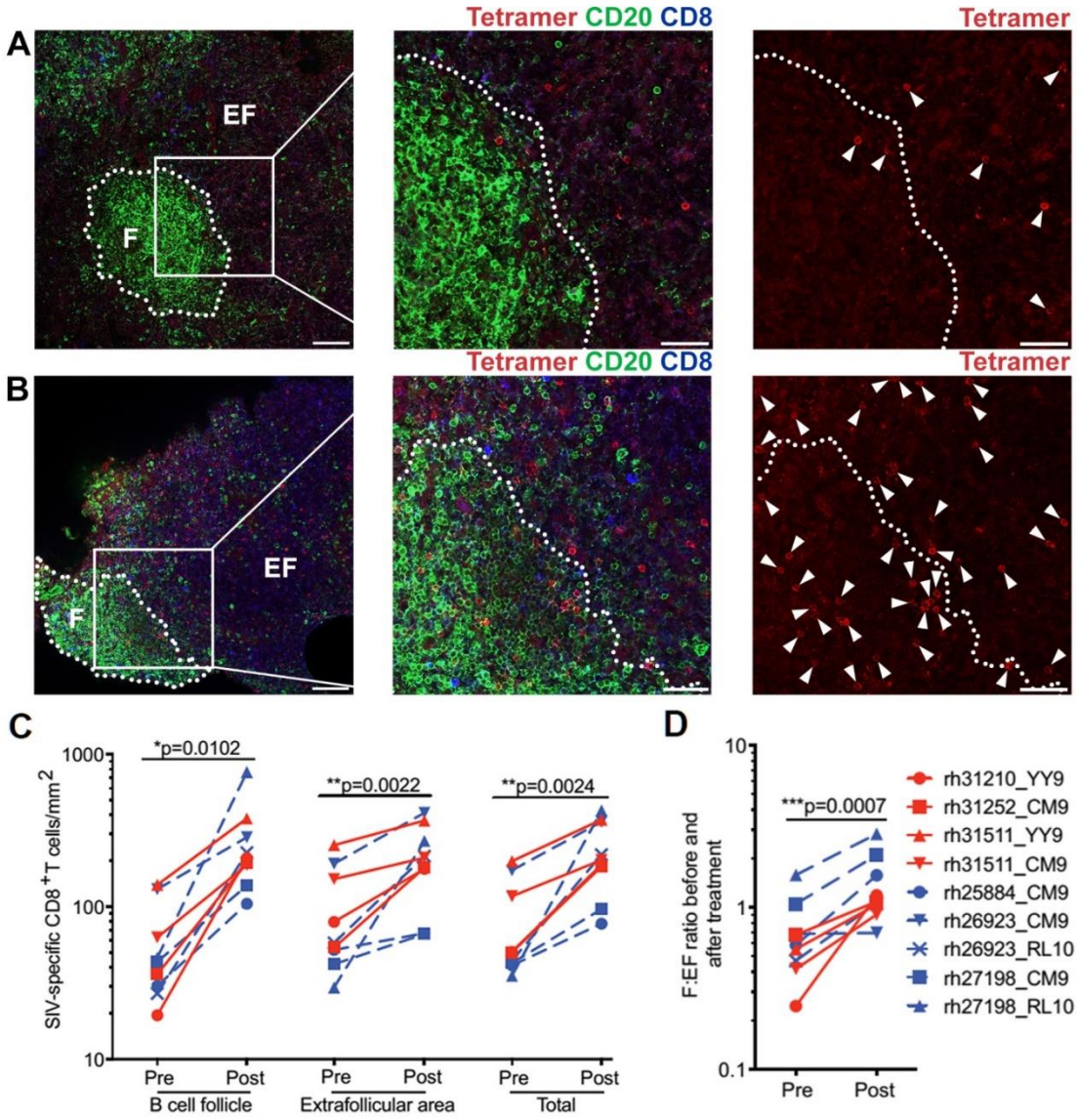
A
EF
F
Tetramer CD20 CD8
Tetramer
B
EF
F
Tetramer CD20 CD8
Tetramer
C
SIV-specific CD8+T cells/mm2
1000
100
10
*p=0.0102
**p=0.0022
**p=0.0024
Pre
Post
B cell follicle
Extrafollicular area
Total
D
F:EF ratio before and after treatment
10
1
0.1
***p=0.0007
Pre
Post
rh31210_YY9
rh31252_CM9
rh31511_YY9
rh31511_CM9
rh25884_CM9
rh26923_CM9
rh26923_RL10
rh27198_CM9
rh27198_RL10

FIG 4 ALT-803 stimulates significant accumulation of SIV-specific CD8 T cells within B cell follicles. Representative images of Mamu-A*001/GagCM9-specific tetramer positive cells (red), CD20+ cells (green) and CD8+ cells (blue) in lymph node sections taken from SIV-infected animal Rh31252 before (**A**) and 5 days after (**B**) ALT-803 treatment. CD20 staining is used to define B cell follicles (F) and extrafollicular regions (EF) outside B cell follicles. The images on the far right show the same field as presented in middle panel with only the red tetramer staining shown. Each tetramer-binding cell is indicated with a white arrow. Bars indicate 100 μm. (**C**) Numbers of tetramer positive SIV-specific CD8 T cells per mm^2 inside and outside of the B cell follicle as well as total tissue before and after ALT-803 treatment. (**D**) F:EF ratio of tetramer positive SIV-specific CD8 T cells per mm^2 before and after ALT-803 treatment.

Figure 5

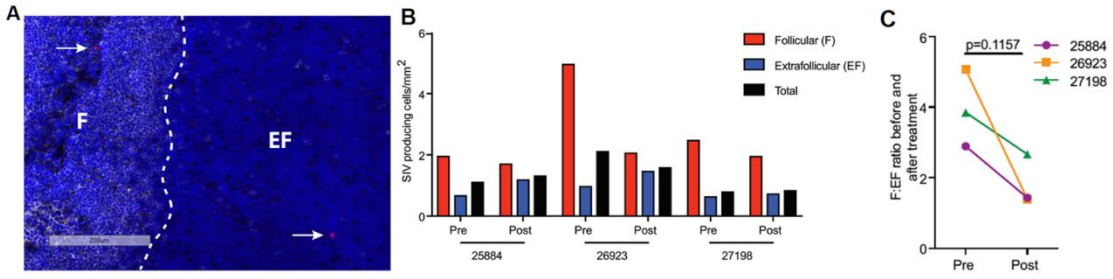

FIG 5 ALT-803 reduces the number of SIV-producing cells within B cell follicles. SIV-infected controller rhesus macaques (n=3) were administered 100 ug/kg of ALT-803 intravenously. Lymph nodes were sampled before ALT-803 treatment and 5 days post-treatment and RNAscope analysis was used to determine the number of SIV-producing cells in lymph nodes. (**A**) Representative image of RNAscope analysis of lymph node. RNA+ cells (red, white arrows) detected in lymph node tissue section of SIVmac239-infected rhesus macaque prior to treatment with ALT-803 (animal 25884). B cell follicle (demarcated by white line) was determined morphologically by staining with CD20 (white). Tissue was counterstained with DAPI (blue) to identify cell nuclei. Bar indicates 200 μm. (**B**) Compiled data of the number of SIV-producing cells in B cell follicular (F) or extrafollicular (EF) space within lymph nodes of SIV-infected controller rhesus macaques pre and post ALT-803 treatment. (**C**) F:EF ratio of SIV-producing cells evaluated in lymph nodes before and after ALT-803 treatment.

Figure 6

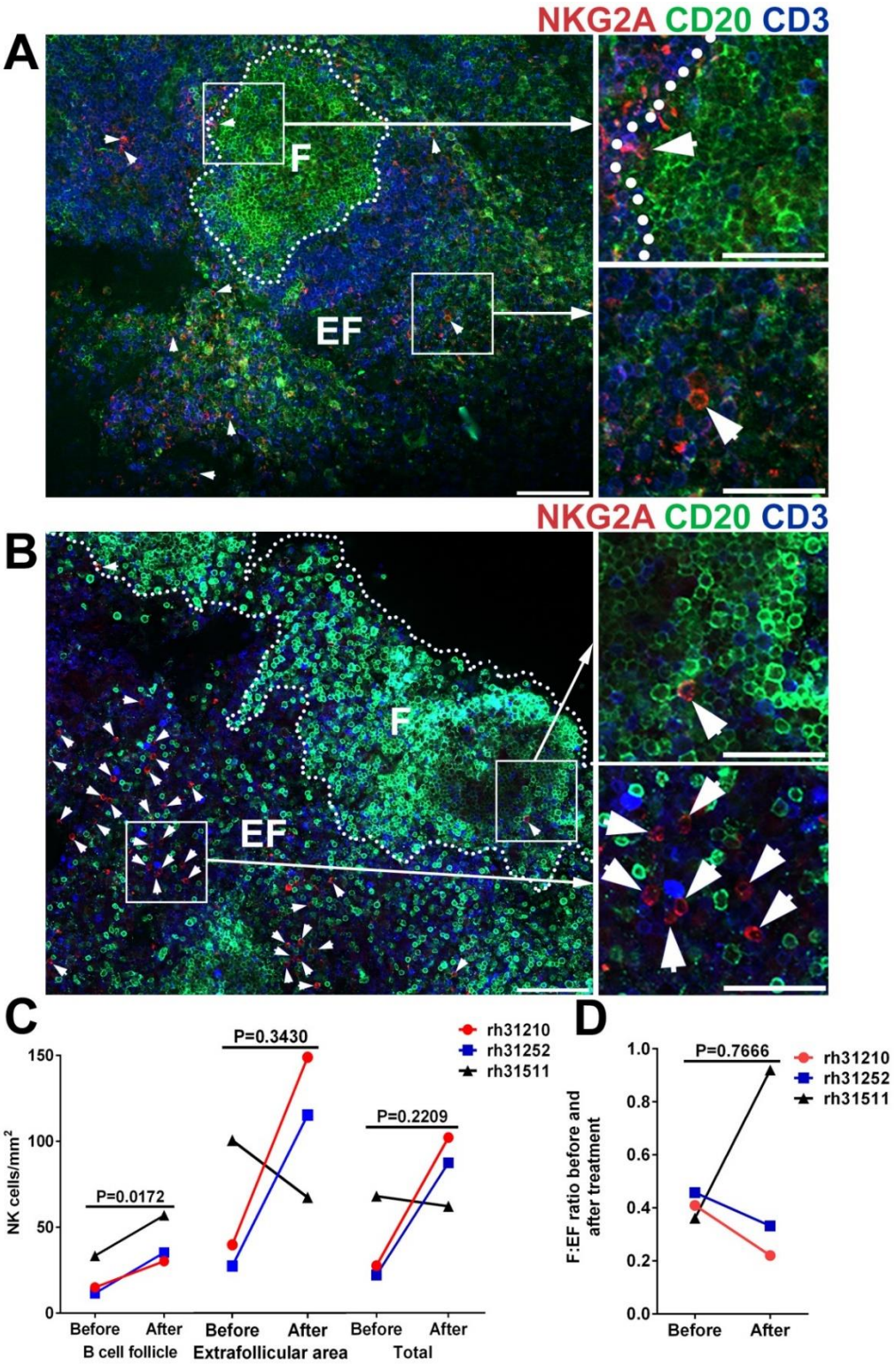
NKG2A CD20 CD3
A
F
EF
NKG2A CD20 CD3
B
F
EF
C
rh31210
rh31252
rh31511
P=0.3430
P=0.2209
P=0.0172
NK cells/mm2
150
100
50
0
Before
After
Before
After
Before
After
B cell follicle
Extrafollicular area
Total
D
P=0.7666
F:EF ratio before and after treatment
1.0
0.8
0.6
0.4
0.2
0.0
Before
After

FIG 6 ALT-803 drives moderate increase of NK cells in B cell follicles. Representative images of NKG2A+ cells (red), CD20+ cells (green) and CD3+ cells (blue) in lymph node sections taken from SIV-infected animal Rh31210 before (**A**) and 5 days after (**B**) ALT-803 treatment. CD20 staining is used to define B cell follicles (F) and extrafollicular regions (EF) outside B cell follicles. Each NKG2A+ cell is indicated with a white arrow. Bars indicate 100 μm. (**C**) Numbers of NKG2A+ cells per mm^2 inside and outside of the B cell follicle as well as total tissue before and after ALT-803 treatment. (**D**) F:EF ratio of NKG2A+ cells per mm^2 before and after ALT-803 treatment.

Chapter 5

Conclusion and future direction

HIV/AIDS has caused severe global pandemic since it was first identified in 1980s. Currently, more than 36 million people worldwide are living with HIV and there are still around 1.8 million new infections annually ("Fact sheet – Latest statistics on the status of the AIDS epidemic". UNAIDS.org. 2016). Even though the wide clinical application of cART substantially reduced the morbidity and mortality of HIV infection, and prolonged the longevity of HIV infected individuals (88), their unpleasant side effects, high cost and low availability for patients in underdeveloped area can't be ignored (84, 92, 246, 247). Moreover, cART can suppress HIV replication, but can't eliminate it. Thus there is still an urgent need for development of an effective HIV vaccine and a functional cure to fully eradicate HIV infection.

Many studies have demonstrated that CD8 T cells have critical role in controlling both HIV and SIV infection (147, 148, 150, 151, 168-170, 203). However, these cells fail to fully suppress viral replication. HIV- and SIV-producing cells are highly concentrated in B cell follicles during chronic HIV and SIV infection (73-75, 77). Moreover, residual productive HIV/SIV infection also preferentially localized in B cell follicles in the setting of long-term, suppressive cART (78, 136). In contrast to the concentrated HIV/SIV replication in B cell follicles, virus-specific CD8 T cells fail to accumulate in large number in these sites (73, 74, 79, 80). The relative paucity of effective anti-viral CD8 T cell responses inside B cell follicles is an important mechanism that, at least partially, accounts for the persistent HIV and SIV infection. The main objective here is to study the follicular virus-specific CD8 T cells during HIV infection using SIV-infected rhesus macaques.

First, our data suggest that during chronic SIV infection, despite the expression of inhibitory receptor PD-1 and likely inhibition by Foxp3+ Tregs, a subset of follicular SIV-specific CD8 T cells are activated and proliferating, and expressing cytolytic molecule perforin. Results from CD8 depletion study indicate that follicular SIV-specific CD8 T cells are functional in suppressing viral replication in B cell follicles. These findings are consistent with recent

studies about follicular CXCR5+ CD8 T cells during chronic viral infection in different species (174, 229, 237, 238). Together, these studies strongly support HIV cure strategies that boosting functional follicular virus-specific CD8 T cells to enhance viral control.

Many studies demonstrate that CD8 T cells are critical in initial viral control during primary HIV and SIV infection (147, 148, 169, 170, 234). However, this control is incomplete. We found that, similar to chronic infection, SIV-specific CD8 T cells in early infection are also largely excluded from B cell follicles, and these cells are likely possess cytolytic potential. Importantly, low levels of follicular SIV-specific CD8 T cells from GCs during early infection may pave the road for persistent chronic infection. These findings may help inform future HIV vaccine design.

IL-15 plays a crucial role in mediating T cells and NK cells activation and proliferation. ALT-803, a novel IL-15 superagonist, demonstrates stronger biological activity and prolonged half-life in serum than free IL-15. Last, we found that ALT-803 stimulated substantial proliferation of SIV-specific CD8 T cells in lymph nodes, and drove significant accumulation of these cells inside B cell follicles and more evenly distribution of these cells in the follicular and extrafollicular regions. Additionally, frequency of SIV-producing cells was reduced in all rhesus macaques. These data suggest that ALT-803 is a good candidate to promote progress in seeking HIV eradication.

Taken together, these data describe the dynamics of follicular SIV-specific CD8 T cells during early and chronic infection, and effect of ALT-803 treatment on these cells. The model we propose based on these findings is shown in Figure 1. After HIV infection, a large number of CD4 T cells are infected and there is little difference in the frequencies of virus-producing cells inside and outside B cell follicles in secondary lymphoid tissues prior to the emergence of anti-viral CD8 T cell responses (Fig. 1A). When CD8 T cell responses are initiated in early infection,

a small population of virus-specific CD8 T cells with cytolytic potential migrate into B cell follicles while most of these cells locate in T cell zones. At this moment, because HIV-specific CD8 T cells just emerge, relatively even distribution of virus-producing cells inside and outside B cell follicles has not been changed in spite of quantitative advantage of HIV-specific CD8 T cells in T cell zones compare to those in follicles (Fig. 1B). Both follicular and extrafollicular HIV-specific CD8 T cells are able to kill virus-producing cells. However, because of their low frequency, follicular HIV-specific CD8 T cells fail to eliminate virus-producing cells as efficiently as their counterparts outside the follicles. As time goes on, most virus-producing cells are gradually confined inside B cell follicles (Fig. 1C). These virus-producing cells still generate new infectious virions. If the host immune system is highly functional, HIV-producing cells are largely trapped in follicles. However, when host immune systems compromises and lose the control on HIV replication, HIV-producing cells come out from the B cell follicles and diffuse into T cell zones, blood and other non-lymphoid tissues progressively. Administration of ALT-803 not only stimulates large expansion of HIV-specific CD8 T cells in lymph nodes, but also drives significant accumulation of SIV-specific CD8 T cells inside B cell follicle, more evenly distribution of these cells in the follicular and extrafollicular regions and reduced virus-producing cells inside B cell follicles (Fig. 1D).

However, despite the striking characteristics of follicular CXCR5+ CD8 T cells have been revealed, more information is required to fully explore the potential of these cells for functional cure efforts in HIV research. First, we characterized the phenotypes of follicular SIV-specific CD8 T cells during early infection, but didn't directly evaluate their function in suppressing viral replication. Further comprehensive functional analyses are required. Vaccine design to establish large pool of virus-specific CD8 T cells inside B cell follicles deserves further exploration in seeking prevention of HIV infection. This strategy is supported by a recent study which demonstrated that transfer of large number of molecularly engineered SIV-specific T cells

limit the establishment of infection (248). Second, although ALT-803 stimulates significant accumulation of follicular SIV-specific CD8 T cells, comprehensive analyses about phenotype and functionality of these induced cells have not been done. Whether they are able to potently suppress viral replication remain inconclusive. There is a heterogeneity exists within follicular CXCR5+ CD8 T cells which may lead to divergent function in suppressing viral replication (207). A population of follicular regulatory CD8 T cells has been recently reported in HIV and SIV infection (249). The proportion of follicular regulatory CD8 T cells among total follicular SIV-specific CD8 T cells after ALT-803 treatment, and the positive and negative effect of this cell population in HIV infection deserve further evaluation. Besides, stimulation of ALT-803 on lymphocytes proliferation is transient, which is an obstacle that needs to be taken into account to pursue long-term viral control. Third, application of bispecific antibodies and blockade of PD-1/PD-L1 pathway have been demonstrated to potentiate the anti-viral effect of follicular CXCR5+ CD8 T cells (174, 237). Therapeutic administration of IL-27 may could enhance the reactivity of follicular CXCR5+ CD8 T cells along with blocking suppression from Tregs, as shown in cancer research (250). Exploration of ALT-803 administration in conjunction with other potentiators will be needed to further improve immune responses and eradication of HIV infection.

In addition to ALT-803 treatment, adoptive transfer of autologous HIV-specific CXCR5+ CD8 T cells has been proposed to increase levels of HIV-specific CXCR5+ CD8 T cells in follicles and reduce follicular viral replication. However, given the low percentage of CXCR5+ HIV-specific CD8 T cells in infected individuals, producing sufficient numbers of autologous CXCR5+ HIV-specific CD8 T cells for infusion is a big challenge to be considered. Genetic modification of CD8 T cells is a feasible solution for this obstacle. A recent study successfully introduced engineered CXCR5+ CD8 T cells into B cell follicles in SIV-infected rhesus macaques (251). Besides, transduction of SIV-specific TCR gene into CD8 T cells confers the ability to suppress SIV replication on these cells (252). Application of chimeric antigen

receptors (253-257) may be another possible way to produce large number of HIV-specific CD8 T cells. Moreover, potential enhancers are able to improve immune responses mentioned above can be applied in adoptive cell transfer too. Different studies about adoptive transfer of autologous SIV-specific CD8 T cells all observed high frequencies of infused cells localized in lung instead of lymphoid tissues which reduce the efficacy of cell transfer (248, 251, 258, 259). While CXCR5 has been demonstrated to be necessary for localization of CD8 T cells in B cell follicles (174, 229, 251), whether CXCR5 alone is sufficient for entry of these cells into B cell follicles remain controversial. A better understanding about the network that drives trafficking of CD8 T cells into B cell follicles may further enhance the efficacy of adoptive transfer of autologous CD8 T cells. Therefore, adoptive cell transfer is another promising measure to augment HIV-specific CXCR5+ CD8 T cells in lymphoid follicles, but more exploration is required to further enhance its efficiency.

Notably, besides HIV-producing cells, there exist a population of latently HIV-infected resting cells which serve as important viral reservoirs that can't be efficiently eliminated by anti-viral CD8 T cells. Antigen-specific stimulation of CD8 T cells results in efficient killing of reactivated viral infected cells (260, 261). Therefore, the strategies that combine augment of follicular HIV-specific CD8 T cells, antigen-specific stimulation and latency reverse agent would be required for eradication of HIV infection.

In conclusion, our findings strongly support the HIV cure strategies that augment functional follicular virus-specific CD8 T cells to enhance viral control and demonstrate that ALT-803 is a potential candidate for this strategy.

Figure 1

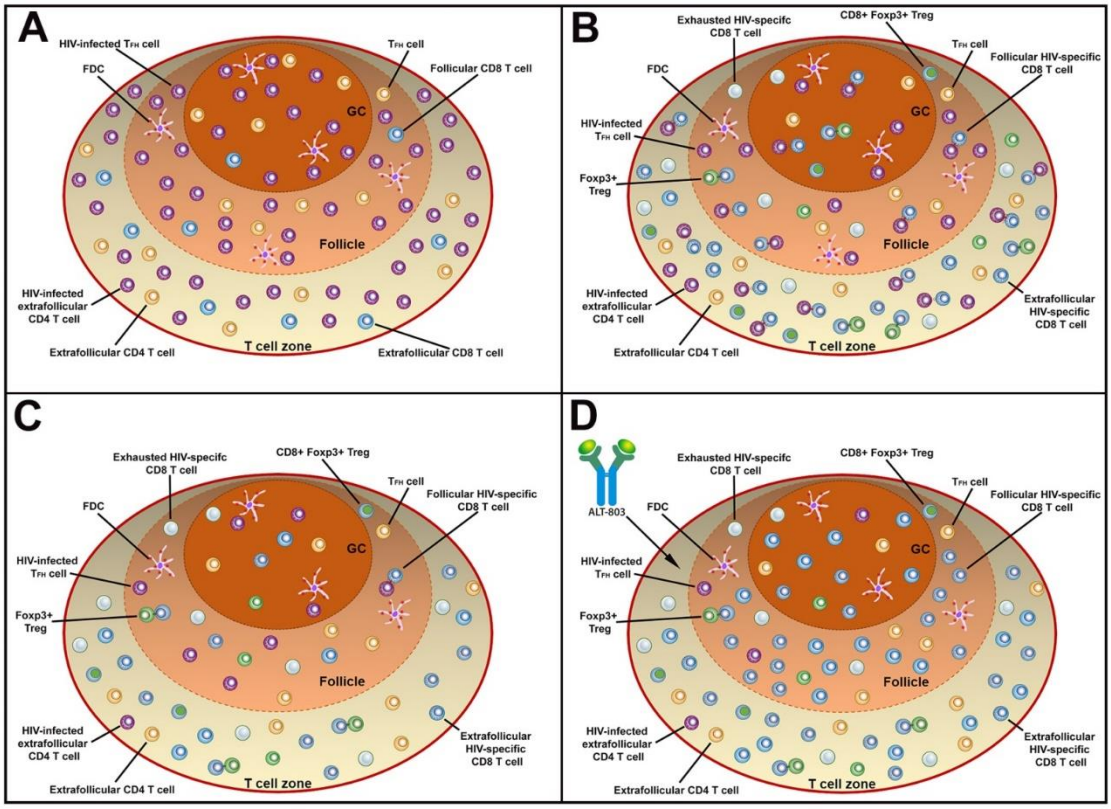
A
HIV-infected TFH cell
TFH cell
FDC
Follicular CD8 T cell
GC
Follicle
HIV-infected extrafollicular CD4 T cell
Extrafollicular CD4 T cell
T cell zone
Extrafollicular CD8 T cell
B
Exhausted HIV-specifc CD8 T cell
CD8+ Foxp3+ Treg
TFH cell
Follicular HIV-specific CD8 T cell
FDC
HIV-infected TFH cell
Foxp3+ Treg
GC
Follicle
HIV-infected extrafollicular CD4 T cell
Extrafollicular CD4 T cell
T cell zone
Extrafollicular HIV-specific CD8 T cell
C
Exhausted HIV-specifc CD8 T cell
CD8+ Foxp3+ Treg
TFH cell
Follicular HIV-specific CD8 T cell
FDC
HIV-infected TFH cell
Foxp3+ Treg
GC
Follicle
HIV-infected extrafollicular CD4 T cell
Extrafollicular CD4 T cell
T cell zone
Extrafollicular HIV-specific CD8 T cell
D
ALT-803
Exhausted HIV-specifc CD8 T cell
CD8+ Foxp3+ Treg
TFH cell
Follicular HIV-specific CD8 T cell
FDC
HIV-infected TFH cell
Foxp3+ Treg
GC
Follicle
HIV-infected extrafollicular CD4 T cell
Extrafollicular CD4 T cell
T cell zone
Extrafollicular HIV-specific CD8 T cell

FIG 1 The dynamics of follicular HIV-specific CD8 T cells during early and chronic infection. (**A**) After HIV infection, a large number of CD4 T cells are infected and there is little difference in the frequencies of virus-producing cells inside and outside B cell follicles in secondary lymphoid tissues prior to the emergence of anti-viral CD8 T cell responses. (**B**) When anti-viral CD8 T cell responses are first initiated in early infection, a small population of HIV-specific CD8 T cells with cytolytic potential express CXCR5 and migrate into B cell follicles. Despite high level of exhaustion and likely inhibition by Foxp3+ Tregs, these cells effectively kill HIV-infected T_{FH} cells through secretion of cytolytic molecules and/or contact dependent killing mechanism. Simultaneously, a significantly higher level of HIV-specific CD8 T cells locate in T cell zones and exert strong cytotoxicity as well. At this time point, because HIV-specific CD8 T cells just emerge, relatively even distribution of virus-producing cells inside and outside B cell follicles has not been changed in spite of quantitative advantage of HIV-specific CD8 T cells in T cell zones compare to those in follicles. (**C**) During chronic infection, HIV-producing cells in T cell zones are effectively suppressed by extrafollicular HIV-specific CD8 T cells. However, because of their low frequency, follicular HIV-specific CD8 T cells fail to control HIV replication as efficiently as their counterparts in T cell zones. As time goes on, most HIV-producing cells are gradually confined inside B cell follicles. (**D**) Administration of ALT-803 not only stimulates large expansion of HIV-specific CD8 T cells in lymph nodes, but also drives significant accumulation of SIV-specific CD8 T cells inside B cell follicle, more evenly distribution of these cells in the follicular and extrafollicular regions and reduced virus-producing cells inside B cell follicles.

References:

1. **HIV/AIDS JUNPo.** 2010. AIDS scorecards: overview: UNAIDS report on the global AIDS epidemic 2010. UNAIDS.
2. **Embretson J, Zupancic M, Ribas JL, Burke A, Racz P, Tenner-Racz K, Haase AT.** 1993. Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. Nature **362:**359-362.
3. **Schnittman SM, Lane HC, Greenhouse J, Justement JS, Baseler M, Fauci AS.** 1990. Preferential infection of CD4+ memory T cells by human immunodeficiency virus type 1: evidence for a role in the selective T-cell functional defects observed in infected individuals. Proceedings of the National Academy of Sciences **87:**6058-6062.
4. **Douek DC, Brenchley JM, Betts MR, Ambrozak DR, Hill BJ, Okamoto Y, Casazza JP, Kuruppu J, Kunstman K, Wolinsky S.** 2002. HIV preferentially infects HIV-specific CD4+ T cells. Nature **417:**95-98.
5. **Gottlieb MS, Schanker HM, Fan PT, Saxon A, Weisman JD, Pozalski I.** 1981. Pneumocystis pneumonia--Los Angeles. MMWR Morbidity and mortality weekly report **30:**250-252.
6. **Friedman-Kien AE.** 1981. Disseminated Kaposi's sarcoma syndrome in young homosexual men. Journal of the American Academy of Dermatology **5:**468-471.
7. **Hymes K, Greene J, Marcus A, William D, Cheung T, Prose N, Ballard H, Laubenstein L.** 1981. Kaposi's sarcoma in homosexual men—a report of eight cases. The Lancet **318:**598-600.
8. **Barre-Sinoussi F, Chermann J, Rey F, Nugeyre M, Chamaret S, Gruest J, Dauguet C.** 2004. Isolation of T-lymphotropic retrovirus from a patient at risk for acquired immune defficiency syndrome (AIDS). Revista de investigación clínica **56:**126-129.

9. **Gallo RC, Sarin PS, Gelmann E, Robert-Guroff M, Richardson E, Kalyanaraman V, Mann D, Sidhu GD, Stahl RE, Zolla-Pazner S.** 1983. Isolation of human T-cell leukemia virus in acquired immune deficiency syndrome (AIDS). Science **220:**865-867.

10. **Levy JA, Hoffman AD, Kramer SM, Kandis JA, Shimabururo JM, Oshiro LS.** 1984. Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. Science **225:**840-843.

11. **Coffin J, Haase A, Levy JA, Montagnier L, Oroszlan S, Teich N, Temin H, Toyoshima K, Varmus H, Vogt P.** 1986. What to call the AIDS virus? Nature **321:**10-10.

12. **Louwagie J, McCutchan FE, Peeters M, Brennan TP, Sanders-Buell E, Eddy GA, Van Der Groen G, Fransen K, Gershy-Damet G-M, Deleys R.** 1993. Phylogenetic analysis of gag genes from 70 international HIV-1 isolates provides evidence for multiple genotypes. Aids **7:**769-780.

13. **Myers G.** 1994. HIV: between past and future. AIDS research and human retroviruses **10:**1317-1324.

14. **Reeves JD, Doms RW.** 2002. Human immunodeficiency virus type 2. Journal of general virology **83:**1253-1265.

15. **Foley BT, Leitner TK, Apetrei C, Hahn B, Mizrachi I, Mullins J, Rambaut A, Wolinsky S, Korber BTM.** 2015. HIV Sequence Compendium 2015. Los Alamos National Lab.(LANL), Los Alamos, NM (United States),

16. **Leis J, Baltimore D, Bishop J, Coffin J, Fleissner E, Goff S, Oroszlan S, Robinson H, Skalka A, Temin Ha.** 1988. Standardized and simplified nomenclature for proteins common to all retroviruses. Journal of virology **62:**1808-1809.

17. **Chan DC, Fass D, Berger JM, Kim PS.** 1997. Core structure of gp41 from the HIV envelope glycoprotein. Cell **89:**263-273.

18. **Scarlata S, Carter C.** 2003. Role of HIV-1 Gag domains in viral assembly. Biochimica et Biophysica Acta (BBA)-Biomembranes **1614:**62-72.

19. **Chan DC, Kim PS.** 1998. HIV entry and its inhibition. Cell **93:**681-684.

20. **Greene WC, Peterlin BM.** 2002. Charting HIV's remarkable voyage through the cell: Basic science as a passport to future therapy. Nature medicine **8:**673-680.

21. **Sugimoto C, Tadakuma K, Otani I, Moritoyo T, Akari H, Ono F, Yoshikawa Y, Sata T, Izumo S, Mori K.** 2003. nef gene is required for robust productive infection by simian immunodeficiency virus of T-cell-rich paracortex in lymph nodes. Journal of virology **77:**4169-4180.

22. **Münch J, Rajan D, Schindler M, Specht A, Rücker E, Novembre FJ, Nerrienet E, Müller-Trutwin MC, Peeters M, Hahn BH.** 2007. Nef-mediated enhancement of virion infectivity and stimulation of viral replication are fundamental properties of primate lentiviruses. Journal of virology **81:**13852-13864.

23. **Kuang XT, Li X, Anmole G, Mwimanzi P, Shahid A, Le AQ, Chong L, Qian H, Miura T, Markle T.** 2014. Impaired Nef function is associated with early control of HIV-1 viremia. Journal of virology **88:**10200-10213.

24. **Miller JH, Presnyak V, Smith HC.** 2007. The dimerization domain of HIV-1 viral infectivity factor Vif is required to block virion incorporation of APOBEC3G. Retrovirology **4:**81.

25. **Ronsard L, Raja R, Panwar V, Saini S, Mohankumar K, Sridharan S, Padmapriya R, Chaudhuri S, Ramachandran VG, Banerjea AC.** 2015. Genetic and functional characterization of HIV-1 Vif on APOBEC3G degradation: First report of emergence of B/C recombinants from North India. Scientific reports **5:**15438.

26. **Richter S, Frasson I, Palu G.** 2009. Strategies for inhibiting function of HIV-1 accessory proteins: a necessary route to AIDS therapy? Current medicinal chemistry **16:**267-286.

27. **Bukrinsky M, Adzhubei A.** 1999. Viral protein R of HIV-1. Reviews in medical virology **9:**39-49.

28. **Kwong PD, Wyatt R, Robinson J, Sweet RW.** 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature **393:**648.

29. **Deng H, Liu R, Ellmeier W, Choe S.** 1996. Identification of a major co-receptor for primary isolates of HIV-1. Nature **381:**661.

30. **Choe H, Farzan M, Sun Y, Sullivan N, Rollins B, Ponath PD, Wu L, Mackay CR, LaRosa G, Newman W.** 1996. The β-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. Cell **85:**1135-1148.

31. **Rizzuto CD, Wyatt R, Hernández-Ramos N, Sun Y, Kwong PD, Hendrickson WA, Sodroski J.** 1998. A conserved HIV gp120 glycoprotein structure involved in chemokine receptor binding. Science **280:**1949-1953.

32. **Feng Y, Broder CC, Kennedy PE, Berger EA.** 2011. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein–coupled receptor. Journal of immunology (Baltimore, Md: 1950) **186:**6076.

33. **Miller MD, Farnet CM, Bushman FD.** 1997. Human immunodeficiency virus type 1 preintegration complexes: studies of organization and composition. Journal of virology **71:**5382-5390.

34. **Adams M, Sharmeen L, Kimpton J, Romeo JM, Garcia JV, Peterlin BM, Groudine M, Emerman M.** 1994. Cellular latency in human immunodeficiency virus-infected individuals with high CD4 levels can be detected by the presence of promoter-proximal transcripts. Proceedings of the National Academy of Sciences **91:**3862-3866.

35. **Nguyen DH, Hildreth JE.** 2000. Evidence for budding of human immunodeficiency virus type 1 selectively from glycolipid-enriched membrane lipid rafts. Journal of virology **74:**3264-3272.

36. **Freed EO.** 2015. HIV-1 assembly, release and maturation. Nature Reviews Microbiology **13:**484-496.

37. **Nath BM, Schumann KE, Boyer JD.** 2000. The chimpanzee and other non-human-primate models in HIV-1 vaccine research. Trends in microbiology **8:**426-431.

38. **Chakrabarti L, Isola P, Cumont M-C, Claessens-Maire M-A, Hurtrel M, Montagnier L, Hurtrel B.** 1994. Early stages of simian immunodeficiency virus infection in lymph nodes. Evidence for high viral load and successive populations of target cells. The American journal of pathology **144:**1226.

39. **Chakrabarti L, Guyader M, Alizon M, Daniel MD, Desrosiers RC, Tiollais P, Sonigo P.** 1987. Sequence of simian immunodeficiency virus from macaque and its relationship to other human and simian retroviruses. Nature **328:**543-547.

40. **Miller C, Alexander N, Sutjipto S, Lackner A, Gettie A, Hendrickx A, Lowenstine L, Jennings M, Marx P.** 1989. Genital mucosal transmission of simian immunodeficiency virus: animal model for heterosexual transmission of human immunodeficiency virus. Journal of virology **63:**4277-4284.

41. **Daniel M, Letvin N, King N, Kannagi M, Sehgal P, Hung R.** 1985. Isolation of T-cell tropic HTLV-III-like retrovirus from macaques. Science **228:**1201-1205.

42. **Letvin NL, King NW.** 1990. Immunologic and pathologic manifestations of the infection of rhesus monkeys with simian immunodeficiency virus of macaques. JAIDS Journal of Acquired Immune Deficiency Syndromes **3:**1023-1040.

43. **Rosenberg Y, Shafferman A, White B, Papermaster S, Leon E, Eddy G, Benveniste R, Burke D, Lewis M.** 1992. Variation in the CD4+ and CD8+ populations in lymph nodes does not reflect that in the blood during SIVMNE/E11S infection of macaques. Journal of medical primatology **21:**131-137.

44. **JOLING P, VAN WICHEN DF, PARMENTIER HK, BIBERFELD P, BÖTTIGER D, TSCHOPP J, RADEMAKERS LH, SCHUURMAN H-J.** 1992. Simian

immunodeficiency virus (SIVsm) infection of cynomolgus monkeys: effects on follicular dendritic cells in lymphoid tissue. AIDS research and human retroviruses **8:**2021-2030.

45. **Zhang Z-Q, Schuler T, Zupancic M, Wietgrefe S, Staskus K, Reimann K, Reinhart T, Rogan M, Cavert W, Miller C.** 1999. Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells. Science **286:**1353-1357.

46. **Zhang Z-Q, Wietgrefe SW, Li Q, Shore MD, Duan L, Reilly C, Lifson JD, Haase AT.** 2004. Roles of substrate availability and infection of resting and activated CD4+ T cells in transmission and acute simian immunodeficiency virus infection. Proceedings of the National Academy of Sciences of the United States of America **101:**5640-5645.

47. **Mowat AM, Viney JL.** 1997. The anatomical basis of intestinal immunity. Immunological reviews **156:**145-166.

48. **Haase AT.** 2005. Perils at mucosal front lines for HIV and SIV and their hosts. Nature reviews Immunology **5:**783.

49. **Li Q, Duan L, Estes JD, Zhong-Min M.** 2005. Peak SIV replication in resting memory CD4+ T cells depletes gut lamina propris CD4+ T cells. Nature **434:**1148.

50. **Lavreys L, Thompson ML, Martin Jr HL, Mandaliya K, Ndinya-Achola JO, Bwayo JJ, Kreiss J.** 2000. Primary human immunodeficiency virus type 1 infection: clinical manifestations among women in Mombasa, Kenya. Clinical infectious diseases **30:**486-490.

51. **Schacker TW, Hughes JP, Shea T, Coombs RW, Corey L.** 1998. Biological and virologic characteristics of primary HIV infection. Annals of internal medicine **128:**613-620.

52. **Kinloch-de Loës S, de Saussure P, Saurat J-H, Stalder H, Hirschel B, Perrin LH.** 1993. Symptomatic primary infection due to human immunodeficiency virus type 1: review of 31 cases. Clinical Infectious Diseases **17:**59-65.

53. **Cooper D, Maclean P, Finlayson R, Michelmore H, Gold J, Donovan B, Barnes T, Brooke P, Penny R, Group SAS.** 1985. Acute AIDS retrovirus infection: definition of a clinical illness associated with seroconversion. The Lancet **325:**537-540.

54. **Evian C.** 2007. Primary HIV/AIDS Care: A Practical Guide for Primary Care Personnel in a Clinical and Supportive Setting. Jacana Media.

55. **Baert A.** 2012. Radiology of AIDS. Springer Science & Business Media.

56. **Elliott T, Casey A, Lambert PA, Sandoe J.** 2012. Lecture Notes: Medical Microbiology and Infection. John Wiley & Sons.

57. **Overbaugh J, Morris L.** 2012. The antibody response against HIV-1. Cold Spring Harbor perspectives in medicine **2:**a007039.

58. **Burton DR.** 1997. A vaccine for HIV type 1: the antibody perspective. Proceedings of the National Academy of Sciences **94:**10018-10023.

59. **Johnson WE, Desrosiers RC.** 2002. Viral persistence: HIV's strategies of immune system evasion. Annual review of medicine **53:**499-518.

60. **Lu L, Yu F, Du L, Xu W, Jiang S.** 2013. Tactics used by HIV-1 to evade host innate, adaptive, and intrinsic immunities. Chin Med J (Engl) **126:**2374-2379.

61. **Richman DD, Wrin T, Little SJ, Petropoulos CJ.** 2003. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. Proceedings of the National Academy of Sciences **100:**4144-4149.

62. **Friedrich TC, Dodds EJ, Yant LJ, Vojnov L, Rudersdorf R, Cullen C, Evans DT, Desrosiers RC, Mothé BR, Sidney J.** 2004. Reversion of CTL escape–variant immunodeficiency viruses in vivo. Nature medicine **10:**275-281.

63. **Leslie A, Pfafferott K, Chetty P, Draenert R, Addo M, Feeney M, Tang Y, Holmes E, Allen T, Prado J.** 2004. HIV evolution: CTL escape mutation and reversion after transmission. Nature medicine **10:**282.

64. **Sakaguchi S, Sakaguchi N.** 2005. Regulatory T cells in immunologic self-tolerance and autoimmune disease. International reviews of immunology **24:**211-226.

65. **Sakaguchi S, Ono M, Setoguchi R, Yagi H, Hori S, Fehervari Z, Shimizu J, Takahashi T, Nomura T.** 2006. Foxp3 CD25 CD4 natural regulatory T cells in dominant self-tolerance and autoimmune disease. Immunological reviews **212:**8-27.

66. **Sakaguchi S, Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T.** 2009. Regulatory T cells: how do they suppress immune responses? International immunology **21:**1105-1111.

67. **Schmidt A, Oberle N, Krammer PH.** 2012. Molecular mechanisms of treg-mediated T cell suppression. Frontiers in immunology **3:**51.

68. **Li S, Gowans EJ, Chougnet C, Plebanski M, Dittmer U.** 2008. Natural regulatory T cells and persistent viral infection. Journal of virology **82:**21-30.

69. **Estes JD, Li Q, Reynolds MR, Wietgrefe S, Duan L, Schacker T, Picker LJ, Watkins DI, Lifson JD, Reilly C.** 2006. Premature induction of an immunosuppressive regulatory T cell response during acute simian immunodeficiency virus infection. The Journal of infectious diseases **193:**703-712.

70. **Kinter AL, Horak R, Sion M, Riggin L, McNally J, Lin Y, Jackson R, O'Shea A, Roby G, Kovacs C.** 2007. CD25+ regulatory T cells isolated from HIV-infected individuals suppress the cytolytic and nonlytic antiviral activity of HIV-specific CD8+ T cells in vitro. AIDS research and human retroviruses **23:**438-450.

71. **Miller CJ, Li Q, Abel K, Kim E-Y, Ma Z-M, Wietgrefe S, La Franco-Scheuch L, Compton L, Duan L, Shore MD.** 2005. Propagation and dissemination of infection after vaginal transmission of simian immunodeficiency virus. Journal of virology **79:**9217-9227.

72. **Reynolds MR, Rakasz E, Skinner PJ, White C, Abel K, Ma Z-M, Compton L, Napoé G, Wilson N, Miller CJ.** 2005. CD8+ T-lymphocyte response to major

immunodominant epitopes after vaginal exposure to simian immunodeficiency virus: too late and too little. Journal of virology **79:**9228-9235.

73. **Connick E, Folkvord JM, Lind KT, Rakasz EG, Miles B, Wilson NA, Santiago ML, Schmitt K, Stephens EB, Kim HO, Wagstaff R, Li S, Abdelaal HM, Kemp N, Watkins DI, MaWhinney S, Skinner PJ.** 2014. Compartmentalization of simian immunodeficiency virus replication within secondary lymphoid tissues of rhesus macaques is linked to disease stage and inversely related to localization of virus-specific CTL. Journal of immunology (Baltimore, Md: 1950) **193:**5613-5625.

74. **Connick E, Mattila T, Folkvord JM, Schlichtemeier R, Meditz AL, Ray MG, McCarter MD, Mawhinney S, Hage A, White C, Skinner PJ.** 2007. CTL fail to accumulate at sites of HIV-1 replication in lymphoid tissue. Journal of immunology (Baltimore, Md: 1950) **178:**6975-6983.

75. **Folkvord JM, Armon C, Connick E.** 2005. Lymphoid follicles are sites of heightened human immunodeficiency virus type 1 (HIV-1) replication and reduced antiretroviral effector mechanisms. AIDS Research & Human Retroviruses **21:**363-370.

76. **Hufert FT, van Lunzen J, Janossy G, Bertram S, Schmitz J, Haller O, Racz P, von Laer D.** 1997. Germinal centre CD4 T cells are an important site of HIV replication in vivo. Aids **11:**849-857.

77. **Brenchley JM, Vinton C, Tabb B, Hao XP, Connick E, Paiardini M, Lifson JD, Silvestri G, Estes JD.** 2012. Differential infection patterns of CD4+ T cells and lymphoid tissue viral burden distinguish progressive and nonprogressive lentiviral infections. Blood **120:**4172-4181.

78. **Fukazawa Y, Lum R, Okoye AA, Park H, Matsuda K, Bae JY, Hagen SI, Shoemaker R, Deleage C, Lucero C.** 2015. B cell follicle sanctuary permits persistent productive simian immunodeficiency virus infection in elite controllers. Nature medicine **21:**132-139.

79. **Tjernlund A, Zhu J, Laing K, Diem K, McDonald D, Vazquez J, Cao J, Ohlen C, McElrath MJ, Picker LJ, Corey L.** 2010. In situ detection of Gag-specific CD8+ cells in the GI tract of SIV infected Rhesus macaques. Retrovirology **7:**12-4690-4697-4612.

80. **Sasikala-Appukuttan AK, Kim HO, Kinzel NJ, Hong JJ, Smith AJ, Wagstaff R, Reilly C, Piatak Jr M, Lifson JD, Reeves RK.** 2013. Location and dynamics of the immunodominant CD8 T cell response to SIVΔnef immunization and SIVmac251 vaginal challenge.

81. **Kilby JM, Hopkins S, Venetta TM, DiMassimo B, Cloud GA, Lee JY, Alldredge L, Hunter E, Lambert D, Bolognesi D.** 1998. Potent suppression of HIV-1 replication in humans by T-20, a peptide inhibitor of gp41-mediated virus entry. Nature medicine **4**.

82. **Baggiolini M, Moser B.** 1997. Blocking chemokine receptors. Journal of Experimental Medicine **186:**1189-1191.

83. **Simon V, Ho DD, Karim QA.** 2006. HIV/AIDS epidemiology, pathogenesis, prevention, and treatment. The Lancet **368:**489-504.

84. **Shulman N, Winters M.** 2003. A review of HIV-1 resistance to the nucleoside and nucleotide inhibitors. Current Drug Targets-Infectious Disorders **3:**273-281.

85. **de Béthune M-P.** 2010. Non-nucleoside reverse transcriptase inhibitors (NNRTIs), their discovery, development, and use in the treatment of HIV-1 infection: a review of the last 20 years (1989–2009). Antiviral research **85:**75-90.

86. **Mitsuya H, Maeda K, Das D, Ghosh AK.** 2008. Development of Protease Inhibitors and the Fight with Drug-Resistant HIV-1 Variants. Advances in pharmacology **56:**169-197.

87. **Serrao E, Odde S, Ramkumar K, Neamati N.** 2009. Raltegravir, elvitegravir, and metoogravir: the birth of" me-too" HIV-1 integrase inhibitors. Retrovirology **6:**25.

88. **Palella Jr FJ, Delaney KM, Moorman AC, Loveless MO, Fuhrer J, Satten GA, Aschman DJ, Holmberg SD, Investigators HOS.** 1998. Declining morbidity and

mortality among patients with advanced human immunodeficiency virus infection. New England Journal of Medicine **338:**853-860.

89. **Stebbing J, Bower M, Mandalia S, Nelson M, Gazzard B.** 2006. Highly active anti-retroviral therapy (HAART)-induced maintenance of adaptive but not innate immune parameters is associated with protection from HIV-induced mortality. Clinical & Experimental Immunology **145:**271-276.

90. **Gulick RM, Meibohm A, Havlir D, Eron JJ, Mosley A, Chodakewitz JA, Isaacs R, Gonzalez C, McMahon D, Richman DD.** 2003. Six-year follow-up of HIV-1-infected adults in a clinical trial of antiretroviral therapy with indinavir, zidovudine, and lamivudine. Aids **17:**2345-2349.

91. **Gulick RM, Mellors JW, Havlir D, Eron JJ, Gonzalez C, McMahon D, Richman DD, Valentine FT, Jonas L, Meibohm A.** 1997. Treatment with indinavir, zidovudine, and lamivudine in adults with human immunodeficiency virus infection and prior antiretroviral therapy. New England Journal of Medicine **337:**734-739.

92. **Montessori V, Press N, Harris M, Akagi L, Montaner JS.** 2004. Adverse effects of antiretroviral therapy for HIV infection. Canadian Medical Association Journal **170:**229-238.

93. **Reust CE.** 2011. Common adverse effects of antiretroviral therapy for HIV disease. American family physician **83**.

94. **Mosmann TR, Coffman R.** 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annual review of immunology **7:**145-173.

95. **Shortman K, Egerton M, Spangrude GJ, Scollay R.** The generation and fate of thymocytes, p 3-12. *In* (ed),

96. **Evans HG, Suddason T, Jackson I, Taams LS, Lord GM.** 2007. Optimal induction of T helper 17 cells in humans requires T cell receptor ligation in the context of Toll-like

receptor-activated monocytes. Proceedings of the National Academy of Sciences **104:**17034-17039.

97. **Awasthi A, Murugaiyan G, Kuchroo VK.** 2008. Interplay between effector Th17 and regulatory T cells. Journal of clinical immunology **28:**660.

98. **Martinez GJ, Nurieva RI, Yang XO, Dong C.** 2008. Regulation and function of proinflammatory TH17 cells. Annals of the New York Academy of Sciences **1143:**188-211.

99. **Ouyang W, Kolls JK, Zheng Y.** 2008. The biological functions of T helper 17 cell effector cytokines in inflammation. Immunity **28:**454-467.

100. **Favre D, Lederer S, Kanwar B, Ma Z-M, Proll S, Kasakow Z, Mold J, Swainson L, Barbour JD, Baskin CR.** 2009. Critical loss of the balance between Th17 and T regulatory cell populations in pathogenic SIV infection. PLoS pathogens **5:**e1000295.

101. **Sakaguchi S.** 2005. Naturally arising Foxp3-expressing CD25+ CD4+ regulatory T cells in immunological tolerance to self and non-self. Nature immunology **6:**345.

102. **Tenner-Racz K, Racz P.** 1995. Follicular dendritic cells initiate and maintain infection of the germinal centers by human immunodeficiency virus, p 141-159, An Antigen Depository of the Immune System: Follicular Dendritic Cells. Springer.

103. **Wolint P, Betts MR, Koup RA, Oxenius A.** 2004. Immediate cytotoxicity but not degranulation distinguishes effector and memory subsets of CD8+ T cells. The Journal of experimental medicine **199:**925-936.

104. **Kurschus FC, Jenne DE.** 2010. Delivery and therapeutic potential of human granzyme B. Immunological reviews **235:**159-171.

105. **Trapani JA, Smyth MJ.** 2002. Functional significance of the perforin/granzyme cell death pathway. Nature reviews Immunology **2:**735.

106. **Bots M, Medema JP.** 2006. Granzymes at a glance. Journal of cell science **119:**5011-5014.

107. **Shiver JW, Su L, Henkart PA.** 1992. Cytotoxicity with target DNA breakdown by rat basophilic leukemia cells expressing both cytolysin and granzyme A. Cell **71:**315-322.

108. **Strasser A, O'Connor L, Dixit VM.** 2000. Apoptosis signaling. Annual review of biochemistry **69:**217-245.

109. **Chavez-Galan L, Arenas-Del Angel M, Zenteno E, Chavez R, Lascurain R.** 2009. Cell death mechanisms induced by cytotoxic lymphocytes. Cellular and molecular Immunology **6:**15.

110. **HARTSHORN KL, NEUMEYER D, VOGT MW, SCHOOLEY RT, HIRSCH MS.** 1987. Activity of interferons alpha, beta, and gamma against human immunodeficiency virus replication in vitro. AIDS research and human retroviruses **3:**125-133.

111. **Schroder K, Hertzog PJ, Ravasi T, Hume DA.** 2004. Interferon-γ: an overview of signals, mechanisms and functions. Journal of leukocyte biology **75:**163-189.

112. **Levy JA, Mackewicz CE, Barker E.** 1996. Controlling HIV pathogenesis: the role of the noncytotoxic anti-HIV response of CD8+ T cells. Immunology today **17:**217224-217222.

113. **Mackewicz CE, Garovoy MR, Levy JA.** 1998. HLA compatibility requirements for CD8+-T-cell-mediated suppression of human immunodeficiency virus replication. Journal of virology **72:**10165-10170.

114. **Cocchi F, DeVico AL, Garzino-Demo A, Arya SK, Gallo RC, Lusso P.** 1995. Identification of RANTES, MIP-1α, and MIP-1β as the Major HIV-Suppressive Factors Produced by CD8 ^+ T Cells. Science**:**1811-1815.

115. **Oberlin E, Amara A, Bachelerie F, Bessia C, Virelizier J-L, Arenzana-Seisdedos F, Schwartz O, Heard J-M, Legler DF, Moser B.** 1996. The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. Nature **382:**833-835.

116. **Mackewicz CE, Blackbourn DJ, Levy JA.** 1995. CD8+ T cells suppress human immunodeficiency virus replication by inhibiting viral transcription. Proceedings of the National Academy of Sciences **92:**2308-2312.

117. **Rojo JM, Bello R, Portolés P.** 2008. T-cell receptor. Multichain Immune Recognition Receptor Signaling**:**1-11.

118. **Spiliotis ET, Osorio M, Zúñiga MC, Edidin M.** 2000. Selective export of MHC class I molecules from the ER after their dissociation from TAP. Immunity **13:**841-851.

119. **Bishop GA, Hostager BS.** 2001. B lymphocyte activation by contact-mediated interactions with T lymphocytes. Current opinion in immunology **13:**278-285.

120. **Carter LL, Dutton RW.** 1996. Type 1 and type 2: a fundamental dichotomy for all T-cell subsets. Current opinion in immunology **8:**336-342.

121. **Stavnezer J.** 2000. A touch of antibody class. Science **288:**984-985.

122. **Schacker T, Little S, Connick E, Gebhard K, Zhang ZQ, Krieger J, Pryor J, Havlir D, Wong JK, Schooley RT, Richman D, Corey L, Haase AT.** 2001. Productive infection of T cells in lymphoid tissues during primary and early human immunodeficiency virus infection. The Journal of infectious diseases **183:**555-562.

123. **Reinhart TA, Rogan MJ, Huddleston D, Rausch DM, Eiden LE, Haase AT.** 1997. Simian immunodeficiency virus burden in tissues and cellular compartments during clinical latency and AIDS. The Journal of infectious diseases **176:**1198-1208.

124. **Willard-Mack CL.** 2006. Normal structure, function, and histology of lymph nodes. Toxicologic pathology **34:**409-424.

125. **Ansel KM, Harris RB, Cyster JG.** 2002. CXCL13 is required for B1 cell homing, natural antibody production, and body cavity immunity. Immunity **16:**67-76.

126. **MacLennan I, Liu Y, Oldfield S, Zhang J, Lane P.** 1990. The evolution of B-cell clones, p 37-63, Immunological Memory. Springer.

127. **Rajewsky K.** 1996. Clonal selection and learning in the antibody system. Nature **381:**751.

128. **Hess J, Laumen H, Müller KB, Wirth T.** 1998. Molecular genetics of the germinal center reaction. Journal of cellular physiology **177:**525-534.

129. **Natkunam Y.** 2007. The biology of the germinal center. ASH Education Program Book **2007:**210-215.

130. **Meyer-Hermann M.** 2002. A mathematical model for the germinal center morphology and affinity maturation. Journal of theoretical Biology **216:**273-300.

131. **Crotty S.** 2014. T follicular helper cell differentiation, function, and roles in disease. Immunity **41:**529-542.

132. **Miles B, Connick E.** 2016. T FH in HIV latency and as sources of replication-competent virus. Trends in microbiology **24:**338-344.

133. **Cyster JG, Ansel KM, Reif K, Ekland EH, Hyman PL, Tang HL, Luther SA, Ngo VN.** 2000. Follicular stromal cells and lymphocyte homing to follicles. Immunological reviews **176:**181-193.

134. **Streeck H.** 2015. AIDS virus seeks refuge in B cell follicles. Nature medicine **21:**111-112.

135. **Pallikkuth S, Sharkey M, Babic DZ, Gupta S, Stone GW, Fischl MA, Stevenson M, Pahwa S.** 2016. Peripheral T follicular helper cells are the major HIV reservoir within central memory CD4 T cells in peripheral blood from chronically HIV-infected individuals on combination antiretroviral therapy. Journal of virology **90:**2718-2728.

136. **Banga R, Procopio FA, Noto A, Pollakis G, Cavassini M, Ohmiti K, Corpataux J-M, de Leval L, Pantaleo G, Perreau M.** 2016. PD-1+ and follicular helper T cells are responsible for persistent HIV-1 transcription in treated aviremic individuals. Nature medicine **22:**754-761.

137. **Perreau M, Savoye A-L, De Crignis E, Corpataux J-M, Cubas R, Haddad EK, De Leval L, Graziosi C, Pantaleo G.** 2013. Follicular helper T cells serve as the major CD4 T cell compartment for HIV-1 infection, replication, and production. Journal of Experimental Medicine **210:**143-156.

138. **Lindqvist M, van Lunzen J, Soghoian DZ, Kuhl BD, Ranasinghe S, Kranias G, Flanders MD, Cutler S, Yudanin N, Muller MI.** 2012. Expansion of HIV-specific T follicular helper cells in chronic HIV infection. The Journal of clinical investigation **122:**3271.

139. **Petrovas C, Yamamoto T, Gerner MY, Boswell KL, Wloka K, Smith EC, Ambrozak DR, Sandler NG, Timmer KJ, Sun X.** 2012. CD4 T follicular helper cell dynamics during SIV infection. The Journal of clinical investigation **122:**3281.

140. **Chowdhury A, Del Rio PME, Tharp GK, Trible RP, Amara RR, Chahroudi A, Reyes-Teran G, Bosinger SE, Silvestri G.** 2015. Decreased T follicular regulatory cell/T follicular helper cell (TFH) in simian immunodeficiency virus–infected rhesus macaques may contribute to accumulation of TFH in chronic infection. The Journal of Immunology **195:**3237-3247.

141. **Spiegel H, Herbst H, Niedobitek G, Foss H-D, Stein H.** 1992. Follicular dendritic cells are a major reservoir for human immunodeficiency virus type 1 in lymphoid tissues facilitating infection of CD4+ T-helper cells. The American journal of pathology **140:**15.

142. **Haase AT, Henry K, Zupancic M, Sedgewick G, Faust RA, Melroe H, Cavert W, Gebhard K, Staskus K, Zhang Z-Q.** 1996. Quantitative image analysis of HIV-1 infection in lymphoid tissue. Science**:**985-989.

143. **Heath SL, Tew JG, Tew JG, Szakal AK, Burton GF.** 1995. Follicular dendritic cells and human immunodeficiency virus infectivity. Nature **377:**740-744.

144. **Thacker TC, Zhou X, Estes JD, Jiang Y, Keele BF, Elton TS, Burton GF.** 2009. Follicular dendritic cells and human immunodeficiency virus type 1 transcription in CD4+ T cells. Journal of virology **83:**150-158.

145. **Kannagi M, Chalifoux LV, Lord CI, Letvin NL.** 1988. Suppression of simian immunodeficiency virus replication in vitro by CD8+ lymphocytes. Journal of immunology (Baltimore, Md: 1950) **140:**2237-2242.

146. **Walker CM, Moody DJ, Stites DP, Levy JA.** 1986. CD8+ lymphocytes can control HIV infection in vitro by suppressing virus replication. Science (New York, NY) **234:**1563-1566.

147. **Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MB.** 1994. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. Journal of virology **68:**6103-6110.

148. **Koup RA, Safrit JT, Cao Y, Andrews CA, McLeod G, Borkowsky W, Farthing C, Ho DD.** 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. Journal of virology **68:**4650-4655.

149. **Li Q, Skinner PJ, Ha SJ, Duan L, Mattila TL, Hage A, White C, Barber DL, O'Mara L, Southern PJ, Reilly CS, Carlis JV, Miller CJ, Ahmed R, Haase AT.** 2009. Visualizing antigen-specific and infected cells in situ predicts outcomes in early viral infection. Science (New York, NY) **323:**1726-1729.

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

151. **Migueles SA, Laborico AC, Shupert WL, Sabbaghian MS, Rabin R, Hallahan CW, Van Baarle D, Kostense S, Miedema F, McLaughlin M.** 2002. HIV-specific CD8 T

cell proliferation is coupled to perforin expression and is maintained in nonprogressors. Nature immunology **3:**1061-1068.

152. **Walker BD, Goulder PJ.** 2000. AIDS. Escape from the immune system. Nature **407:**313-314.

153. **Barouch DH, Kunstman J, Kuroda MJ, Schmitz JE, Santra S, Peyerl FW, Krivulka GR, Beaudry K, Lifton MA, Gorgone DA.** 2002. Eventual AIDS vaccine failure in a rhesus monkey by viral escape from cytotoxic T lymphocytes. Nature **415:**335-339.

154. **Chopera DR, Mlotshwa M, Woodman Z, Mlisana K, de Assis Rosa D, Martin DP, Abdool Karim S, Gray CM, Williamson C, Team CS.** 2011. Virological and immunological factors associated with HIV-1 differential disease progression in HLA-B 58:01-positive individuals. Journal of virology **85:**7070-7080.

155. **Goulder PJR, Phillips RE, Colbert RA, McAdam S, Ogg G, Giangrande P, Luzzi G, Morgan B, Edwards A, McMichael A.** 1997. Late escape from an immunodominant cytotoxic T lymphocyte response associated with progression to AIDS. Immunology letters **56:**25.

156. **Kelleher AD, Long C, Holmes EC, Allen RL, Wilson J, Conlon C, Workman C, Shaunak S, Olson K, Goulder P, Brander C, Ogg G, Sullivan JS, Dyer W, Jones I, McMichael AJ, Rowland-Jones S, Phillips RE.** 2001. Clustered mutations in HIV-1 gag are consistently required for escape from HLA-B27-restricted cytotoxic T lymphocyte responses. The Journal of experimental medicine **193:**375-386.

157. **Ferre AL, Hunt PW, Critchfield JW, Young DH, Morris MM, Garcia JC, Pollard RB, Yee HF, Jr., Martin JN, Deeks SG, Shacklett BL.** 2009. Mucosal immune responses to HIV-1 in elite controllers: a potential correlate of immune control. Blood **113:**3978-3989.

158. **Deeks SG, Walker BD.** 2007. Human immunodeficiency virus controllers: mechanisms of durable virus control in the absence of antiretroviral therapy. Immunity **27:**406-416.

159. **Lambotte O, Boufassa F, Madec Y, Nguyen A, Goujard C, Meyer L, Rouzioux C, Venet A, Delfraissy JF, Group S-HS.** 2005. HIV controllers: a homogeneous group of HIV-1-infected patients with spontaneous control of viral replication. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America **41:**1053-1056.

160. **Mudd PA, Watkins DI.** 2011. Understanding animal models of elite control: windows on effective immune responses against immunodeficiency viruses. Current opinion in HIV and AIDS **6:**197-201.

161. **Saez-Cirion A, Lacabaratz C, Lambotte O, Versmisse P, Urrutia A, Boufassa F, Barre-Sinoussi F, Delfraissy JF, Sinet M, Pancino G, Venet A, Agence Nationale de Recherches sur le Sida EPHIVCSG.** 2007. HIV controllers exhibit potent CD8 T cell capacity to suppress HIV infection ex vivo and peculiar cytotoxic T lymphocyte activation phenotype. Proceedings of the National Academy of Sciences of the United States of America **104:**6776-6781.

162. **Walker BD, Xu GY.** 2013. Unravelling the mechanisms of durable control of HIV-1. Nature Reviews Immunology **13:**487-498.

163. **Skinner PJ, Connick E.** 2014. Overcoming the immune privilege of B cell follicles to cure HIV-1 infection. JHumVirolRetrovirol **1:**00001-00003.

164. **Bailey JR, Williams TM, Siliciano RF, Blankson JN.** 2006. Maintenance of viral suppression in HIV-1-infected HLA-B*57+ elite suppressors despite CTL escape mutations. The Journal of experimental medicine **203:**1357-1369.

165. **Goulder PJR, Watkins DI.** 2004. HIV and SIV CTL escape: implications for vaccine design. Nature Reviews Immunology **4:**630-640.

166. **Mens H, Kearney M, Wiegand A, Shao W, Schonning K, Gerstoft J, Obel N, Maldarelli F, Mellors JW, Benfield T, Coffin JM.** 2010. HIV-1 continues to replicate

and evolve in patients with natural control of HIV infection. Journal of virology **84:**12971-12981.

167. **Chowdhury A, Hayes TL, Bosinger SE, Lawson BO, Vanderford T, Schmitz JE, Paiardini M, Betts M, Chahroudi A, Estes JD, Silvestri G.** 2015. Differential Impact of In Vivo CD8+ T Lymphocyte Depletion in Controller versus Progressor Simian Immunodeficiency Virus-Infected Macaques. Journal of virology **89:**8677-8686.

168. **Jin X, Bauer DE, Tuttleton SE, Lewin S, Gettie A, Blanchard J, Irwin CE, Safrit JT, Mittler J, Weinberger L, Kostrikis LG, Zhang L, Perelson AS, Ho DD.** 1999. Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. The Journal of experimental medicine **189:**991-998.

169. **Matano T, Shibata R, Siemon C, Connors M, Lane HC, Martin MA.** 1998. Administration of an anti-CD8 monoclonal antibody interferes with the clearance of chimeric simian/human immunodeficiency virus during primary infections of rhesus macaques. Journal of virology **72:**164-169.

170. **Schmitz JE, Kuroda MJ, Santra S, Sasseville VG, Simon MA, Lifton MA, Racz P, Tenner-Racz K, Dalesandro M, Scallon BJ, Ghrayeb J, Forman MA, Montefiori DC, Rieber EP, Letvin NL, Reimann KA.** 1999. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. Science (New York, NY) **283:**857-860.

171. **Brodie SJ, Lewinsohn DA, Patterson BK, Jiyamapa D, Krieger J, Corey L, Greenberg PD, Riddell SR.** 1999. In vivo migration and function of transferred HIV-1-specific cytotoxic T cells. Nature medicine **5**.

172. **Koenig S, Conley AJ, Brewah YA, Jones GM, Leath S, Boots LJ, Davey V, Pantaleo G, Demarest JF, Carter C.** 1995. Transfer of HIV-1-specific cytotoxic T lymphocytes

to an AIDS patient leads to selection for mutant HIV variants and subsequent disease progression. Nature medicine **1:**330-336.

173. **Mitsuyasu RT, Anton PA, Deeks SG, Scadden DT, Connick E, Downs MT, Bakker A, Roberts MR, June CH, Jalali S.** 2000. Prolonged survival and tissue trafficking following adoptive transfer of CD4ζ gene-modified autologous CD4+ and CD8+ T cells in human immunodeficiency virus–infected subjects. Blood **96:**785-793.

174. **He R, Hou S, Liu C, Zhang A, Bai Q, Han M, Yang Y, Wei G, Shen T, Yang X.** 2016. Follicular CXCR5-expressing CD8 T cells curtail chronic viral infection. Nature.

175. **Schacker T, Little S, Connick E, Gebhard-Mitchell K, Zhang Q, Krieger J.** 2000. Rapid accumulation of HIV in lymphatic tissue reservoirs during acute and early HIV infection: implications for timing of antiretroviral therapy. J Infect Dis **181:**354-357.

176. **Elahi S, Dinges WL, Lejarcegui N, Laing KJ, Collier AC, Koelle DM, McElrath MJ, Horton H.** 2011. Protective HIV-specific CD8 T cells evade Treg cell suppression. Nature medicine **17:**989-995.

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

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

179. **Day CL, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, Mackey EW, Miller JD, Leslie AJ, DePierres C.** 2006. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. Nature **443:**350-354.

180. **Sharpe AH, Wherry EJ, Ahmed R, Freeman GJ.** 2007. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. Nature immunology **8:**239-245.

181. **Förster R, Mattis AE, Kremmer E, Wolf E, Brem G, Lipp M.** 1996. A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. Cell **87:**1037-1047.

182. **Ansel KM, Ngo VN, Hyman PL, Luther SA.** 2000. A chemokine-driven positive feedback loop organizes lymphoid follicles. Nature **406:**309.

183. **Haynes NM, Allen CD, Lesley R, Ansel KM, Killeen N, Cyster JG.** 2007. Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1high germinal center-associated subpopulation. The Journal of Immunology **179:**5099-5108.

184. **Burton JD, Bamford RN, Peters C, Grant AJ, Kurys G, Goldman CK, Brennan J, Roessler E, Waldmann TA.** 1994. A lymphokine, provisionally designated interleukin T and produced by a human adult T-cell leukemia line, stimulates T-cell proliferation and the induction of lymphokine-activated killer cells. Proceedings of the National Academy of Sciences **91:**4935-4939.

185. **Gilboa E, Yewdell J, Bennink J, Schwartz R.** 1994. Cloning of a T Cell Growth Factor That Interacts with the 1B Chain of the lnterleukin-2 Receptor. Science **264:**965.

186. **Steel JC, Waldmann TA, Morris JC.** 2012. Interleukin-15 biology and its therapeutic implications in cancer. Trends in pharmacological sciences **33:**35-41.

187. **Di Sabatino A, Calarota SA, Vidali F, MacDonald TT, Corazza GR.** 2011. Role of IL-15 in immune-mediated and infectious diseases. Cytokine & growth factor reviews **22:**19-33.

188. **Waldmann T, Tagaya Y.** 1999. The multifaceted regulation of interleukin-15 expression and the role of this cytokine in NK cell differentiation and host response to intracellular pathogens. Annual review of immunology **17:**19-49.

189. **Kennedy MK, Glaccum M, Brown SN, Butz EA, Viney JL, Embers M, Matsuki N, Charrier K, Sedger L, Willis CR.** 2000. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15–deficient mice. Journal of Experimental Medicine **191:**771-780.

190. **Berger C, Berger M, Hackman RC, Gough M, Elliott C, Jensen MC, Riddell SR.** 2009. Safety and immunologic effects of IL-15 administration in nonhuman primates. Blood **114:**2417-2426.

191. **Marks-Konczalik J, Dubois S, Losi JM, Sabzevari H, Yamada N, Feigenbaum L, Waldmann TA, Tagaya Y.** 2000. IL-2-induced activation-induced cell death is inhibited in IL-15 transgenic mice. Proceedings of the National Academy of Sciences **97:**11445-11450.

192. **Mueller YM, Petrovas C, Bojczuk PM, Dimitriou ID, Beer B, Silvera P, Villinger F, Cairns JS, Gracely EJ, Lewis MG.** 2005. Interleukin-15 increases effector memory CD8+ t cells and NK Cells in simian immunodeficiency virus-infected macaques. Journal of Virology **79:**4877-4885.

193. **Picker LJ, Reed-Inderbitzin EF, Hagen SI, Edgar JB, Hansen SG, Legasse A, Planer S, Piatak M, Lifson JD, Maino VC.** 2006. IL-15 induces CD4+ effector memory T cell production and tissue emigration in nonhuman primates. Journal of Clinical Investigation **116:**1514.

194. **Dubois S, Mariner J, Waldmann TA, Tagaya Y.** 2002. IL-15Rα recycles and presents IL-15 in trans to neighboring cells. Immunity **17:**537-547.

195. **Stonier SW, Schluns KS.** 2010. Trans-presentation: a novel mechanism regulating IL-15 delivery and responses. Immunology letters **127:**85-92.

196. **Lodolce JP, Boone DL, Chai S, Swain RE, Dassopoulos T, Trettin S, Ma A.** 1998. IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. Immunity **9:**669-676.

197. **Zhu X, Marcus WD, Xu W, Lee H-i, Han K, Egan JO, Yovandich JL, Rhode PR, Wong HC.** 2009. Novel human interleukin-15 agonists. The Journal of Immunology **183:**3598-3607.

198. **Rhode PR, Egan JO, Xu W, Hong H, Webb GM, Chen X, Liu B, Zhu X, Wen J, You L.** 2015. Comparison of the superagonist complex, ALT-803, to IL15 as cancer immunotherapeutics in animal models. Cancer immunology research.

199. **Xu W, Jones M, Liu B, Zhu X, Johnson CB, Edwards AC, Kong L, Jeng EK, Han K, Marcus WD.** 2013. Efficacy and mechanism-of-action of a novel superagonist interleukin-15: interleukin-15 receptor αSu/Fc fusion complex in syngeneic murine models of multiple myeloma. Cancer research **73:**3075-3086.

200. **Seay K, Church C, Zheng JH, Deneroff K, Ochsenbauer C, Kappes JC, Liu B, Jeng EK, Wong HC, Goldstein H.** 2015. In vivo activation of human NK cells by treatment with an interleukin-15 superagonist potently inhibits acute in vivo HIV-1 infection in humanized mice. Journal of virology **89:**6264-6274.

201. **Jones RB, Mueller S, O'Connor R, Rimpel K, Sloan DD, Karel D, Wong HC, Jeng EK, Thomas AS, Whitney JB.** 2016. A subset of latency-reversing agents expose HIV-infected resting CD4+ T-cells to recognition by cytotoxic T-lymphocytes. PLoS pathogens **12:**e1005545.

202. **Schacker T, Little S, Connick E, Gebhard-Mitchell K, Zhang ZQ, Krieger J, Pryor J, Havlir D, Wong JK, Richman D, Corey L, Haase AT.** 2000. Rapid accumulation of human immunodeficiency virus (HIV) in lymphatic tissue reservoirs during acute and early HIV infection: implications for timing of antiretroviral therapy. The Journal of infectious diseases **181:**354-357.

203. **Hersperger AR, Pereyra F, Nason M, Demers K, Sheth P, Shin LY, Kovacs CM, Rodriguez B, Sieg SF, Teixeira-Johnson L, Gudonis D, Goepfert PA, Lederman MM, Frank I, Makedonas G, Kaul R, Walker BD, Betts MR.** 2010. Perforin expression directly ex vivo by HIV-specific CD8 T-cells is a correlate of HIV elite control. PLoS pathogens **6:**e1000917.

204. **Migueles SA, Sabbaghian MS, Shupert WL, Bettinotti MP, Marincola FM, Martino L, Hallahan CW, Selig SM, Schwartz D, Sullivan J, Connors M.** 2000. HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. Proceedings of the National Academy of Sciences of the United States of America **97:**2709-2714.

205. **Hong JJ, Reynolds MR, Mattila TL, Hage A, Watkins DI, Miller CJ, Skinner PJ.** 2009. Localized populations of CD8 MHC class I tetramer SIV-specific T cells in lymphoid follicles and genital epithelium. PloS one **4:**e4131.

206. **Xu H, Wang X, Lackner AA, Veazey RS.** 2013. CD8 down-regulation and functional impairment of SIV-specific cytotoxic T lymphocytes in lymphoid and mucosal tissues during SIV infection. Journal of leukocyte biology **93:**943-950.

207. **Quigley MF, Gonzalez VD, Granath A, Andersson J, Sandberg JK.** 2007. CXCR5 CCR7–CD8 T cells are early effector memory cells that infiltrate tonsil B cell follicles. European journal of immunology **37:**3352-3362.

208. **Abdelaal HM, Kim HO, Wagstaff R, Sawahata R, Southern PJ, Skinner PJ.** 2015. Comparison of Vibratome and Compresstome sectioning of fresh primate lymphoid and genital tissues for in situ MHC-tetramer and immunofluorescence staining. Biological Procedures Online **17:**2-014-0012-0014. eCollection 2015.

209. **Skinner PJ, Daniels MA, Schmidt CS, Jameson SC, Haase AT.** 2000. Cutting edge: In situ tetramer staining of antigen-specific T cells in tissues. Journal of immunology (Baltimore, Md: 1950) **165:**613-617.

210. **Allen TM, Sidney J, del Guercio MF, Glickman RL, Lensmeyer GL, Wiebe DA, DeMars R, Pauza CD, Johnson RP, Sette A, Watkins DI.** 1998. Characterization of the peptide binding motif of a rhesus MHC class I molecule (Mamu-A*01) that binds an immunodominant CTL epitope from simian immunodeficiency virus. Journal of immunology (Baltimore, Md: 1950) **160:**6062-6071.

211. **Loffredo JT, Sidney J, Bean AT, Beal DR, Bardet W, Wahl A, Hawkins OE, Piaskowski S, Wilson NA, Hildebrand WH, Watkins DI, Sette A.** 2009. Two MHC class I molecules associated with elite control of immunodeficiency virus replication, Mamu-B*08 and HLA-B*2705, bind peptides with sequence similarity. Journal of immunology (Baltimore, Md: 1950) **182:**7763-7775.

212. **Loffredo JT, Sidney J, Wojewoda C, Dodds E, Reynolds MR, Napoe G, Mothe BR, O'Connor DH, Wilson NA, Watkins DI, Sette A.** 2004. Identification of seventeen new simian immunodeficiency virus-derived CD8+ T cell epitopes restricted by the high frequency molecule, Mamu-A*02, and potential escape from CTL recognition. Journal of immunology (Baltimore, Md: 1950) **173:**5064-5076.

213. **Parsons NR, Costa ML, Achten J, Stallard N.** 2009. Repeated measures proportional odds logistic regression analysis of ordinal score data in the statistical software package R. Computational Statistics & Data Analysis **53:**632-641.

214. **Parsons NR, Edmondson RN, Gilmour SG.** 2006. A generalized estimating equation method for fitting autocorrelated ordinal score data with an application in horticultural research. Journal of the Royal Statistical Society: Series C (Applied Statistics) **55:**507-524.

215. **Parsons NR.** 2013. Proportional-odds models for repeated composite and long ordinal outcome scales. Statistics in medicine **32:**3181-3191.

216. **Team RC.** 2014. R: A language and environment for statistical computingR Foundation for Statistical Computing, Vienna, Austria2013.

217. **Trautmann L, Janbazian L, Chomont N, Said EA, Gimmig S, Bessette B, Boulassel M-R, Delwart E, Sepulveda H, Balderas RS.** 2006. Upregulation of PD-1 expression on HIV-specific CD8 T cells leads to reversible immune dysfunction. Nature medicine **12:**1198-1202.

218. **Velu V, Kannanganat S, Ibegbu C, Chennareddi L, Villinger F, Freeman GJ, Ahmed R, Amara RR.** 2007. Elevated expression levels of inhibitory receptor programmed death 1 on simian immunodeficiency virus-specific CD8 T cells during chronic infection but not after vaccination. Journal of virology **81:**5819-5828.

219. **Velu V, Titanji K, Zhu B, Husain S, Pladevega A, Lai L, Vanderford TH, Chennareddi L, Silvestri G, Freeman GJ.** 2009. Enhancing SIV-specific immunity in vivo by PD-1 blockade. Nature **458:**206-210.

220. **Hori S, Nomura T, Sakaguchi S.** 2003. Control of regulatory T cell development by the transcription factor Foxp3. Science (New York, NY) **299:**1057-1061.

221. **Fontenot JD, Gavin MA, Rudensky AY.** 2003. Foxp3 programs the development and function of CD4 CD25 regulatory T cells. Nature immunology **4:**330-336.

222. **Kim H-J, Verbinnen B, Tang X, Lu L, Cantor H.** 2010. Inhibition of follicular T-helper cells by CD8 regulatory T cells is essential for self tolerance. Nature **467:**328-332.

223. **Suzuki M, Jagger AL, Konya C, Shimojima Y, Pryshchep S, Goronzy JJ, Weyand CM.** 2012. CD8+CD45RA+CCR7+FOXP3+ T cells with immunosuppressive properties: a novel subset of inducible human regulatory T cells. Journal of immunology (Baltimore, Md: 1950) **189:**2118-2130.

224. **Nigam P, Velu V, Kannanganat S, Chennareddi L, Kwa S, Siddiqui M, Amara RR.** 2010. Expansion of FOXP3+ CD8 T cells with suppressive potential in colorectal mucosa following a pathogenic simian immunodeficiency virus infection correlates with diminished antiviral T cell response and viral control. Journal of immunology (Baltimore, Md: 1950) **184:**1690-1701.

225. **Park HJ, Park JS, Jeong YH, Son J, Ban YH, Lee BH, Chen L, Chang J, Chung DH, Choi I, Ha SJ.** 2015. PD-1 upregulated on regulatory T cells during chronic virus infection enhances the suppression of CD8+ T cell immune response via the interaction with PD-L1 expressed on CD8+ T cells. Journal of immunology (Baltimore, Md: 1950) **194:**5801-5811.

226. **Scholzen T, Gerdes J.** 2000. The Ki-67 protein: from the known and the unknown. Journal of cellular physiology **182:**311-322.

227. **Soares A, Govender L, Hughes J, Mavakla W, de Kock M, Barnard C, Pienaar B, van Rensburg EJ, Jacobs G, Khomba G.** 2010. Novel application of Ki67 to quantify antigen-specific in vitro lymphoproliferation. Journal of immunological methods **362:**43-50.

228. **Sallusto F, Geginat J, Lanzavecchia A.** 2004. Central memory and effector memory T cell subsets: function, generation, and maintenance. AnnuRevImmunol **22:**745-763.

229. **Leong YA, Chen Y, Ong HS, Wu D, Man K, Deleage C, Minnich M, Meckiff BJ, Wei Y, Hou Z.** 2016. CXCR5 follicular cytotoxic T cells control viral infection in B cell follicles. Nature immunology.

230. **Pavlakis GN, Bergamaschi C, Li J, Valentin A, Chen S, Ng SS, Beach REK, Bear J, Rosati M, Alicea C.** 2013. Heterodimeric IL-15 regulates the differentiation and survival of different populations of memory T cells and the balance of effector and regulatory cells. Journal for immunotherapy of cancer **1:**1-1.

231. **Salcedo R, Hixon JA, Stauffer JK, Jalah R, Brooks AD, Khan T, Dai RM, Scheetz L, Lincoln E, Back TC, Powell D, Hurwitz AA, Sayers TJ, Kastelein R, Pavlakis GN, Felber BK, Trinchieri G, Wigginton JM.** 2009. Immunologic and therapeutic synergy of IL-27 and IL-2: enhancement of T cell sensitization, tumor-specific CTL reactivity and complete regression of disseminated neuroblastoma metastases in the liver and bone marrow. Journal of immunology (Baltimore, Md: 1950) **182:**4328-4338.

232. **Gratton S, Cheynier R, Dumaurier M-J, Oksenhendler E, Wain-Hobson S.** 2000. Highly restricted spread of HIV-1 and multiply infected cells within splenic germinal centers. Proceedings of the National Academy of Sciences **97:**14566-14571.

233. **Mylvaganam GH, Velu V, Hong J-J, Sadagopal S, Kwa S, Basu R, Lawson B, Villinger F, Amara RR.** 2014. Diminished Viral Control during SIV Infection is Associated with Aberrant PD-1hi CD4 T cell Enrichment in the Lymphoid Follicles of the Rectal Mucosa. Journal of immunology (Baltimore, Md: 1950) **193:**4527.

234. **Ndhlovu ZM, Kamya P, Mewalal N, Kløverpris HN, Nkosi T, Pretorius K, Laher F, Ogunshola F, Chopera D, Shekhar K.** 2015. Magnitude and kinetics of CD8+ T cell activation during hyperacute HIV infection impact viral set point. Immunity **43:**591-604.

235. **Streeck H, Lu R, Beckwith N, Milazzo M, Liu M, Routy J-P, Little S, Jessen H, Kelleher AD, Hecht F.** 2014. Emergence of individual HIV-specific CD8 T cell responses during primary HIV-1 infection can determine long-term disease outcome. Journal of virology **88:**12793-12801.

236. **Li S, Folkvord JM, Rakasz EG, Abdelaal HM, Wagstaff RK, Kovacs KJ, Kim HO, Sawahata R, MaWhinney S, Masopust D.** 2016. Simian immunodeficiency virus-producing cells in follicles are partially suppressed by CD8+ cells in vivo. Journal of virology **90:**11168-11180.

237. **Petrovas C, Ferrando-Martinez S, Gerner MY, Casazza JP, Pegu A, Deleage C, Cooper A, Hataye J, Andrews S, Ambrozak D.** 2017. Follicular CD8 T cells accumulate in HIV infection and can kill infected cells in vitro via bispecific antibodies. Science translational medicine **9:**eaag2285.

238. **Mylvaganam GH, Rios D, Abdelaal HM, Iyer S, Tharp G, Mavinger M, Hicks S, Chahroudi A, Ahmed R, Bosinger SE.** 2017. Dynamics of SIV-specific CXCR5+ CD8 T cells during chronic SIV infection. Proceedings of the National Academy of Sciences **114:**1976-1981.

239. **Sakaguchi S.** 2005. Naturally arising Foxp3-expressing CD25 CD4 regulatory T cells in immunological tolerance to self and non-self. Nature immunology **6:**345-352.

240. **Yu S-W, Andrabi SA, Wang H, Kim NS, Poirier GG, Dawson TM, Dawson VL.** 2006. Apoptosis-inducing factor mediates poly (ADP-ribose)(PAR) polymer-induced cell death. Proceedings of the National Academy of Sciences **103:**18314-18319.

241. **Lugli E, Goldman CK, Perera LP, Smedley J, Pung R, Yovandich JL, Creekmore SP, Waldmann TA, Roederer M.** 2010. Transient and persistent effects of IL-15 on lymphocyte homeostasis in nonhuman primates. Blood **116:**3238-3248.

242. **Vandergeeten C, DaFonseca S, Fromentin R, Sekaly R, Chomont N.** Differential impact of IL-7 and IL-15 on HIV reservoir persistence, p. *In* (ed),

243. **Rosario M, Liu B, Kong L, Collins LI, Schneider SE, Chen X, Han K, Jeng EK, Rhode PR, Leong JW.** 2016. The IL-15-based ALT-803 complex enhances FcγRIIIa-triggered NK cell responses and in vivo clearance of B cell lymphomas. Clinical Cancer Research **22:**596-608.

244. **Kim PS, Kwilas AR, Xu W, Alter S, Jeng EK, Wong HC, Schlom J, Hodge JW.** 2016. IL-15 superagonist/IL-15RαSushi-Fc fusion complex (IL-15SA/IL-15RαSu-Fc; ALT-803) markedly enhances specific subpopulations of NK and memory CD8+ T cells, and mediates potent anti-tumor activity of murine breast and colon carcinomas. AACR.

245. **Naranbhai V, Altfeld M, Karim SSA, Ndung'u T, Karim QA, Carr WH.** 2013. Changes in Natural Killer cell activation and function during primary HIV-1 Infection. PloS one **8:**e53251.

246. **Burgoyne RW, Tan DH.** 2008. Prolongation and quality of life for HIV-infected adults treated with highly active antiretroviral therapy (HAART): a balancing act. Journal of antimicrobial chemotherapy **61:**469-473.

247. **Chen LF, Hoy J, Lewin SR.** 2007. Ten years of highly active antiretroviral therapy for HIV infection. Medical Journal of Australia **186:**146.

248. **Ayala VI, Trivett MT, Barsov EV, Jain S, Piatak M, Trubey CM, Alvord WG, Chertova E, Roser JD, Smedley J.** 2016. Adoptive transfer of engineered rhesus simian immunodeficiency virus-specific CD8+ T cells reduces the number of transmitted/founder viruses established in rhesus macaques. Journal of virology **90:**9942-9952.

249. **Miles B, Miller SM, Folkvord JM, Kimball A, Chamanian M, Meditz AL, Arends T, McCarter MD, Levy DN, Rakasz EG.** 2015. Follicular regulatory T cells impair follicular T helper cells in HIV and SIV infection. Nature communications **6**.

250. **Salcedo R, Hixon JA, Stauffer JK, Jalah R, Brooks AD, Khan T, Dai R-M, Scheetz L, Lincoln E, Back TC.** 2009. Immunologic and therapeutic synergy of IL-27 and IL-2: enhancement of T cell sensitization, tumor-specific CTL reactivity and complete regression of disseminated neuroblastoma metastases in the liver and bone marrow. The Journal of Immunology **182:**4328-4338.

251. **Ayala VI, Deleage C, Trivett MT, Jain S, Coren LV, Breed MW, Kramer JA, Thomas JA, Estes JD, Lifson JD.** 2017. CXCR5-Dependent Entry of CD8 T Cells into Rhesus Macaque B-Cell Follicles Achieved through T-Cell Engineering. Journal of Virology **91:**e02507-02516.

252. **Barsov EV, Trivett MT, Minang JT, Sun H, Ohlen C, Ott DE.** 2011. Transduction of SIV-specific TCR genes into rhesus macaque CD8+ T cells conveys the ability to suppress SIV replication. PLoS One **6:**e23703.

253. **Phan GQ, Rosenberg SA.** 2013. Adoptive cell transfer for patients with metastatic melanoma: the potential and promise of cancer immunotherapy. Cancer Control **20:**289-297.

254. **Barrett DM, Grupp SA, June CH.** 2015. Chimeric antigen receptor–and TCR-modified T cells enter main street and wall street. The Journal of Immunology **195:**755-761.

255. **Zhang L, Morgan RA.** 2012. Genetic engineering with T cell receptors. Advanced drug delivery reviews **64:**756-762.

256. **Morgan RA, Dudley ME, Yik Y, Zheng Z, Robbins PF, Theoret MR, Wunderlich JR, Hughes MS, Restifo NP, Rosenberg SA.** 2003. High efficiency TCR gene transfer into primary human lymphocytes affords avid recognition of melanoma tumor antigen glycoprotein 100 and does not alter the recognition of autologous melanoma antigens. The Journal of Immunology **171:**3287-3295.

257. **Morgan RA, Dudley ME, Wunderlich JR, Hughes MS, Yang JC, Sherry RM, Royal RE, Topalian SL, Kammula US, Restifo NP.** 2006. Cancer regression in patients after transfer of genetically engineered lymphocytes. Science **314:**126-129.

258. **Minang JT, Trivett MT, Bolton DL, Trubey CM, Estes JD, Li Y, Smedley J, Pung R, Rosati M, Jalah R.** 2010. Distribution, persistence, and efficacy of adoptively transferred central and effector memory-derived autologous simian immunodeficiency virus-specific CD8+ T cell clones in rhesus macaques during acute infection. The journal of immunology **184:**315-326.

259. **Bolton DL, Minang JT, Trivett MT, Song K, Tuscher JJ, Li Y, Piatak M, O'Connor D, Lifson JD, Roederer M.** 2010. Trafficking, persistence, and activation state of adoptively transferred allogeneic and autologous Simian Immunodeficiency Virus-specific CD8+ T cell clones during acute and chronic infection of rhesus macaques. The journal of immunology **184:**303-314.

260. **Siliciano JD, Kajdas J, Finzi D, Quinn TC, Chadwick K, Margolick JB, Kovacs C, Gange SJ, Siliciano RF.** 2003. Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting CD4+ T cells. Nature medicine **9:**727.

261. **Shan L, Deng K, Shroff NS, Durand CM, Rabi SA, Yang H-C, Zhang H, Margolick JB, Blankson JN, Siliciano RF.** 2012. Stimulation of HIV-1-specific cytolytic T

lymphocytes facilitates elimination of latent viral reservoir after virus reactivation. Immunity **36:**491-501.

Appendix

Publications:

1. **Shengbin Li**, Joy M. Folkvord, Katalin J Kovacs, Reece K Wagstaff , Gwantwa Mwakalundwa, Eva G Rakasz, Elizabeth Connick and Pamela J Skinner, Low levels of SIV-specific CD8 T cells in germinal centers during early infection may set the stage for persistent chronic infection (Manuscript in preparation).
2. **Li S**, Kovacs KJ, Kiniry BE, Wagstaff RK, Ferre AL, Hunt PW, Somsouk M, Shacklet BL, Skinner PJ, Distribution and phenotype of HIV-specific CD8 T cells in situ in the rectal mucosa of HIV^+ elite controllers (Manuscript in preparation).
3. Gabriela M. Webb*, **Shengbin Li***, Justin M. Greene, Jason S. Reed, Jeffery J. Stanton, Alfred W. Legasse, Byung S. Park, Michael K. Axthelm, Emily K. Jeng, Hing C. Wong, James B. Whitney, R. Brad Jones, Douglas F. Nixon, Pamela J. Skinner, Jonah B. Sacha. The human IL-15 superagonist ALT-803 directs SIV-specific $CD8^+$ T cells into B cell follicles. Blood Advances 2018 2:76-84; doi: https://doi.org/10.1182/bloodadvances.2017012971 (**Selected as the cover image**)
4. Brenna E. Kiniry, **Shengbin Li**, Anupama Ganesh, Peter W. Hunt, Ma Somsouk, Pamela Skinner, Steven G. Deeks, Barbara L. Shacklett. Functionality of Gastrointestinal Tissue Resident $CD8^+$ T-cells in Chronic HIV-1 Infection. Mucosal Immunol. 2017 Nov 15. doi: 10.1038/mi.2017.96.
5. **Shengbin Li**, Gwantwa Mwakalundwa, Pamela J. Skinner. *In Situ* MHC-tetramer Staining and Quantitative Analysis to Determine the Location, Abundance, and Phenotype of Antigen-specific T Cells in Tissues. JoVE. 2017 Sep 22;(127). doi: 10.3791/56130.
6. **Shengbin Li**, Joy M. Folkvord, Eva G. Rakasz, Hadia M. Abdelaal, Reece K. Wagstaff, Katalin J. Kovacs, Hyeon O. Kim, Ryoko Sawahata, Samantha MaWhinney, David Masopust, Elizabeth Connick, and Pamela J. Skinner. Simian immunodeficiency virus-producing cells in follicles are partially suppressed by CD8+ cells in vivo. J Virol. 2016 Oct 5. pii: JVI.01332-16. **(Selected as a JVI spotlight feature)**
7. Elizabeth Connick, Joy M. Folkvord, Katherine T. Lind, Eva G. Rakasz, Brodie Miles, Nancy A. Wilson, Mario L. Santiago, Kimberly Schmitt, Edward B. Stephens, Hyeon O. Kim, Reece Wagstaff, **Shengbin Li**, Hadia M. Abdelaal, Nathan Kemp, David I. Watkins, Samantha MaWhinney, and Pamela J. Skinner. Compartmentalization of simian immunodeficiency virus replication within secondary lymphoid tissues of rhesus macaques is linked to disease stage and inversely related to localization of virus-specific CTL. J Immunol. 2014 Dec 1;193(11):5613-25. doi: 10.4049/jimmunol.1401161.